BIOCHEMISTRY AND MOLECULAR BIOLOGY OF AMIDASES
FROM METHYLOPHILUS METHYLOTROPHUS

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ABSTRACT

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF AMIDASES FROM METHYLOPHILUS METHYLOTROPHUS

Neil R. Wyborn

The biochemistry and molecular biology of amidases from *M. methylotrophus* was investigated. Acetamidase purification from whole cells exhibiting low specific activities yielded pure low-activity acetamidase (specific activity 6-15 μmol min⁻¹ [mg protein]⁻¹), the activity of which could be reactivated to a level approaching that of the high-activity acetamidase by heating (1-6 h, 60°C) with an activator component. Identical purifications from whole cells with high specific activities produced pure 'high-activity' acetamidases exhibiting a wide range of generally diminished activities (19-108 μmol min⁻¹ [mg protein]⁻¹) and an unexpected propensity for heat-reactivation similar to that of low-activity acetamidase. It was concluded that high-activity acetamidases underwent varying degrees of 'switch-off' of activity both pre- and post-purification.

The physico-chemical properties of purified acetamidases were investigated in vitro to elucidate the nature of the putative acetamidase post-transcriptional modification and its role in the reversible regulation of acetamidase activity. High- and low-activity acetamidases exhibited significantly different properties, although their respective MW values differed only by ≤ 52Da. Low-activity acetamidase was significantly more stable than high-activity acetamidases which were labile. Results suggested that high- and low-activity acetamidases existed in different conformational states and that the regulation of acetamidase activity probably involved an allosteric mechanism.

Cloning of the *M. methylotrophus* acetamidase structural gene (amiE) was unsuccessful, but the formamidase structural gene (fmd) was successfully cloned and heterologously-expressed in *E. coli*. The DNA sequences of fmd and three putative ORFs showed that (i) the formamidase primary sequence exhibited 57% strict identity with that of the *Mycobacterium smegmatis* 'acetamidase' (Mahenthiralingam et al., 1993), and (ii) ORF2 and ORF3 apparently respectively encoded a zinc finger DNA-binding protein and an AmiC-type regulatory protein. The evolutionary and regulatory implications of these findings were discussed.
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This work is respectfully dedicated to Iwe who gave up a lot to ensure that I got this far, and to my Gramp who was sadly unable to witness the completion of this work.
ABBREVIATIONS

ANS - 1-anilinonaphthalene-8-sulphonate

CIP - Calf intestinal alkaline phosphatase

CRP - Cyclic AMP receptor protein

CTAB - Cetyltrimethylammonium bromide

D - Dilution rate (h⁻¹)

ddNTPs - 2', 3'-dideoxynucleoside 5'-triphosphates

DHA - Dihydroxyacetone

DMF - Dimethylformamide

DMSO - Dimethylsulphoxide

DRAG - Dinitrogenase reductase-activating glycohydrolase

DRAT - Dinitrogenase reductase ADP-ribosyltransferase

DTNB - 5, 5'-Dithio-6A-(2-nitrobenzoic acid)

DTT - 1,4-Dithiothreitol

EDTA - Ethylene-diaminetetra-acetic acid

EMS - Ethyl methanesulphonate

ESMS - Electrospray mass spectrometry

F6P - Fructose 6-phosphate

FPLC - Fast protein liquid chromatography

GDH - Glutamate dehydrogenase

GOGAT - Glutamate synthase (glutamine amide-2-oxoglutarate aminotransferase)

GS - Glutamine synthetase

GTE - Glucose/tris-Cl/EDTA (buffer)

HPLC - High pressure liquid chromatography

Hu6P - Hexulose 6-phosphate

IPTG - Isopropyl-β-D-thiogalactopyranoside

KDPG - 2-keto, 3-deoxy, 6-phosphogluconate

LB - Luria-Bertani (medium)

LDMS - Laser desorption mass spectrometry

M9 - Mineral salts medium

M13mp19 - Polylinker multiple cloning site, used in E. coli cloning vectors (based on M13 phage)

MALDI-TOF - Matrix-assisted laser desorption-time of flight spectrometry

MDH - Methanol dehydrogenase

MOPS - Morpholinopropanesulphonic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PQQ</td>
<td>Pyrrolo-quinoline quinone</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>Ru5P</td>
<td>Ribulose 5-phosphate</td>
</tr>
<tr>
<td>Rubisco</td>
<td>Ribulose 1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>RuBP</td>
<td>Ribulose bisphosphate</td>
</tr>
<tr>
<td>S</td>
<td>Svedberg unit (sedimentation coefficient)</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE II</td>
<td>Seed II (minimal medium)</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline sodium citrate (buffer)</td>
</tr>
<tr>
<td>TA</td>
<td>Transaldolase</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA (buffer)</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate EDTA (buffer)</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA (buffer)</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N', N''-tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>V₀</td>
<td>Elution volume</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>Δ</td>
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INTRODUCTION

1.1 The microbiology and biochemistry of M. methylotrophus

1.1.1 Aspects of Methylotrophy

Methylotrophs are organisms that are capable of growth on C1-compounds, i.e. reduced carbon compounds containing one or more C atoms, but no C-C bonds (Anthony, 1982), and both pro- and eukaryotic methylotrophs (the latter are predominantly yeasts) have been isolated and studied. They are essentially ubiquitous in nature and can be readily isolated from activated sludge, pond water, river water and mud. Methylotrophs exhibit significant metabolic diversity and are capable of utilizing a relatively wide range of C and N growth substrates.

Certain methylotrophic organisms, the methanotrophs (e.g. Methylosinus sp., Methylococcus sp. & Methylomonas sp.), can utilize methane as a C and energy source and are characterized by the presence of a complex intracellular membranous structure, methane mono-oxygenase and the ability to form differentiated resistant resting bodies (spores or cysts). Methanotrophs assimilate C either via the ribulose monophosphate pathway (Type I organisms) or the serine pathway (Type II organisms) and generally use either ammonia, nitrate or nitrite as N-sources. Furthermore, all Type II (and some Type I) organisms are able to use atmospheric N₂ as their sole N-source; however, the methanotrophs will not be described in detail in this study.

The remaining methylotrophic organisms (e.g. Methylophilus sp., Pseudomonas sp. & Bacillus sp.) do not possess methane mono-oxygenase and cannot grow on methane; neither do they exhibit the specialized methanotrophic structures described above. In contrast to this though, the general biochemical properties of these two different types of bacteria are essentially similar. Methylophilus methylotrophus NCIB 10515 (literally meaning 'methyl radical loving, methyl radical consuming'; Jenkins et al., 1987), the amidases of which were investigated in this study, is one such organism that was originally isolated from activated sludge, and whose taxonomic properties have been described in detail previously (Jenkins et al., 1987). This bacterium is an aerobic Gram-negative rod that occurs singly or in pairs, and which possesses a relatively thick cell wall. Its DNA G + C content has been estimated at approximately 50%, and the organism may be motile by virtue of a polar flagellum; however, it is the ability of M. methylotrophus to grow rapidly and with high yield on methanol as a C substrate that is one of its most striking features, and this property was of prime importance to the initial use of this organism as a source of single cell protein (see Vasey & Powell, 1984; Sharp, 1989; see also below).

Methylotrophs unable to use methane as a C-source can be classified as 'obligate' or 'facultative', depending on the range of C substrates which can be utilized for growth by a given organism; obligate organisms cannot use non-C₁ compounds, whereas restricted
facultative methylotrophs either exhibit very slow growth on a very narrow range of non-C₁ substrates (e.g. glucose and fructose) (Type M), or show much faster growth on a slightly more diverse range of C substrates (Type L). Indeed, on occasions this classification system sometimes appears slightly arbitrary, as the boundaries delimiting facultative or obligate growth are not necessarily clear-cut. *M. methylotrophus* is most accurately described as a restricted facultative methylotroph due to its ability to grow on glucose (and, to a lesser degree, fructose) as a sole C and energy source (Jenkins *et al.*, 1987), although it is often referred to as an obligate methylotroph in the literature.

1.1.2 The nature and occurrence of *M. methylotrophus* C substrates

*M. methylotrophus* exhibits growth on only a narrow range of C and energy substrates (Anthony, 1982). The preferred carbon source is methanol, but good growth also occurs on methylated amines (e.g. methylamine and trimethylamine) (Large & Haywood, 1981) which can also act as N-sources (see below). These compounds are found in abundance in soil, water and sewage as a result of the decomposition of organic compounds. Methanol is produced by the hydrolysis of plant-derived methyl ethers and esters which are constituents of pectin and lignin, and by the combined catalytic activities of methanogenic and methanotrophic bacteria, which respectively generate methane (via the anaerobic reduction of CO₂) and oxidize methane to methanol. Similarly, methylamine is a component of plant material and is a product of the oxidation of all other methylated amines; dimethylamine is liberated during the oxidation of trimethylamine, and results from the decomposition of certain pesticides; and trimethylamine is produced by the microbial decomposition of choline derivatives. Formate is also formed in nature from the oxidation of C₁-compounds, and as a major end-product of bacterial fermentation. *M. methylotrophus* is unable to utilize formate as its sole C-source, but it readily dissimilates formate to CO₂ by virtue of its formate dehydrogenase (Patchett *et al.*, 1985; Jones *et al.*, 1987), and it has been suggested that this system contributes to the energy economy of the cell.

1.1.3 The oxidation of C₁-compounds by *M. methylotrophus*

Methanol can be formed from the oxidation of methane catalysed by methane mono-oxygenase in a remarkable reaction utilizing O₂ and either NADH or NAD(P)H as a reductant, which cannot be effected chemically in a single step (Anthony, 1982). It is this enzyme that is the key factor underpinning methanotrophic growth. *M. methylotrophus* does not contain this enzyme and cannot therefore grow directly on methane; however, it does possess an NAD⁺-independent alcohol dehydrogenase which allows this organism to grow readily on methanol (Anthony, 1982; Anthony *et al.*, 1993). All methanol-oxidizing bacteria exhibit this enzyme, which is generally present intracellularly at ≥ 5% of the total cell protein, and which is induced to higher levels after growth under methanol-limitation. Although this dehydrogenase has a wide substrate specificity its principal function resides in the oxidation
of methanol to formaldehyde and the enzyme is therefore referred to as methanol dehydrogenase (MDH). Furthermore, MDH also catalyses the oxidation of formaldehyde to formate at a similar rate to that of methanol oxidation (although it is unknown whether or not this enzyme ever functions as a formaldehyde dehydrogenase in vivo), and is activated by ammonia and primary amines when prepared aerobically. However, there is some evidence to suggest that the latter MDH property may be due to an alteration to the enzyme which occurs during its purification in the presence of oxygen.

The *M. methylotrophus* MDH has been shown to be a periplasmic quinoprotein (containing a pyrrolo-quinoline quinone [PQQ] prosthetic group) which catalyses the transfer of electrons from methanol to its specific electron acceptor cytochrome c\textsubscript{553}, and thence into the respiratory chain via cytochrome c\textsubscript{552}. A periplasmic modifier protein (M-protein) has also been described for this MDH (Long & Anthony, 1991) which alters the MDH substrate specificity by an unknown mechanism, despite the presence of MDH in at least a five-fold excess over the amount of the M-protein. *M. methylotrophus* also contains the enzymes trimethylamine dehydrogenase, dimethylamine mono-oxygenase and methylamine dehydrogenase, which collectively allow growth on methylated amines, by catalysing the oxidation of these substrates to formaldehyde and ammonia (Burton et al., 1983).

The formaldehyde produced from the oxidation of methanol and methylated amines can either be oxidized completely to CO\textsubscript{2} (dissimilation) or assimilated by the RuMP cycle (see below). Dissimilation of formaldehyde can occur linearly by way of methanol dehydrogenase to formate and thence to CO\textsubscript{2} by formate dehydrogenase, or in a cyclic fashion (Fig. 1.1) utilizing the assimilatory enzymes of the KDPG aldolase/TA variant of the RuMP pathway, in addition to the dissimilatory enzyme 6-phosphogluconate dehydrogenase. Both dissimilatory routes are coupled to the production of reducing equivalents (NADH or NAD[P]H), and therefore both routes of formaldehyde metabolism are able to generate metabolic energy.

### 1.1.4 Carbon assimilation by methylotrophic bacteria

The aerobic methylotrophic bacteria assimilate carbon into biomass by one of three different metabolic pathways, viz. the ribulose bisphosphate (RuBP; Calvin cycle), ribulose monophosphate (RuMP) and serine pathways, each of which is characterized by an alternative discrete complement of enzymes and at least two biochemical variants (Anthony, 1982). A fourth pathway, the dihydroxyacetone (DHA) pathway, which will not be considered further, operates in methylotrophic yeasts. These pathways all essentially fix a C\textsubscript{1}-compound (CO\textsubscript{2} and/or formaldehyde) to eventually generate a C\textsubscript{3}-compound from which cell biomass can be produced.

The RuBP pathway supports autotrophic growth (that which occurs using CO\textsubscript{2} as the primary C-source) and is utilized by photosynthetic plants and by both photosynthetic (e.g. *Rhodospirillum rubrum*) and non-photosynthetic (e.g. *Paracoccus denitrificans*) bacteria. Cellular carbon is assimilated at the oxidation level of CO\textsubscript{2} in this pathway, and at least some of this CO\textsubscript{2} is initially provided by the oxidation of reduced C\textsubscript{1}-compounds in the
The enzymes of this cycle are those of the assimilatory pathway plus the dissimilatory enzyme 6-phosphogluconate dehydrogenase (see reaction 23). Other enzymes are as follows: (13) hexulose phosphate synthase; (14) hexulose phosphate isomerase; (16) glucose phosphate isomerase; (17) glucose 6-phosphate dehydrogenase (taken from Anthony, 1982).

The summary equation for the cyclic route is as follows:

\[
\text{HCHO} + 2 \text{NAD(P)}^+ \rightarrow \text{CO}_2 + 2 \text{NAD(P)}H + 2 \text{H}^+ 
\]
relatively minor proportion of methylotrophic bacteria that use this pathway. All methylotrophic bacteria (with one exception, viz. *Pseudomonas oxalaticus*) that assimilate C in this way are facultative autotrophs that rely on incident light energy or the oxidation of H₂ to drive the biosynthesis of ATP and hence C-fixation. The key enzymes of this pathway are ribulose bisphosphate carboxylase/oxygenase (RubisCO) and phosphoribulokinase, which respectively catalyse the initial condensation reaction (fixation) in which the C₅-acceptor molecule (ribulose 1,5-bisphosphate) is carboxylated, and the final reaction of the cycle in which the C₅-acceptor molecule is regenerated (occurring after intermediate reductive and rearrangement reactions). The mere presence of these enzymes, and the absence of the key enzymes of alternative assimilatory pathways, has been deemed a sufficiently solid basis on which to infer that a given organism uses the RuBP pathway, in the absence of more concrete evidence for this supposition (e.g. investigation of the passage of a radio-labelled C atom through the pathway with respect to its incorporation into pathway intermediates). Furthermore, certain methylotrophic bacteria (e.g. *Methyllococcus capsulatus* [Bath]) contain enzymes from more than one pathway, suggesting that in some organisms C can be assimilated by alternative mechanisms.

The RuMP pathway is very similar to the RuBP pathway except that C is assimilated at the oxidation level of formaldehyde, and is utilized by organisms such as *Methylobacter methanica*, *Pseudomonas oleovorans* and *Methyllococcus capsulatus* (Anthony, 1982). This cyclic pathway consists of sequential fixation, cleavage and rearrangement reactions and the latter two types of reaction can be effected by different complements of enzymes. Thus, there are potentially four biochemical RuMP pathway variants resulting from the possible presence of two different cleavage enzymes and two different rearrangement systems. These alternatives are not represented equally in nature, neither are the relative energetic efficiencies of these pathways equivalent, although the energetic differences are minimal in comparison to the ATP-expenditure of, for example, the RuBP cycle. Only the KDPG aldolase/TA variant (Fig. 1.2), which predominates in obligate methylotrophs will be described in detail, as this is the pathway utilized by *M. methylotrophus* (Beardsmore et al., 1982).

The initial reaction of this pathway (fixation) is mediated by hexulose phosphate synthase which catalyses the aldol condensation of one molecule of formaldehyde with each of three molecules of the C₅-acceptor, ribulose 5-phosphate (Ru5P; C₅), to form hexulose 6-phosphate (Hu6P) which is subsequently isomerized to fructose 6-phosphate (F6P; three molecules). In the KDPG aldolase variant cleavage reactions one molecule of F6P (C₅) is converted to KDPG, by the action of the Entner-Doudoroff enzymes, which in turn is cleaved by aldolases to yield one molecule each of glyceraldehyde 3-phosphate (C₃) and pyruvate (C₃). The final sequence of rearrangement reactions in the TA pathway variant involves the regeneration of three molecules of the C₅-acceptor molecule (Ru5P) from the remaining two F6P molecules and one molecule of glyceraldehyde 3-phosphate. In addition, phosphoglycerate is synthesized from pyruvate (at the expense of 2 ATP molecules) and both of these molecules constitute precursors for biosynthetic metabolism. Pyruvate can be converted to oxaloacetate which can be fed into the TCA cycle, but since this cycle is
Figure 1.2 The KDPG aldolase/TA variant of the RuMP pathway of C-assimilation

The identities of the pathway enzymes are as follows: (8 a,b) transketolase; (9) pentose phosphate epimerase; (11) pentose phosphate isomerase; (12) transaldolase (TA); (13) hexulose phosphate synthase; (14) hexulose phosphate isomerase; (16) glucose phosphate isomerase; (17) glucose phosphate dehydrogenase; (18) phosphogluconate dehydratase; (19) 2-keto, 3-deoxy, 6-phosphogluconate (KDPG) aldolase; (20) PEP synthetase or equivalent enzyme(s); (21) enolase; (22) phosphoglyceromutase (taken from Anthony, 1982).

\[
3 \text{HCHO} + \text{NAD}^+ + 2 \text{ATP} \rightarrow \text{phosphoglycerate} + \text{NADH} + \text{H}^+ + 2 \text{ADP} + \text{P}_i
\]
incomplete in *M. methylotrophus* it fulfils a biosynthetic role only in this organism, by providing carbon skeletons as precursors for the synthesis of cell material.

The serine pathway is the third C-assimilatory pathway found in methylotrophic bacteria (*e.g.* *Methylobacterium organophilum* XX, *Methylosinus trichosporium* & *Pseudomonas extorquens*) and it cataplyses the fixation of two molecules of formaldehyde and a single molecule of CO₂. This pathway differs from those described above in that the key intermediary step is the formation of serine (from glycine and formaldehyde) and its other intermediates are carboxylic- and amino acids, rather than carbohydrates.

1.1.5 Nitrogen assimilation by *M. methylotrophus*

The growth of *M. methylotrophus* is only supported by a relatively narrow range of nitrogenous compounds, *viz.* ammonia, methylated amines, urea, formamide and aliphatic amides (Anthony, 1982; Burton *et al.*, 1983; Silman, 1990; Silman *et al.*, 1989, 1991). Therefore, this organism cannot grow on the vast majority of organic (*e.g.* amino acids) and inorganic (nitrates, nitriles etc.) nitrogenous compounds that represent N-sources for a variety of other different types of bacteria. *M. methylotrophus* assimilates N at the level of ammonia via the action of the glutamine synthetase/glutamine-oxoglutarate aminotransferase (glutamate synthase) or GS/GOGAT system (Windass *et al.*, 1980). The ammonia can be present as the free base, derived from the oxidation of methylated amines, or formed from the hydrolysis of short-chain aliphatic amides, formamide and urea, by one of the complement of amidases expressed by this organism (see below).

No detailed analysis of the GS/GOGAT system in *M. methylotrophus* has been undertaken, so the discussion of the regulation of N-assimilation that follows is based solely upon the regulation of N-assimilation in enteric bacteria. A vast array of experimental studies have been carried out in this research field (see Tyler, 1978; Merrick, 1988) therefore a complete appraisal of the intricacies of this work is far beyond the scope of this thesis; also, the physiological relevance of enteric N-metabolism to that exhibited by *M. methylotrophus* is also unknown in view of the diverse range of potential N-sources metabolized by *E. coli*, for example, and the restricted range shown by *M. methylotrophus*. However, in virtually all of the systems studied so far glutamate and glutamine serve as the primary cellular intermediates in the subsequent biosynthesis of N-containing compounds (*e.g.* amino acids, purines and pyrimidines etc.) which establishes the central importance of the regulation of three enzymes (GS, GOGAT and glutamate dehydrogenase [GDH]) to the global regulation of the N-pool and the control of cellular N-assimilation.

The biosynthesis of glutamine can only be effected by the amidation of glutamate by ammonia in an energy-dependent reaction catalysed by GS:

\[
\text{L-glutamate + NH}_4^+ + \text{ATP} \rightarrow \text{L-glutamine + ADP + P}_i + H^+ 
\]
Contrasting this, the biosynthesis of glutamate is catalysed either (i) by GOGAT mediating the reductive amination of α-ketoglutarate using glutamine as the N-donor (energy-independent reaction), or (ii) by GDH from NH$_4^+$ and α-ketoglutarate (a TCA cycle intermediate) in a reversible ATP-independent reaction (see below):

(i) α-ketoglutarate + L-glutamine + NAD(P)H + H$^+$ $\rightarrow$ 2 L-glutamate + NAD(P)$^+$

The overall reaction for the GS/GOGAT-catalysed system is as follows:

\[
\text{NH}_4^+ + \alpha\text{-ketoglutarate} + \text{NAD(P)H} + \text{ATP} \rightarrow \text{L-glutamate} + \text{NAD(P)$^+$} + \text{ADP} + \text{Pi}
\]

(ii) \( \text{NH}_4^+ + \alpha\text{-ketoglutarate} + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{L-glutamate} + \text{NAD(P)$^+$} + \text{H}_2\text{O} \)

Furthermore, enteric bacteria can also synthesize glutamate from the amino group of an amino acid other than glutamine and α-ketoglutarate in a transamination reaction, and as a direct product of the degradation of amino acids. From the reactions summarized above, it is clear that the biosynthesis of glutamate using GDH is more energetically-efficient (no ATP-expenditure) than by the combined catalytic activity of GS/GOGAT (requiring the nett hydrolysis of one ATP molecule).

It has been demonstrated in enteric bacteria growing on glucose (the preferred C-substrate) in the presence of excess ammonia (the preferred N-substrate) that the activity of the GS/GOGAT system is low and that glutamate is formed by GDH. However, under conditions of ammonia-limitation, due to the relatively high \( K_m \) of GDH (approximately 1mM; Merrick, 1988) for ammonia and α-ketoglutarate its N-assimilatory function becomes relatively inefficient in comparison to that of the high-affinity GS/GOGAT system (\( K_m << 1\text{mM} \)). Consequently, the latter is activated under N-deficient conditions as it is significantly more efficient at scavenging the available ammonia from a nutrient-limiting environment.

The activities of GS, GOGAT and GDH fulfil a primary role in N-metabolism regardless of the particular characteristics of a given N-source utilized by enteric organisms. In addition, a large number of accessory genes and their products have been implicated in global N-metabolism of the cell. A multitude of different genes are specifically regulated in response to the availability of a diverse range of nitrogenous compounds. This global N-regulation (\( ntr \)) system has been extensively characterized for the enteric bacteria and its presence in other genera of bacteria, or that of an analogous system, is generally predicted, although this has not yet been confirmed for the majority of organisms. Thus, in overall terms, the system appears to regulate ammonia transport and assimilation, and the catabolism of alternative nitrogen substrates, such that under nitrogen-excess conditions cells utilize ammonia preferentially and block alternative nitrogen assimilation pathways.

Regulatory influences exerted on cellular N-metabolism are interwoven in a complex web of transcriptional and post-transcriptional mechanisms involving both positive and negative
effectors. Furthermore, regulation at the protein level occurs by virtue of allosteric interactions and covalent post-translational modifications acting at different points within regulatory cascade systems (see below). The overall activity of the ntr system is therefore able to fulfil both sensory and regulatory functions which serve to coordinate and integrate N-metabolism on multiple levels in response to the nutrient status of the environment.

Studies have shown that of the three primary enzymes of N-assimilation only the activity of GS is both transcriptionally and post-transcriptionally regulated according to the availability of N. The activity of GS is regulated as part of a complex bicyclic cascade system which contains allosteric elements in addition to covalent post-translational modification. Forty different metabolites have been demonstrated to influence the activity of at least one of the enzymes in the GS cascade (Chock et al., 1980) thereby eliciting a remarkable capacity for regulatory flexibility in response to multiple control signals.

The GS of E. coli consists of twelve identical monomers arranged as two hexagonal rings, one stacked on top of the other. Covalent modification of GS occurs by adenylylation (Fig. 1.3), viz. the attachment of a single AMP molecule to a specific tyrosine residue on each monomer; the maximum number of AMP molecules per GS molecule being twelve, and optimal biosynthetic activity resulting from a completely unadenylylated enzyme. Additionally, the susceptibility of GS to cumulative feedback inhibition (see below) by the end-products of glutamine metabolism also increases with adenylylation of GS. There is therefore a cooperative regulatory effect resulting from the coupling of allostery and covalent modification that presumably neither system could achieve independently.

The adenylylation reaction is catalysed reversibly by an adenylyl transferase (ATase) the activity of which is in turn regulated by a low-MW regulatory protein, Pn. The activity of this latter protein is itself regulated by uridylylation, involving the reversible covalent attachment of a UMP molecule to Pn by a uridylyl transferase (UTase). A relationship exists between Pn and GS such that unmodified (deuridylylated) Pn stimulates adenylylation of GS by ATase and hence deactivates GS, and vice versa. A high intracellular glutamine:α-ketoglutarate ratio (indicating conditions of N-sufficiency) stimulates UTase to deuridylylate Pn which down-regulates GS activity, and the opposite occurs when the glutamine:α-ketoglutarate ratio is low. Regulation of GS activity therefore results from a complex cascade of events which are ultimately controlled by the relative intracellular levels of glutamine and α-ketoglutarate. An ntr-mediated transcriptional switch is also operative such that under conditions of N-deficiency the level of GS is ten-fold higher than that of cells grown under conditions of N-excess. Much less information exists at this time concerning links between the ntr system and the regulation of nitrogen fixation. The nitrogen fixation (nif) genes of K. pneumoniae have been shown to be subject to ntr regulation, but similar control is yet to be unequivocally established in other genera of bacteria.

1.2 The genetics and molecular biology of methylotrophs

This section is intended to give a brief summary only of the current state of methylotroph
Figure 1.3 Regulation of glutamine synthetase (GS) activity

The schematic diagram (re-drawn and adapted from Ginsburg & Stadtman, 1973) represents part of the bicyclic cascade controlling the activity of glutamine synthetase in *E. coli*. GS = deadenylylated glutamine synthetase (active); GS-AMP = adenylylated glutamine synthetase (inactive); ATase +/- = adenylyltransferase (adenylylation & deadenylylation modes respectively); $P_{II} =$ deuridylylated $P_{II}$; $P_{II}$-UMP = uridylylated $P_{II}$; UTase +/- = uridylyltransferase (uridylylation & deuridylylation modes respectively).
The study of methylotrophic molecular biology is a relatively new exploratory avenue which has been widened considerably in the past few years due to the continued improvement in manipulative techniques. Thus, sophisticated molecular biological and genetic analyses can be undertaken to investigate a variety of interesting methylotrophic properties (see Lidstom & Stirling, 1990; Murrell, 1992).

The isolation and analysis of 5S and 16S rRNA sequences has improved taxonomic classification methods (Arfman & Dijkhuizen, 1993; Boulygina et al., 1993; Hanson et al., 1993) and dispensed with at least some of the more arbitrary classification systems employed previously. DNA manipulative techniques have also improved. Gene transfer systems are currently quite limited in the methylotrophs as very few transformation systems exist, and those that have been devised are generally inefficient and unreliable. Phage transduction systems are essentially non-existent, and the best gene transfer systems developed to date have involved conjugative and non-conjugative plasmids. However, the recent development of electroporation may improve success in this area. A significant breakthrough in genetic techniques has also been achieved through the development and use of broad host range cloning vectors and cosmid vectors, which has facilitated the cloning and mapping of a number of methylotrophic genes (e.g. Hutchinson & Goodwin, 1993a,b) and the heterologous expression of foreign genes in methylotrophic hosts (e.g. the expression of E. coli GDH [Windass et al., 1980], chicken ovalbumin and mouse dihydrofolate reductase cDNA genes [Hennam et al., 1982] in M. methylotrophus). Newly-developed cosmid, sub-cloning, promoter cloning and expression vectors are also expected to extend success with this work. The characterization of endogenous cryptic methylotrophic plasmids might also yield further insight into the construction of cloning vectors for use in a methylotrophic host.

The generation of mutants is an essential pre-requisite for most genetic analyses and techniques to accomplish this have proven particularly inefficient in the past. Some answers to these problems have been devised with the use of positive selection systems to generate methane and methanol oxidation mutants, for example, but with limited success. More fruitful investigations have involved the optimization of chemical mutagenesis techniques for the isolation of auxotrophic RuMP mutants, and the generation of M. methylotrophus mutants involved with methanol and methylamine metabolism (Dawson & Goodwin, 1990; Hutchinson & Goodwin, 1993a,b). Very limited success has been achieved with transposon mutagenesis.

In comparison to the genetic and molecular biological characterization of E. coli relatively little applied information is currently available to the molecular biologist working with a methylotroph. There is a distinct lack of information concerning codon usage tables, promoter structure and function in both native and heterologous systems, restriction-modification systems, RNA polymerase structure and function, and recombination systems. However, this situation will no doubt be alleviated by the new sequencing data that is continually being generated. Methylotrophic molecular biology is a rapidly expanding research area, particularly with respect to the characterization of key methylotrophic enzymes such as methane mono-
oxygenase (see Murrell, 1992, 1993), methanol dehydrogenase (e.g. Anthony et al., 1993; Harms, 1993) and methylamine dehydrogenase (e.g. Lidstrom & Chistoserdov, 1993).

The heterologous expression of methylotroph genes in foreign hosts was initially largely unsuccessful. Many of these problems have now been overcome and success has been achieved with complementation studies in *E. coli* and *Ps. aeruginosa* in which auxotrophic mutants have been complemented with DNA from *M. methylotrophus* (e.g. Kearney & Holloway, 1987), *M. viscosgenes* and *M. flagellatum*. Unfortunately, these studies were unable to specify with any certainty whether transcription was controlled by a promoter contained within the insert DNA or by the vector promoter. Similarly, examples of the heterologous expression of foreign genes in *M. methylotrophus* (see above) have been described wherein it was assumed that expression was driven by the vector promoter. Finally, detailed work continues to emerge on the fine genetic structure and architecture of genes controlling methanol oxidation (mox genes), nitrogen metabolism (nif and ntr genes), PQQ biosynthesis, methane oxidation (mmo genes), and the serine pathway of C1 metabolism.

### 1.3 Regulation of enzyme activity

#### 1.3.1 Introduction

A number of different systems have evolved to regulate enzyme activity at both genetic and protein levels. Enzyme activity is primarily regulated at the genetic level by induction and repression of gene transcription in response to various environmental stimuli; this then provides 'coarse' control of enzyme activity as a function of the intracellular concentration of a given gene product. The amount of the latter present at a particular point in time can also be modulated by systems that stabilize/destabilize mRNA transcripts, thereby increasing or diminishing the number of copies of each protein translated from a mRNA template, and similarly by systems that positively or negatively influence the intracellular longevity of the protein (e.g. degradation by proteolysis, stabilization by post-translational modification etc.). In addition, a multitude of 'fine' control regulatory systems have been discovered that operate at the protein, or post-translational level, to influence enzymic activity. These regulatory systems are characterized by the covalent or non-covalent interactions of a multitude of effectors (molecules, ions) with the target protein which can provoke an alteration in the activity of the latter. These interactions can be reversible or irreversible, interlinked with other modification systems to form cascade systems or occurring essentially in isolation, and can also operate either transiently or more permanently.

In view of the overwhelming quantity of published material concerning the regulation of enzymic activity, what follows below is a brief distillation of the main points concerning allosteric and covalent regulatory mechanisms, and most of the kinetic elements of the discussion have been omitted. Only reversible systems will be considered below in keeping with the reversible nature of acetamidase regulation in *M. methylotrophus* (J. Mills & C.W. Jones, unpublished; Carver & Jones, 1993).
1.3.2 Allosteric regulation of enzyme activity

1.3.2.1 Allosteric behaviour of haemoglobin

The archetypal allosteric (meaning 'the other site') protein is the oxygen-binding protein, haemoglobin. Christian Bohr was the first to demonstrate (in the 19th century) that haemoglobin bound oxygen in a manner that was described by a sigmoidal curve (see Monod et al., 1963; Koshland, 1973) rather than the 'normal' hyperbolic substrate-binding rate exhibited by most enzymes. Bohr elucidated that binding of the first oxygen molecule to haemoglobin positively enhanced the binding of the second, and so on, such that binding of each O\textsubscript{2} molecule facilitated binding of the next. He termed this effect 'cooperativity'. Since then it has been discovered that sigmoidal binding curves of the reaction velocity, v, versus substrate concentration [s], are important and common features of many regulatory proteins. Later studies (1913-1960) showed that four O\textsubscript{2}-binding sites (haem groups) in haemoglobin interacted cooperatively, and X-ray analysis showed that these sites were topographically separate. These observations implied that interactions between the sites were necessarily 'long-range' interactions (see Engel, 1981), and that haemoglobin must be a flexible molecule.

1.3.2.2 The conformational flexibility of allosteric proteins

The 'induced-fit' theory proposed by Koshland (1958; see also Koshland, 1963) was forwarded to explain the basis of substrate specificity, but it was also probably the first theory which concerned the ability of a protein to undergo a conformational alteration in relation to its functional role as an enzyme. He postulated that instead of the enzyme-substrate relationship operating as a 'lock-and-key' system in which the substrate (the key) had to fit directly into a rigidly-defined and inflexible active site (the lock) for activity to occur, the active site was more analogous to a hand fitting into a glove and altering its shape. Thus, his 'induced-fit' model theorized that (a) a precise orientation of catalytic groups is required for enzyme action; (b) binding of the substrate may cause an appreciable change in the three-dimensional architecture of the amino acids at the active site; and (c) the changes in protein structure caused by a substrate will bring the catalytic groups into the correct orientation for reaction, whereas a non-substrate will not. The seminal paper of Monod et al. (1963) expanded greatly upon the idea of induced conformational changes to protein structure and their regulatory nature by rationalizing the results from a number of different enzyme studies in terms of a qualitative allosteric model for activity regulation. Characteristic regulatory trends were established in this work from a number of different enzyme pathways and used to develop a quantitative model (Monod et al., 1965), the predictions of which were favourably tested against the results from a number of different allosteric enzyme studies.

The original paper (Monod et al., 1963) was concerned initially with observations on feedback inhibition in biosynthetic pathways and the interpretation of these findings in terms of allostery. At that time it was considered generally true that the terminal metabolite
synthesized in a given bacterial biosynthetic pathway was a powerful and specific inhibitor of its own synthesis, and that the enzyme responsible for mediating this effect was usually the first enzyme of a given pathway. Furthermore, it was noted that each regulatory enzyme occurred immediately after a metabolic branching point and that it was only the terminal metabolite that specifically inhibited the regulatory enzyme, and no intermediary metabolites could fulfil this role. Enzymes acting after the regulatory enzyme were not significantly sensitive to inhibition. It was known from consideration of feedback inhibition patterns that effector molecules clearly exhibited different molecular configurations to those of the substrate molecules from the same pathway, *i.e.* substrate and inhibitor molecules were allosteric rather than isosteric with respect to each other. In view of this it was determined that inhibitors must act by forming a stereospecific complex with the enzyme, and it was concluded that substrate and inhibitor binding sites were composed of different groups of substrate binding residues. These conclusions, which were based solely on structural data, were confirmed by the discovery that a regulatory enzyme may lose its sensitivity to the inhibiting metabolite whilst retaining the majority or all of its activity towards its substrate. This process was termed 'desensitization' (see below).

Monod *et al.* (1963) attempted to rationalize the observed patterns of feedback inhibition in terms of three possible types of substrate-inhibitor interactions: (a) the binding sites (defined by different binding groups of amino acid residues) for substrate and inhibitor actually overlapped, indicating that steric hindrance would only allow mutually-exclusive binding of either substrate or inhibitor (see also discussion of feedback regulation below); (b) the binding sites occurred in very close proximity to each other, such that direct interactions (either attractive or repulsive) between substrate and inhibitor were able to occur; and (c) the binding sites were completely separate, thereby preventing direct substrate and inhibitor interactions and suggesting that the inhibitory effect was mediated entirely by the protein, presumably through a conformational shift resulting from the binding of the inhibitor, *i.e.* an allosteric transition. It was concluded that in view of kinetic predictions describing the intrinsic properties of different modes of inhibition, and the observations from desensitization studies and actual inhibitory pathways, that model (a) could not possibly be reconciled with the experimental data, and the same was also true for model (b). In contrast to this, allosteric effects (model [c]) accounted admirably for the observed trends in experimental data.

Studies of desensitization showed that in general the sensitivity of the regulatory enzyme to the inhibitory metabolite was an extremely labile property which could be abolished by treating with mercurials, urea or gentle heating. It was also shown that desensitization of certain enzymes abolished the cooperative substrate effect which essentially transformed their sigmoidal rate-concentration profiles into hyperbolic curves. Furthermore, it was also demonstrated that mutations which produced desensitized enzymes either partially abolished or altered the substrate cooperative effect. It was concluded that both the cooperative effect between substrate binding sites and the interaction between inhibitor and substrate sites were dependent on conservation of the integrity of the native protein state. It therefore followed that
desensitization might result from perturbation of the native protein state, i.e. by disruption of bonds between protein sub-units.

The essential and basic underlying principles of the allosteric model (Monod et al., 1965) have been summarized succinctly in their simplest form (Engel, 1981; Stryer, 1981; Ottaway, 1988). For an allosteric protein consisting of two identical sub-units (each with its own active site) the model proposes that each sub-unit can exist in alternative interconvertible conformations, the R (relaxed) and T (tense) forms (Fig. 1.4.a), in which the former has a relatively high affinity for the substrate and the latter a relatively low affinity. The pivotal assumption of this model is that the symmetry of the dimer must be conserved under all conditions so that the sub-units must always adopt the same conformation. It therefore follows that dimers can exist in the RR or TT forms, but not the energetically unfavourable RT form. In the absence of ligand the conformational equilibrium favours the form that binds the ligand least well (T form). However, when the ligand is added the equilibrium point is shifted towards the R form, which exhibits a higher affinity for the ligand, and since binding of the ligand to the R form must maintain molecular symmetry this event predisposes the second site to ligand binding, which promotes the T to R transition of the second sub-unit. Thus, the transition from T to R is concerted (this model is often referred to as the 'concerted' model) and as the ligand concentration increases the proportion of the R form also increases indicating that ligand binding is cooperative. This process results in a sigmoid saturation curve where the degree of sigmoidicity depends upon the relative affinities of the R and T forms for the ligand, and the position of the equilibrium point in the absence of the ligand. It therefore follows that some allosteric enzymes do not exhibit sigmoid saturation curves, but rather curves that approximate to a hyperbola (as do desensitized allosteric enzymes).

The effects of allosteric inhibitors and activators can be explained readily in terms of this model. Inhibitors bind preferentially to the T form and shift the conformational equilibrium point towards the T form, whereas an activator acts in a diametrically-opposed fashion. Thus, an allosteric inhibitor would stabilize the T form and increase the sigmoidicity of the saturation curve, whereas the opposite would be true for an activator.

Thus the general features of allostery (Monod et al., 1963) are (i) allosteric proteins possess at least two non-overlapping stereospecific receptor sites, the active site and the allosteric site; (ii) the active site binds the substrate and mediates the biological activity of the protein, and the allosteric site (which is complementary to the structure of another metabolite) specifically and reversibly binds the allosteric effector; (iii) the allosteric effector does not participate in the reaction mechanism (except perhaps in certain cases), but serves to effect a discrete reversible conformational change in the molecular configuration of the protein, i.e. an allosteric transition; (iv) the allosteric transition modifies the properties of the active site, changing one or all of the kinetic parameters which characterize the biological activity of the protein. Thus, it is implicit in this summary that the allosteric effector need not bear any chemical or metabolic relation to the substrate itself.
Figure 1.4 The concerted and sequential allosteric models

In the schematic diagrams below (adapted from Stryer, 1981) the binding of a ligand to the low-affinity form of the enzyme (TT) promotes the conformational transition to the high-affinity form (RR) in the concerted model (Fig. 1.4.a), which assumes that the symmetry of the enzyme molecule must be conserved during this process; therefore, a direct TT to RR transition occurs upon binding of the first substrate molecule and no hybrid conformational states (RT) are permitted. Conversely, no such assumptions are made in the sequential model (Fig. 1.4.b) and hybrid conformational states (RT) may exist.

Figure 1.4.a

Figure 1.4.b
1.3.2.3 Implications and significance of molecular cooperativity

Different modes of cooperativity can be defined depending on the nature of the allosteric ligand. Homotropic cooperativity involves interactions between identical ligands (e.g., $O_2$ molecules binding to haemoglobin) and heterotropic cooperativity refers to interactions between different ligands (non-cooperativity also exists whereby the ligand alters the sub-unit to which it is bound, but does not alter interactions between sub-units; if all sub-units express the same properties there is no cooperation between the sub-unit sites [see Koshland, 1973]). In addition, cooperativity can also be positive or negative. The binding of $O_2$ molecules to haemoglobin exemplifies positive (homotropic) cooperativity as the binding of the initial molecule promotes the binding of the second; conversely, negative cooperativity is characterized by an event in which the binding of the first ligand induces a conformational change that discriminates against binding of the second (see Koshland, 1973). The allosteric model proposed by Monod et al. (1963, 1965) is primarily concerned with an interpretation of cooperative homotropic events, but it cannot account for negative homotropic events because the binding of ligand to the enzyme cannot displace the conformational equilibrium in favour of a form that binds the ligand less tightly. This fact highlights an important limitation of the model. The physiological significance of cooperativity has been enunciated by Koshland (1973). A protein exhibiting positive cooperativity is much more sensitive to small fluctuations in the environment than a protein with a normal binding potential, and the increased sensitivity applies equally to all ligands (substrates, inhibitors & activators), thus this system can effect signal amplification, such that a 'small' signal can instigate a 'large' regulatory effect. In contrast, negative cooperativity diminishes the sensitivity of a protein to environmental fluctuations of ligand concentration such that a much greater ligand concentration is required to elicit a significant change in its activity. Hence, this process can dampen the response of such proteins to fluctuations in ligand concentration thereby making them less sensitive to environmental changes.

1.3.2.4 An alternative model for allosteric regulation of enzyme activity

The second major contribution to the interpretation of non-linear enzyme kinetics was proposed by Koshland et al. (1966). This allosteric model (the 'sequential model') was essentially similar to the concerted model, but differed in several important respects, viz. the sequential model does not assume that symmetry must be maintained after an allosteric transition and hybrid protein molecules exhibiting sub-units in alternative conformations are 'allowed'. Thus, reduced to its simplest form the sequential model proposes that there are only two alternative sub-unit conformational states (T & R) that are energetically-favourable (Fig. 1.4.b), and that no equilibrium need exist between T and R forms in the absence of ligand. The conformational change from T to R is induced (cf. the induced fit model [Koshland, 1958] which was proposed to explain an enzyme's substrate specificity) by the binding of ligand to one sub-unit which alters the shape of that sub-unit independently of other sub-
units, *i.e.* there is no significant alteration to the conformation of the other sub-units. The conformational change to the first sub-unit can increase or decrease the affinity of the other sub-units in the same protein for the ligand (see Koshland, 1973; Stryer, 1981; Ottaway, 1988) so that the conformational change in sub-units is sequential. The characteristics of the sequential model saturation curve are determined by the geometry and relative strengths of the sub-unit interactions in the various states of the protein, and by the variable affinities of the different sub-units for the ligand (see Engel, 1981). A particularly noteworthy point concerning the sequential model was its prediction that proteins exhibiting negative cooperativity should exist (*cf.* the concerted model which could not account for such kinetic behaviour), as at the time no such proteins had been identified; however, glyceraldehyde 3-phosphate dehydrogenase later furnished just such an example (see Koshland, 1973), and many other enzymes exhibiting this phenomenon have since been discovered.

In summary, allosteric regulatory proteins exhibit exceptional regulatory properties, allowing the rapid amplification and effective translation of a very low energy signal which results from their intrinsic conformational flexibility. Non-covalent binding of effector molecules at distinct sites on different sub-units of a protein can influence events at the active site and reversibly alter the kinetic parameters of a given protein. The allosteric effect is mediated by a conformational change in the protein molecule itself, which presumably results from alterations to the quaternary protein structure, and its overall effect appears to integrate the regulation of diverse metabolic pathways by effector molecules that are not obliged to exhibit any chemical or metabolic relationship to the substrate of the reaction, and generally do not participate directly in the catalytic mechanism.

1.3.2.5 Feedback regulation

Feedback regulation can exert activatory and inhibitory influences on enzyme activity as a result of the establishment of a rapid equilibrium between enzyme and effector molecules and can be discussed in allosteric terms. Competitive inhibition results from the competition for binding between two different molecules, *i.e.* substrate and end-product, for a single site (the active site) of the enzyme and very often results from the similar molecular configurations of substrates and end-products of a given pathway. Thus, the binding of a substrate or competitor molecule to a single active site is a mutually exclusive event, and this type of inhibition results from a diminution in the catalytic rate, as a result of the reduction in the proportion of enzyme molecules with bound substrate. This particular mode of competitive inhibition does not have an allosteric basis, although this type of inhibition can also occur if an allosteric ligand binds to the enzyme at a site distinct from the active site and alters the conformation of the enzyme in such a way that substrate can no longer bind at the active site (see Engel, 1981).

Non-competitive inhibition involves the binding of a ligand to the enzyme at a topographically distinct site from the active site, which may or may not be present on the catalytic sub-unit, and is a process which can occur simultaneously with substrate binding at
the active site. This type of inhibition arises from the resultant decrease in the turnover number of the enzyme, which in the strictest sense is only a true allosteric effect if the ligand-binding site which mediates the inhibitory interaction is located on a different sub-unit to that of the active site.

Simple competitive and non-competitive modes of inhibition can be easily distinguished kinetically. An enzyme can be released from competitive inhibition by increasing the substrate concentration to a sufficiently high level; this minimizes the number of active sites that bind the competitor molecule and therefore restores maximal enzymic activity. This type of inhibition is characterized by a change in $K_m$, but $V_{\text{max}}$ remains unaltered. Conversely, non-competitive inhibition cannot be overcome by increasing the substrate concentration, and it is characterized by an unaltered $K_m$ and a decrease in $V_{\text{max}}$. However, considerably more bewildering (to the non-kineticist) modes of activity regulation can arise from allosteric interactions involving multiple ligand binding sites.

1.3.2.6 Hysteresis

The term 'hysteretic enzyme' (hysteresis', from the Greek meaning 'to be behind' or 'to lag') has been used by Frieden (1970) to describe enzymes "which respond (in terms of configurational or other changes) slowly to rapid changes in ligand concentration". In this context, the relative terms 'slow' and 'rapid' are related to the time required to measure enzyme activity, and 'ligand' refers to either a substrate or a protein modifier. It is envisaged that the slow change in the properties of a hysteretic enzyme would give rise to a lag period in the response of the enzyme to local changes in the concentration of the ligand, which would ultimately lead to the buffering of certain metabolites in a time-dependent fashion. Since the majority of hysteretic enzymes appear to be regulatory enzymes acting at critical sites in metabolic pathways (which also very often show allosteric kinetic behaviour) this is of potential importance to complex metabolic pathways channelling common intermediates and to those exhibiting multiple branch points. In many cases the 'slow' response correlates with a protein conformational change which may take place in a time period ranging from seconds to hours. Protein conformational changes appear to occur (under certain conditions) by three main mechanisms: (i) ligand-induced isomerization processes, in which it is assumed that the protein may exist in two different conformational states, both of which can bind substrate, but with different affinities; (ii) displacement reactions involving the removal of a tightly bound ligand from an enzyme by another ligand exerting an altered effect on enzymic activity, and (iii) the more complex mechanism of enzymic polymerization/depolymerization, which may contain elements of both of the aforementioned mechanisms. In terms of metabolic significance, hysteresis provides a mechanism by which the activity of an enzyme can be modulated as a function of time for a given ligand concentration. If the ligand induces a slow conformational change which endows different conformations with variable kinetic properties then activity at any given time after ligand addition will be most accurately represented as some weighted average of the activities of the different forms of the enzyme. Thus, in
simplistic terms, hysteretic enzymes would appear to be 'special' types of allosteric enzymes affording yet further regulatory diversity as a result of the relatively long time period (cf. the relatively rapid conformational changes characterizing 'normal' allosteric behaviour) taken to establish a new regulatory state after a change in ligand concentration. Frieden (1970) has postulated that hysteretic buffering systems might be more common in metabolic pathways exhibiting a complex regulatory phenotype than in less complex systems characterized by simple feedback inhibition.

1.3.3 Covalent regulation of enzyme activity

1.3.3.1 Introduction

Allosteric control allows the continuous and rapid revision and modification of the rate of a reaction sequence in response to fluctuating concentrations of effector molecules. In contrast, cellular regulation of enzyme activity that is required over a relatively longer time period is often effected by covalent protein modification. A multitude of different types of covalent modification exist which are described below, although the list is confined to the more common modifications and is not exhaustive.

1.3.3.2 Phosphorylation, ADP-ribosylation and nucleotidylylation

These three types of modification are probably the most common and widely reported covalent modifications to pro- and eukaryotic proteins, and they have been shown to be important to the regulation of activity of a wide range of enzymes. Phosphorylation is catalysed by a protein kinase that transfers a phosphoryl group from a donor molecule (usually ATP) to specific amino acids, most commonly Ser, Thr and Tyr, but His and Lys may also be modified in this way (Wold, 1981). Phosphorylation often elicits a conformational change in the target protein by electrostatic repulsion between the charged groups of its amino acid side chains and the negative charge of the phosphate group. The process is reversible upon removal of the phosphoryl group by a phosphoprotein phosphatase. The donor molecule in ADP-ribosylation is generally NAD$^+$ and the ADP-ribosyl group is specifically attached to Arg, His, Ser or Cys residues; similarly, ATP or UTP act as donors of adenylyl and uridylyl groups during nucleotidylylation of Tyr or Ser residues (Shacter et al., 1986). Examples of these types of reversible modifications include phosphorylation of isocitrate dehydrogenase in *E. coli* (Thorsness & Koshland, 1987), ADP-ribosylation of dinitrogenase reductase in *Rhodospirillum rubrum* (see Ludden & Roberts, 1989), and adenylylation- and uridylylation-mediated control of GS activity in *E. coli* by AMP and UMP (see above).
1.3.3.3 Amidation and carboxylation

The presence of a C-terminal amide rather than a free α-carboxyl group was probably the first post-translational modification to be discovered (see Kreil, 1984). It was first demonstrated in oxytocin and vasopressin which were both found to terminate with glycaminamide, and it is a common cause of the inability to remove C-terminal amino acids with carboxypeptidases (C-terminal ‘blocking’). This type of modification has subsequently been found in a number of different small eukaryotic polypeptides, particularly peptide hormones and toxins and venoms, although there are as yet no reports of prokaryotic amidated proteins. All amino acids, except Arg and Asp have been identified in their amidated form at the C-terminus of various proteins, but no clear evidence exists to indicate the likely biological function of C-terminal amidation at the present time.

Asp and Glu residues can be carboxylated at the β- and γ-carbon atoms respectively. β-carboxyglutaric acid has been identified as an amino acid residue of ribosomal proteins of E. coli (Koch et al., 1984), otherwise very little is known about the biosynthesis and biological function of this modifying group. The only known function of γ-carboxyglutamic acid is to serve in calcium binding (see Wold, 1981; Nelsestuen, 1984) and this is exemplified in the blood coagulation cascade system in eukaryotes, in which vitamin K is involved as a cofactor during the post-translational carboxylation of Glu residues (Gundberg et al., 1984).

1.3.3.4 Methylation

Protein methylation is a widespread reversible post-translational modification which is found in both pro- and eukaryotic organisms (Wold, 1981). The reaction is catalysed by a methyltransferase which transfers a methyl group to the N-terminus of a protein or to the side-chains of various amino acids contained therein, including Lys, Arg and His (see Paik et al., 1980; Paik & Dimaria, 1984). Presumably, a demethylase catalyses the removal of protein-bound methyl groups although only one demethylase has been isolated that is capable of demethylating methyllysine. Since methylated proteins generally exhibit sub-equimolar amounts of methylated amino acid residue per mole of the protein, and methylation results in only a marginal change in protein electrical charge and steric configuration, it is very difficult to detect the physico-chemical differences that exist between methylated and unmethylated forms of the same protein. Proteins that are methylated in vivo include bacterial flagella proteins and eukaryotic histones, myosin, opsins and ribosomal proteins, and detailed studies of protein methylation have concerned the control of bacterial chemotactic phenomena and cytochrome c (Paik et al., 1980).

1.3.3.5 Acylation and glycosylation

Common acylations involve the addition of formyl, acetyl, pyroglutamate and pyruvoyl moieties to either the N-terminus or the amino acid side chains of proteins (Wold, 1981). N-
terminal acylation leads to 'blocking' of the N-terminus which is often characterized by the inability to determine its amino acid sequence by conventional Edman degradation chemistry. Possibly the most abundant side-chain acylation involves acetylation of proteins via the activity of acetyl transferases (acetylases) that catalyse the transfer of acetyl groups from the donor molecule acetyl CoA to the ε-amino group of Lys residues (see Allfrey et al., 1984). This type of modification is particularly common in eukaryotic proteins such as histones and other DNA binding proteins, and is a rapid and reversible modification (de-modification proceeds via the activity of deacetylases). The full side-chain acetylation potential is not realized in all histone molecules so that populations of these molecules may exhibit marked micro-heterogeneity. In this system acetylation serves to neutralize a positive charge with the concomitant weakening of the electrostatic attraction between the DNA and the protein. Acetylation appears to be considerably less common in prokaryotes.

Glycosylation involves the attachment of mono-, oligo- and polysaccharides to the amino acids Asn, Ser, Thr and Cys (Wold, 1981), all of which may be involved with a variety of different biological functions, including protease resistance and establishing structural integrity. However, the reversible regulation of prokaryotic enzyme activity by glycosylation is not a common regulatory mechanism.

1.3.3 Miscellaneous modifications

Proteolytic cleavage is a universal modification effecting enzyme precursor (zymogen) activation in the eukaryotic gut, prohormone activation (e.g. human preproinsulin is cleaved to yield proinsulin which is itself cleaved to liberate insulin), virus protein maturation and protein translocation across membranes (Wold, 1981). However, this type of modification is highly unlikely to be involved with reversible regulatory systems. The formation of cystine from the oxidation of two cysteine residues is important in the stabilization of the three-dimensional structure of multi-sub-unit proteins by intra-chain cross-linking (Wold, 1981), and this modification has also been demonstrated in the reversible regulation of protein disulphide isomerase activity in E. coli (Wunderlich et al., 1993).

Hydroxylation of Asp, Pro and Lys residues has also been identified in certain proteins. Hydroxyproline-containing proteins were discovered in plants (see Chrispeels, 1984), and have also been found in animals. The reaction is catalysed by a prolyl hydroxylase, but little information is available concerning the biological role of this modification.

The oxidation of His residues has been proposed to function as a marker for protein degradation, in the prevention of cellular autolysis and is also of importance to oxygen toxicity mechanisms. Bacterial GS has been shown to be subject to modification in this way via the action of a mixed-function oxidation system, which promotes the loss of GS activity and also renders it more susceptible to degradation (see Levine, 1984). The biological roles outlined above for oxidation of His suggest that this modification is unlikely to function in reversible regulatory systems.
Finally, halogenation of amino acids (iodination, bromination and chlorination; see Hunt, 1984), which primarily involves Tyr residues is widespread in eukaryotic scleroproteins and has been implicated in the functioning of the thyroid gland in vertebrates. However, there are no reports of halogenation mechanisms effecting reversible regulation of prokaryotic enzyme activity.

1.3.4 Metabolic cascade systems

The regulation and integration of metabolism is of prime importance for the continued functioning of any cell, and the integrative processes that have evolved are both exceptionally complex and highly-ordered. The covalent modification and demodification reactions that act upon key regulatory enzymes are catalysed by converter enzymes acting in opposing directions, and since each enzyme acts upon another the process is defined as a cascade system. The action of a pair of converter enzymes upon a single regulatory enzyme is defined as a monocyclic cascade (e.g. the regulation of mammalian pyruvate dehydrogenase). Further to this, when the active form of the interconvertible enzyme acts as a converter enzyme for the modification of a second interconvertible enzyme, different enzymic systems can become inter-linked to form a bicyclic cascade (e.g. regulation of GS activity in E. coli). Furthermore, the inter-linking of different cascade systems can become much more complex with multiple interactions to form polycyclic cascades. Thus, cyclic cascade systems play a key role in the integration of metabolism and they involve both allosteric and covalent regulatory elements (Chock et al., 1980, 1985).

The concept of covalent modification acting solely as a discrete on/off 'switch' to activate or deactivate a given enzyme has been essentially invalidated by in vitro and in vivo analysis, although under certain circumstances cascade systems do appear to function in this way (e.g. regulation of dinitrogenase reductase activity from Rhodospirillum rubrum; see Ludden & Roberts, 1989). This generally out-dated notion has been superseded by the idea of a cascade system as a dynamic regulatory process which mediates the conversion of an enzyme between active and inactive forms and leads to the establishment of a steady-state activity under the direct influence of the converter enzymes (Chock et al., 1980). The activity of the interconvertible enzyme at any given time is therefore envisaged to be a composite function of the proportion of modified versus unmodified enzyme.

Cascade systems can sense the fluctuating concentrations of a vast range of metabolites by interactions with allosteric effectors; this sensory function can elicit an immediate regulatory response by automatically altering the activity of the converter enzymes, and hence the steady-state level of modified regulatory enzyme. Thus, the activity of an interconvertible enzyme can be smoothly and continuously modulated over a wide range, and these systems facilitate exceptional metabolic flexibility. The key regulatory properties of cyclic cascades have been summarized (Chock et al., 1980), thus: (i) signal amplification, which allows a relatively small fractional activation of the converter enzyme to effect a large change in the fractional modification of the interconvertible enzyme; (ii) the ability to modulate the amplitude of the
interconversion of the target enzyme under saturating conditions of the primary effector, i.e. to
determine the relative proportions of modified and unmodified enzyme; (iii) to modulate the
sensitivity of the interconversion process in response to the concentration of the primary
effector, i.e. cascades are capable of eliciting both positive and negative cooperative effects in
response to increasing concentrations of an allosteric effector; (iv) they can serve as biological
integration systems which can sense simultaneous fluctuations in the intracellular
concentrations of numerous metabolites and adjust the specific activity of the interconvertible
enzyme accordingly; (v) they are extremely flexible systems with respect to allosteric
regulation and are capable of exhibiting, both qualitatively and quantitatively, various
responses to primary allosteric stimuli; and (vi) they serve as rate amplifiers, and are therefore
capable of responding very rapidly (in the millisecond range) to changes in metabolite levels.

1.4 Enzymic hydrolysis of amide bonds

1.4.1 The diversity of amidases

Amidases (amidohydrolases) are enzymes that are able to hydrolyse the amide bonds of
various substrates. In general, enzymes exhibiting amidase activity are quite specific with
respect to the type of amide bonds that they cleave, although in many cases different amidases
show a limited degree of overlapping substrate specificity. However, certain proteases in
particular can attack a very wide range of amide bonds. Very few amidases that are specific
for aliphatic amides have been identified (Clarke, 1970).

Amidases can be classified into broad categories depending on the type of substrate with
which their catalytic activity is maximal (see Maestracci et al., 1988; Clarke, 1970). These
categories include: (i) microbial cell wall hydrolases (exhibiting \(N\)-acetylmuramoyl-\(L\)-alanine
amidase activity), (ii) proteases exhibiting amidase activity towards peptide or amino acid
amide groups (serine proteases, aminopeptidases, asparaginase, glutaminase and \(\alpha\)-amidase),
(iii) acylamide amidohydrolases, (iv) ureases, (v) biotinidases, (vi) nicotinamide deaminases,
(vii) 5-aminovaleramidases, (viii) allantoinase, and (ix) penicillin amidases (see Cole, 1969a,b;
Valle et al., 1991). In addition to their hydrolytic activity, amidases often exhibit acyl
transferase activity, i.e. they are able to catalyse the transfer of the acyl moiety of the amide to
hydroxylamine to form an acyl hydroxamate, and this property of the amidases has been used
in addition to the hydrolase reaction to measure the activity of certain of these enzymes
(Brammar & Clarke, 1964; Kelly & Kornberg, 1964; Clarke, 1970; Thiéry et al., 1986a).

The activities of various amidases (aminopeptidases, cell wall hydrolases, and the \(L\)-\(\alpha\)-
aminoamidase of *Brevibacterium* sp. R312) are sensitive to the concentration of particular
cations (e.g. \(Mg^{2+}\), \(Ca^{2+}\) and \(Zn^{2+}\)) which may exhibit either inhibitory or activatory
influences. However, other amidases (e.g. the 'amidase' of *Ps. aeruginosa*, see below) appear
typically to be subject to activity regulation at the genetic level only (induction versus
repression; catabolite repression). In addition, the inhibition of amidase activity by thiol
reagents suggests that thiol groups are important for the biological activity in a number of
amidases (Asano et al., 1982a,b; Maestracci, et al., 1986; Silman, 1990); indeed a reaction mechanism involving thiol groups has been proposed for the *Brevibacterium* sp. R312 'wide-spectrum' amidase, and thiol groups have also been implicated in aminopeptidase catalytic mechanisms (see Maestracci et al., 1988).

In general, amidases are apparently quite abundant in nature, and more specifically a number of acylamidases have been identified in bacteria, yeasts and fungi (see Thiéry et al., 1986b). Enzymes exhibiting very limited acylamidase activity have also been identified in higher eukaryotes. A number of different bacterial species contain amidases (Maestracci et al., 1988) including Corynebacteria, Mycobacteria, Pseudomonas, Bacillus, Micrococcus, Brevibacterium, Nocardia, Rhodococcus, Streptomyces, Arthrobacter, Rhodococcus, Alcaligenes and Methylophilus. A considerable amount of research time has been directed at the characterization of microbial acylamidases (acylamide amidohydrolase EC 3.5.1.4), particularly enzymes most active towards short-chain aliphatic amides, which catalyse the hydrolysis of the amide to the corresponding organic acid plus ammonia. The remainder of this section will be primarily concerned with this type of amidase.

1.4.2 The amidase of *Ps. aeruginosa*

1.4.2.1 Characterization of amidase activity

The short-chain aliphatic acylamidase of *Ps. aeruginosa* (hereafter referred to as 'amidase') has been the subject of intense investigation since the early 1960's and has been used as a model system for the study of the evolution of enzymes with novel and altered properties (Clarke, 1970, 1984; Clarke & Drew, 1988). Very early work established that the wild-type organism grew well in a mineral salts medium containing acetamide or propionamide as the sole source of C and N by virtue of a discrete amidase that exhibited both amidohydrolase and acyl transferase activities (Kelly & Clarke, 1962; Kelly & Kornberg, 1962a,b, 1964). This enzyme exhibited a narrow hydrolase substrate specificity and rapidly hydrolysed aliphatic amides containing four or less C atoms, viz. propionamide, acetamide, glycollamide and acrylamide. Butyramide and formamide were also hydrolysed albeit at a significantly diminished rate in comparison to propionamide (the best substrate); however, formamide could not be used as a carbon source for growth as *Ps. aeruginosa* cannot assimilate C1-compounds, and acrylamide strongly inhibited amidase activity. Amidase inducer-specificity was demonstrated to be distinct from its substrate specificity, *i.e.* all substrates except butyramide induced amidase (acetamide > propionamide >> formamide), but some N-substituted amides (e.g. N-methylacetamide and N-acetylacetamide) and lactamide acted as gratuitous inducers which provided a tool with which to study the kinetics of amidase synthesis in the absence of substrate metabolism. Acetate also induced amidase activity for reasons that remain obscure although W.J. Brammar (see Clarke, 1970) has suggested that sufficient acetamide can be formed from acetate and ammonia by spontaneous chemical reaction to effect induction under certain conditions. Furthermore, it was also shown that
thioacetamide and cyanoacetamide, which were neither substrates or inducers prevented the induction of amidase by inducers, as did formamide and butyramide (Brammar, 1965). More detailed studies (Brammar & Clarke, 1964) showed that cyanoacetamide repressed amidase synthesis after induction by acetamide or $N$-acetylamidamide. This phenomenon was termed 'amide analogue repression' and was later interpreted in terms of the competition for binding between inducing and repressing amides for the inducer-binding site which was also involved with the initiation of amidase synthesis. It was assumed that this binding site was present on the regulatory protein (AmiR; see below) and that binding of an inducing amide at this site resulted in AmiR adopting an active conformation (Farin & Clarke, 1978). Conversely, the binding of cyanoacetamide and butyramide or other amide analogue repressors to this site was assumed to prevent AmiR from adopting an active regulatory conformation. This study highlighted the similar degree of catabolite repression of amidase synthesis (approximately 60-70%) that was effected by several TCA cycle intermediates and metabolically related compounds (propionate > citrate/malate/pyruvate > acetate), as was relief from this phenomenon by increasing the concentration of the inducer (acetamide). Further work by Brammar et al. (1966) suggested the presence of a constitutive permease responsible for the internalization of amides. This finding apparently precluded the inhibition of amide uptake from involvement with amide analogue repression, and it was concluded from later more detailed studies (Farin, 1976) that there was no requirement for an active amide-uptake system, and no evidence to support the existence of such a system in *P. aeruginosa*.

### 1.4.2.2 Investigation of amidase evolution and regulation through the isolation of mutant strains

The characterization of amidase regulatory properties allowed the design of specific media for the selection of particular types of mutant. Succinate/formamide (S/F) medium was used to select constitutive (regulatory) mutants (Brammar et al., 1966), the rationale being that formamide was both a poor substrate (able to provide an N-source only, as *P. aeruginosa* cannot grow on formate) and a poor inducer, so that if constitutive mutants arose they would be able to outgrow the wild-type since they would not require induction by formamide. In addition, the carbon source, succinate, exerted a strong selection pressure due to its role in catabolite repression. This selection regime yielded three types of regulatory mutants all of which synthesized the wild-type enzyme: (i) magno-constitutive mutants that synthesized high amidase levels independently of inducers, (ii) semi-constitutive mutants with reduced activities that could be elevated to higher levels by induction, and (iii) formamide-inducible strains that had undergone an alteration in inducer-specificity. Many of these mutants were also very sensitive to catabolite repression by succinate, and one mutant could even be repressed by an amide that induced the wild-type strain, suggesting that this mutation changed amidase regulation from being amide-inducible to amide-repressible. Phage transduction studies, in which crosses were made between constitutive or formamide-inducible mutants (donor strains) and amidase-negative mutants (recipient strain; see Skinner & Clarke, 1965), showed...
exceptionally high co-transduction frequencies for the regulator gene (amiR) and the structural gene (amiE) indicating that these two genes were closely linked (Brammar et al., 1967). The generation of these new mutant strains provided additional scope for the selection of extended mutant phenotypes. Large numbers of such mutants were subsequently generated using carefully chosen parent (wild-type or mutant) strains and selective media, and some of the more interesting mutants are described below.

Both mutant C11 (a magno-constitutive strain selected on S/F medium) and the wild-type strain are unable to grow on butyramide, due to the very high $K_m$ for this substrate and to the fact that butyramide represses amidase synthesis. It was suggested that further mutation of strain C11 by chemical-mutagenesis might generate a mutant exhibiting an altered substrate specificity (Brown et al., 1969). The validity of this notion was proven when six mutants were isolated that exhibited good growth on butyramide. These organisms were shown to produce a different type of amidase (B amidase) which exhibited altered kinetic properties (ten-fold lower $K_m$, ten-fold higher $V_{max}$, altered substrate specificity) and electrophoretic properties to the wild-type (A amidase). It was concluded from these results that the double mutation (regulatory plus structural gene mutations) harboured by these strains represented positive evolutionary events as they extended the range of potential growth substrates beyond that of the wild-type. This type of mutant was therefore able to grow on butyramide because the B amidase exhibited kinetic advantages over the A amidase and hence greater activity towards the substrate. In contrast, constitutive mutants that were also resistant to butyramide repression were generated from the same parent mutant strain (C11), which were able to grow on butyramide, but expressed the (wild-type) A amidase. The ability of the mutant to utilize butyramide resulted not from altered kinetic properties, but rather due to the over-expression of the wild-type amidase (in the absence of butyramide repression of amidase synthesis due to a second different regulatory gene mutation) so that despite the low activity of the enzyme towards this substrate the enhanced amidase levels were sufficient to support growth (Brown & Clarke, 1970). Furthermore, one of the mutants expressing B amidase was used to select various mutants that were able to grow on valeramide (V mutants), a substrate on which the wild-type was unable to grow. Many of the V mutants had lost the ability to grow on acetamide and these mutations represented a shift in substrate specificity to organisms expressing optimal activity with longer aliphatic amide side chains. Amidases from these strains were significantly less stable than either A or B amidases, which in conjunction with other evidence suggested that the double structural gene mutation defining the extended substrate specificity of these mutants also conferred a deleterious structural penalty.

The substrate specificity of the amidase was altered still-further by the selection of mutant strains able to use phenylacetamide as an N-source (Ph mutants; Betz & Clarke, 1972) or N-phenylacetamide as a C-source (AI3 amidase; Brown & Clarke, 1972), both of which were inaccessible as substrates for growth by the wild-type. The latter type of mutant was able to use phenylacetamide as a C-source as a result of its constitutivity, which allowed it to produce large quantities of amidase, and due to a second mutation in the structural gene which caused Ile to be substituted for Thr in the enzyme. This substitution abolished the ability of mutant
AI3 to grow on butyramide which suggested that the substitution probably affected the substrate binding residues. Similarly, it was later shown (Paterson & Clarke, 1979) that certain of the B and V mutants contained a Phe residue in place of a Ser residue which probably promoted a minor conformational change in the enzyme; this was plausibly invoked to account for the altered kinetic properties of these mutants. A later series of urea-resistant amidases was also selected from mutant AI3 (Brown et al., 1978), and additional Ph mutants were selected from various different parent strains and characterized. All Ph mutants were regulatory mutants containing at least one structural gene mutation and their mutant amidases were generally unstable. Study of these mutants indicated that a novel (phenylacet)amidase could evolve along several different mutational routes (Clarke & Drew, 1988).

The selection of amidase-negative mutants was first achieved (Skinner & Clarke, 1965, 1968) after chemical mutagenization of the wild-type strain. EMS-treated cells were plated on to mineral salts medium containing acetamide as the major C-source and traces of succinate to enhance growth of mutant colonies. Mutants were identified as minute colonies after growth resulting from the sequestration of acetate from the medium around wild-type colonies. Some of these mutants were defective in enzymes concerning acetate metabolism and others in the amidase structural gene. Other amidase-defective mutants were selected with much more complex phenotypes on media designed to select catabolite-resistant mutants (Brown, 1969; see Clarke, 1970). Amidase-negative mutants were also selected using fluoroacetamide (Clarke & Tata, 1973) and glycollamide as selective agents (Brown & Tata, 1987). The rationale underpinning these positive selection methods involved the inhibition of colony growth by the toxic/inhibitory products of fluoroacetamide and glycollamide hydrolysis, fluoroacetate and glycollate respectively; therefore only amidase-defective mutants exhibiting reduced amidase activity were expected to grow on these media.

The growth of the wild-type organism on lactamide (a poor substrate, but a very good inducer) plus succinate (a strong catabolite repressor) allowed the selection of both inducible and constitutive catabolite-resistant mutants as a result of the high inducing and poor substrate activity of lactamide. However, these mutations were not linked to amiE and amiR (Smyth & Clarke, 1975a). Smyth and Clarke (1975b) predicted that if catabolite repression affected transcription initiation, as in E. coli, then it should be possible to isolate a (new) class of promoter mutants that were resistant to catabolite repression and insensitive to the stimulation of amidase synthesis by c-AMP (see Smyth & Clarke, 1975a,b; Clarke, 1984). This notion was confirmed using a complex selection rationale from which a catabolite repression-resistant 'up-promoter' mutant was isolated. This mutant expressed the wild-type enzyme and it was demonstrated that the mutation which conferred resistance to catabolite repression was co-transduced at a very high frequency with the amiER genes, indicating that the site of the mutation was very close to these genes.

The generation of amidase-negative mutants was of considerable importance to genetic analysis of the amidase regulatory system. Phage transduction studies using amidase-negative recipient strains and carefully-selected donor strains allowed linkage and mapping analysis of wild-type and mutant genotypes conferring altered amidase phenotypes (see Clarke, 1970,
Thus, genetic analysis enabled further dissection of mutant phenotypes and clarified their genetic basis in several cases. That the regulation of amidase synthesis in *Ps. aeruginosa* was under positive control rather than negative control, as described by the Jacob-Monod model of gene expression, was demonstrated by Farin and Clarke (1978). It had been initially assumed that amidase regulation was probably under negative control by a *lacI*-type repressor, so attempts were made to isolate temperature-sensitive mutants that expressed thermolabile repressor proteins that would be inducible at low temperatures, but constitutive at higher temperatures, as for the *lac* system. The search for a mutant expressing this phenotype was unsuccessful, although a large number of mutants were screened. This apparent inability to isolate such a mutant hinted that the amidase was not necessarily under negative control. This prompted a search for a mutant expressing a thermolabile regulator protein which synthesized amidase at lower temperatures, but ceased synthesis at higher temperatures, which would have been indicative of a positive control mechanism. Two such mutants were isolated that were shown to synthesize constitutively the wild-type amidase at 28°C (which proved that the mutant phenotype did not result from a temperature-sensitive amidase), but which ceased amidase synthesis almost immediately after a temperature increase to 41°C; amidase synthesis was resumed upon re-incubation of cultures at 28°C. Since this culture exhibited an amidase that was physico-chemically identical to that of the wild-type, it was concluded that this phenotype resulted from a mutant regulatory protein. Further evidence that a positive amidase control system was operative was furnished by analysis of amidase-negative mutant revertant strains. Positive regulation of amidase was later confirmed (Drew, 1984), and its mechanism (anti-termination of transcription) elucidated in greater detail using a molecular biological approach (Drew & Lowe, 1989), as was the physical association of *amiER* on the *Ps. aeruginosa* chromosome (Cousens & Drew, 1984) and the DNA sequences of the amidase structural gene (*amiE*) (Brammar et al., 1987) and a regulatory gene (*amiR*) (Lowe et al., 1989). Molecular biological studies also indicated the presence of an amidase operon containing other regulatory genes, and was able to infer their likely mode of regulatory action. These studies allowed the integration of information concerning regulation of amidase activity at the molecular level with that determined previously concerning the physiology and genetics of mutant strains (see Chapter 5).

In summary, a vast number of *Ps. aeruginosa* mutants were isolated and characterized. This was achieved after consideration of the desired mutant phenotype in relation to the likely properties of the selective route necessary to generate such mutants. The latter required the careful selection of a parent strain (mutant or wild-type) and its matching to a selective medium capable of exploiting its particular metabolic properties in order to direct the subsequent selection of new mutations. Three basic types of mutants were isolated: (i) regulatory mutants, exhibiting constitutivity, an alteration in inducer-specificity or resistance to catabolite repression; (ii) amidase-negative mutants, exhibiting point mutations or deletions that abolished amidase activity, temperature-sensitive (regulatory) amidase mutants, down-promoter amidase mutants and mutants severely altered in substrate specificity that were...
unable to grow on acetamide, and (iii) mutants with altered enzymes, exhibiting altered
substrate specificities and some of which also contained unstable amidases or amidases that
were resistant to inhibitors of amidase activity.

1.4.3 The amidases of *Brevibacterium* sp. R312

*Brevibacterium* sp. R312 is able to transform a wide range of nitriles into their
corresponding organic acids via the sequential action of a nitrile hydratase which converts the
nitrile into an amide, and an acylamidase that hydrolyses the amide to the organic acid plus
ammonia. That two distinct enzymes catalysed this conversion was demonstrated by the
isolation of two spontaneous mutant strains, one of which had lost the ability to hydrate
nitriles into amides, but retained acylamidase activity, and the other exhibited the reciprocal
phenotype (Thiéry et al., 1986b). Amidase-negative mutants were selected on fluoroacetamide
as described above for *Ps. aeruginosa*. The acylamidase primarily involved in these reactions
was shown to hydrolyse a wide range of amides (aromatic and aliphatic; Thiéry et al., 1986b)
and has been designated the 'wide-spectrum' amidase. The organism also contains an L-\(\alpha\)-
aminoimidase and is thought to harbour several specific enzymes for the hydrolysis of urea,
formamide, nicotinamide and L-glutamine (Maestracci et al., 1988).

The wide-spectrum amidase was constitutive at a low-level and further-inducible by
acetamide and methylacetamide only, which was in marked contrast to the acylamidase of *Ps.
aeruginosa* (see above); it was also repressed by organic acids, but not by ammonia or
glucose. Acetamide, propionamide and butyramide could be used by this organism as its sole
C and N-source, and this enzyme also exhibited acyl transferase activity. Furthermore,
Mayaux et al. (1990) have concluded on the basis of the exceptional biochemical similarities
between *Brevibacterium* sp. R312 and *Rhodococcus* sp. strain N-774 that these organisms are
probably identical. The physico-chemical and molecular biological properties of this
acylamidase are further described in Chapters 3 and 5 respectively.

1.4.4 The amidases of *M. smegmatis*

Draper (1965, 1967) investigated the amidase activity of *Mycobacterium smegmatis* whole
cells and cell-free extracts and demonstrated that this organism grew well on acetamide as sole
C and energy source in minimal medium, and that acetamide, butyramide and acetate (as for
*Ps. aeruginosa*) induced the synthesis of an aliphatic acylamidase(s). *N*-methylacetamide and
*N*-acetylacetamide acted as gratuitous inducers of aliphatic acylamidase activity (also as
for *Ps. aeruginosa*). Cell extracts were active towards a wide range of substrates including
acetamide, propionamide, formamide, glycollamide, *n*-butyramide and acrylamide, but activity
was approximately 40-fold higher with formamide than with the next best substrate (*n*-
butyramide). The simplest interpretation of results was that two distinct enzymes gave rise to
the observed profile, one of which was specific for formamide (*i.e.* a formamidase) whilst the
other exhibited a broader specificity and was maximally active with *n*-butyramide. However, a
partial (2.2-fold) purification of this amidase activity did not separate formamidase and
butyramidase activities, and both activities responded similarly to pH variation, heat-treatment
and exposure to dithio-bis-nitrobenzoic acid. The cloning and DNA sequencing of an
'acetamidase' from *M. smegmatis* has recently been reported (Mahenthiralingam *et al.*, 1993;
see Chapter 7), but the substrate profile of this enzyme has not been reported.

*M. smegmatis* also exhibited acyl transferase activity which was catalysed by an enzyme
distinct from that responsible for the hydrolase activity, and benzamidase activity was also
evident; induction of the latter varied independently of the aliphatic acylamidase(s), suggesting
that a discrete aryl acylamidase(s) was expressed by this organism.

### 1.4.5 The amidases of *M. methylotrophus*

#### 1.4.5.1 Introduction

Previous studies have shown that *M. methylotrophus* contains a short-chain aliphatic
referred to as 'acetamidase'), a formamidase (formamide amidohydrolase EC 3.5.1.49) (D.J.
Scherr, N.J. Silman, & C.W. Jones, unpublished; Wyborn *et al.*, 1994). A urease and
possibly a fourth amidase exhibiting activity towards butyramide have also been detected.

#### 1.4.5.2 Regulation of acetamidase activity: the discovery of 'switch-off'

The conditions under which induction and repression of acetamidase occurred in *M.
methylotrophus* were defined by ZENECA Bio Products in pilot studies geared towards the
commercial production of this enzyme (see below). This enzyme was induced by acetamide
and acrylamide and repressed by ammonia. Acetamidase was found to be maximally induced
under nitrogen-limited (acetamide-limited) growth conditions, but with carbon in excess the
organism synthesized sufficient amounts of a viscous exopolysaccharide to render this
method uneconomical for the commercial production of acetamidase. Similarly, growth of *M.
methylotrophus* under carbon-limited growth conditions, with acetamide as nitrogen source,
was unsatisfactory as acetamidase synthesis was subject to strong repression (intimating that
under these conditions repression by ammonia was dominant to induction by acetamide) and
was present therefore only at a very low level in these cells. Growth of the organism under
dual methanol-acetamide limitation produced cultures exhibiting maximal derepression of
acetamidase synthesis, but the whole cell specific activity was unexpectedly low in comparison
to the activity expressed by cultures grown either acetamide-limited or methanol-limited with
acetamide supplied as nitrogen source. Analysis of the acetamidase from low-activity cells
showed that the reduction in whole cell specific activity was due to the synthesis of an
acetamidase with a significantly decreased *K*_**<sub>cat</sub>** for acetamide in comparison to the enzyme
synthesized by amide-limited cultures (< 45 s<sup>-1</sup> cf. 128 s<sup>-1</sup>; see Carver & Jones, 1993). It was
concluded that growth of *M. methylotrophus* under conditions of dual carbon-nitrogen
limitation enforced the down-regulation of high-activity acetamidase which was mediated by a
cryptic biochemical process. This anomalous reduction in specific activity was termed 'switch-off' by analogy to the work of Zumft and Castillo (1978) who were the first workers to coin this phrase in relation to the inhibition of nitrogenase activity by ammonia \textit{in vivo}, and it was suggested that the mechanism of switch-off of acetamidase might also involve post-translational modification of high- or low-activity acetamidase as was evident for nitrogenase (Ludden & Roberts, 1989). In a manner consistent with the new terminology cells grown under amide-limitation exhibiting high-activity acetamidase were termed 'switched-on' and this phrase was generally used to describe whole cell specific activities of approximately 1.5-2.0 μmol min\(^{-1}\) (mg dry wt cells\(^{-1}\)) in contrast to the switched-off activity of approximately 0.5 μmol min\(^{-1}\) (mg dry wt cells\(^{-1}\)). Hence the enzymes later purified from switched-on and switched-off cells, respectively exhibiting specific activities of 49.52 and 15.02 μmol min\(^{-1}\) (mg protein\(^{-1}\)) as reported by Silman (1990) and Silman et al. (1989, 1991) became known colloquially as 'switched-on amidase' and 'switched-off amidase'. The further serendipitous observation that (switched-off) low-activity cells exhibited an increase in specific activity of up to 5000% after a period of heating at 60°C ('heat-reactivation'; M.A. Carver & J. Hinton, personal communication; Carver & Jones, 1993) allowed the large-scale continuous culture of the organism under dual methanol-acetamide limitation at high cell density to be adopted as the basis for the commercial production of acetamidase.

More recent work concerning growth of \textit{M. methylotrophus} in continuous culture at low cell density under true dual methanol-acetamide limitation (under conditions in which no ammonia was detectable in culture supernatants; J. Mills & C.W. Jones, unpublished; and where it was also assumed that no acetate was present in culture supernatants [M.A. Carver, personal communication]) showed that these cultures exhibited fully derepressed acetamidase synthesis and a fully switched-on whole cell specific activity equivalent to that of an identical low cell density culture grown under amide-limitation. This finding was in marked contrast to the switched-off activities of dual C/N limited cultures grown by ZENECA Bio Products. Further work with low cell density dual C/N limited cultures showed that the high acetamidase activity of these cultures could be transiently switched-off by pulsing a relatively high concentration of exogenous ammonia into the culture (J. Mills & C.W. Jones, unpublished), such that the final ammonia concentration in the culture was 2mM, in an essentially identical fashion to that described by Carver and Jones (1993). Scrutiny of the industrial-scale 'dual' methanol-acetamide limitation growth regime shows that it is, in fact, not strictly dual-limited. It is more accurately described as pseudo-dual limitation, in contradistinction to the true dual C/N limitation evident in low cell density cultures, as a result of the system devised to manage the accumulation of potentially toxic acetate in industrial culture vessels (see below).

Acetate and ammonia are the end-products of the hydrolysis of acetamide catalysed by acetamidase, and it is known that \textit{M. methylotrophus} cannot utilize acetate as its sole carbon and energy source (Carver & Jones, 1993). However, the conspicuous absence of acetate in culture supernatants of this organism grown under acetamide-limitation or true dual methanol-acetamide limitation at low cell density indicated that this organism expresses a limited ability
to metabolize acetate, otherwise this compound would be expected to be discharged into the
growth medium. It has been shown previously that M. methylotrophus converts acetate to
acetyl CoA using its acetyl CoA synthetase (Lloyd, 1990), but lacks a complete TCA cycle.
The overall biochemical mechanism by which acetate is cleared from cultures of M.
methylotrophus, including the metabolic fate of acetyl CoA therefore remains obscure. Acetate
spillage into the culture originally presented a problem to the industrial-scale culture of this
organism at high cell density. Acetate appeared in the culture medium at a time presumably
corresponding to the saturation of the organism's limited acetate-clearing potential and after
this time the concentration of acetate in the culture increased rapidly as a consequence of
continued acetamide hydrolysis. Silman (1990) and Silman et al. (1989) showed that
extracellular acetate concentrations in the range 30-40 mM exerted a toxic effect on the culture
(by a mechanism which is not yet understood), causing depression of \( \mu_{\text{max}} \) below the
imposed dilution rate and the eventual resultant culture wash-out. The problem of acetate
toxicity in high cell density continuous culture was circumvented by alternating acetamide and
ammonia (or urea) as nitrogen source. Cultures were maximally derepressed by growth under
dual methanol-acetamide limitation until acetate had accumulated to a concentration slightly
less than that necessary to adversely affect the growth rate of the culture. At this point the
acetamide supply was replaced by ammonia in such a way that the extracellular ammonia
concentration experienced only minor fluctuations. During this time acetate levels were found
to decay to sub-toxic levels at a rate faster than could be accounted for by simple dilution
from the culture and this suggested that the culture was metabolizing acetate. An upper limit
on the replacement of the acetamide feed by ammonia of approximately 2 h before the
reintroduction of acetamide as nitrogen source acceptably minimized repression of
acetamidase synthesis without concomitant deleterious effect on either culture acetamidase
activity or biomass (Carver & Jones, 1993). The initiation of the switch-off of acetamidase
activity in pseudo-dual C/N limited cultures was found to correlate with the replacement of
acetamide as nitrogen source by ammonia which occurred in the presence of high acetate
concentrations. As stated above, the extracellular ammonia concentration remained
approximately constant during this time at a level insufficient to repress acetamidase.
Similarly, switch-off was demonstrated in low cell density cultures grown under true dual
methanol-acetamide limitation by either transiently adding exogenous ammonia to the culture
(see above) or by replacing the acetamide feed with a limiting supply of ammonia (J. Mills &
C.W. Jones, unpublished). Acetate levels were presumably negligible under both sets of
conditions in addition to the observed absence of detectable levels of ammonia in the culture
supernatants.

Interpretation of these findings with respect to identification of the signal(s) that trigger
switch-off of acetamidase activity is difficult. The fact that switch-off correlates with the
cessation of supply of the inducer (acetamide) coupled with its replacement by the repressor
(ammonia) as nitrogen source may in itself be of significance. It appears feasible that a
threshold ammonia concentration exists, above which switch-off can occur, but this level is not
necessarily detectable by routine ammonia assays. This can be deduced from experiments in
which switch-off occurred under conditions of either no detectable ammonia in culture supernatants (after transition of a culture growing at low cell density under true dual methanol-acetamide limitation to true dual methanol-ammonia limitation) or in which ammonia was at relatively high levels in culture supernatants (ammonia-pulsed low cell density true dual methanol-acetamide limited cultures). Both sets of experimental conditions elicited switch-off and both maintained at least a minimum supernatant ammonia concentration which might correspond to the critical threshold value, although in the latter experiment ammonia concentration was much higher than in the former experiment. It is also potentially noteworthy that the switch-off conditions of the former experiment exhibit the greatest similarity to the conditions under which switch-off occurs in pseudo-dual C/N limited cultures.

Discussion of how switch-off occurs so far has largely ignored the potential of acetate to influence the level of switch-off. It is feasible that there exists a critical acetate threshold concentration above which acetate levels predispose cultures to switch-off and that either independently of the effect of ammonia, or in coupling with it, the occurrence and magnitude of switch-off are governed. This putative effect of acetate is plausible in view of the fact that under pseudo-dual C/N limitation when acetate levels are high relative supernatant ammonia concentrations are low; conversely, acetate levels are negligible when an ammonia-pulse into a dual methanol-acetamide culture effects switch-off. Thus, the main differences between high cell density pseudo-dual methanol-acetamide limited cultures and identical low cell density true dual limited cultures, respectively expressing low- and high acetamidase activities, appears to be the periodic removal of the inducer (acetamide) and its replacement as nitrogen source by the repressor (ammonia) in addition to fluctuating levels of potentially toxic acetate in the former growth regime. In the absence of information concerning all culture conditions under which switch-off can occur, including knowledge of the putative critical concentrations of acetate and ammonia effecting switch-off, the cause of switch-off remains unclear. It seems most likely, however, that switch-off is mediated in response to a fluctuating intracellular C/N ratio that might be sensed through the ambient concentrations of acetate and/or ammonia.

1.4.5.3 Isolation of M. methylotrophus acetamidase mutants

Mutant acetamidase strains have been described previously for M. methylotrophus (Silman, 1990; Silman et al., 1989, 1991). The technique of directed evolution was used to select spontaneous and chemically-induced mutants after acetamide- and acrylamide-limited growth in continuous culture at low-dilution rate. Initial growth of this organism under acetamide-limited led to the selection of an apparently genetically stable population of organisms (culture A) that exhibited high acetamidase activity. This culture was shown to harbour significant genetic diversity with respect to induction of acetamidase by acetamide, and a single bacterial colony isolated from it, mutant MM6, was chosen for further investigation. MM6 was then sequentially mutated to yield the (spontaneous) mutant MM8, which in turn was chemically mutagenized and used to select a third mutant strain, MM15, both after
acrylamide-limited growth in continuous culture at 37°C. Mutant characterization showed that both MM6 and MM8 possessed enhanced acetamidase activities due to over-expression of the enzyme; in addition, MM8 exhibited a substantially higher $K_{cat}$ and a reduced $K_m$ for acrylamide in comparison to either MM6 or the wild-type high-activity acetamidase (switched-on acetamidase). Strain MM15 also over-produced acetamidase, but showed an unexpectedly low acetamidase activity coupled with a greatly diminished $K_m$ for acrylamide. Further investigation of mutant acetamidase properties showed that all of the purified enzymes possessed similar $K_m$ values for acetamide (1.2-1.7 mM cf. 1.1mM for the wild-type enzyme), but more diverse affinities for acrylamide (2.1-19.0 mM cf. 16.1mM for the wild-type enzyme). Isoelectric focusing of mutant acetamidases showed that all exhibited isoelectric points identical to the wild-type enzyme (pI = 4.15 ± 0.05), whereas thermostabilities at 60°C were variable with the MM6, MM8 and MM15 enzymes respectively possessing $t_{1/2}$ values of 4.3 h, 1.5 h, and 0.6 h cf. 3.2 h for the wild-type high-activity enzyme. Thus, the MM6 and MM8 enzymes were significantly more and less stable, respectively, than the wild-type enzyme, whereas the MM15 enzyme was very thermolabile. Additionally, a single freeze-thaw cycle was shown to inhibit activity by < 1% for MM6, MM8 and the wild-type high-activity enzymes in comparison with 85% inhibition for the MM15 enzyme. These results suggested that the MM15 enzyme was much more unstable than the other enzymes, and this was confirmed by showing that the loss of activity at 60°C resulted from dissociation of the tetramer into dimers and thence into monomers, and that the greatest degree of dissociation was associated with the MM15 enzyme. Incubation of mutant enzymes with DTNB showed 15-18% inhibition of activity for MM6, MM8 and the wild-type high-activity enzymes purified from cells grown at 37°C, as did the MM15 enzyme (18% inhibition) purified from cells grown at 25°C; however, the level of inhibition increased to 35% for the MM15 enzyme when isolated from a culture grown at 37°C. Spectrophotometric analysis of the reaction of DTNB with MM6, MM8 and wild-type enzymes (37°C), and MM15 (25°C & 37°C) enzymes, showed that DTNB reacted with twice as many cysteine residues in the MM15 (37°C) enzyme only, suggesting that the MM15 acetamidase existed in a slightly looser tetrameric conformation when the organism was grown at 37°C. This hypothesis was supported by thermostability and DTNB inhibition results, and was confirmed by sedimentation velocity ultracentrifugation analysis which showed that corrected S-values were very similar for MM6, MM8 and the wild-type enzymes (37°C), and the MM15 enzyme (25°C), whereas the MM15 (37°C) enzyme sedimented significantly more slowly indicating that in view of its identical MW to the wild-type enzyme it must have been a spatially larger molecule. The MM15 (37°C) enzyme also exhibited a more relaxed substrate specificity which is in keeping with a more open tetrameric structure. Analysis of the effect of growth temperature (25°C & 37°C) on culture acetamidase activity revealed that the unexpectedly low activity of MM15 cultures grown at 37°C was due to the enhanced thermostability of the mutant enzyme at this temperature, such that approximately 80% of the acetamidase existed as inactive monomers. Since mutant MM15 rapidly outgrew mutant MM8 in continuous culture at 37°C, it is clear that under the prevalent growth
conditions the selective advantage conferred by its low $K_m$ favourably offset the deleterious effects of its enhanced thermolability. It was concluded that the molecular alterations encoding the mutant properties of *M. methylotrophus* strains MM6, MM8 and MM15 were analogous with those of *Ps. aeruginosa*, and probably resulted from amino acid substitution(s).

1.5 The commercial exploitation of acetamidase for bioremediation purposes

The commercial exploitation of acetamidase for bioremediation purposes

The development of a single cell protein (SCP) protein product (Pruteen) based on the biomass of *M. methylotrophus* by ZENECA (formerly known as ICI Biological Products) was a major (bio)technological success in the purest sense, although as a commercial venture it ultimately failed (see Sharp, 1989; Vasey & Powell, 1984). During this time intense biochemical and toxicological profiling of this organism was undertaken, and the maintenance of this organism in sterile industrial-scale continuous culture over long periods of time was demonstrated - a remarkable feat of bio-engineering. The experience gained with continuous culture from the Pruteen process contributed to the successful development of the acetamidase (which is actually more active towards propionamide and acrylamide as substrates than towards acetamide; Chapter 3) of *M. methylotrophus* as a bioremediation catalyst for the removal of residual acrylamide (which is a potent neurotoxin and suspected carcinogen) from polyelectrolyte flocculants used in the conditioning of potable water, amongst other applications (Carver & Jones, 1993).

1.6 Objectives

This thesis describes (i) biochemical investigations of the *M. methylotrophus* acetamidase in an attempt to elucidate the nature of the putative modification that mediates reversible *in vivo* regulation of this enzyme, and (ii) biochemical and molecular biological investigations of the *M. methylotrophus* formamidase in order to compare its properties with those of the acetamidase and those of other bacterial amidases.
CHAPTER 2

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MATERIALS AND METHODS

2.1 Growth and maintenance of bacterial cultures

2.1.1 Bacterial strains

*Methylophilus methylotrophus* (NCIB 10515) was kindly provided by ZENECA Bio Products, Billingham, Cleveland, TS23 1YN (formerly known as ICI Biological Products). *Pseudomonas aeruginosa* EF2 and *Escherichia coli* JM109 were gifts from Dr. E.J. Gilbert and Dr. S.G. Williams, respectively.

2.1.2 Preparation of bacterial stocks and growth on solid media

Stock (reference) cultures of *M. methylotrophus*, *E. coli* JM109, and *P. aeruginosa* EF2 were prepared from overnight batch cultures and stored in sterile 15% (v/v) glycerol in deionized water (Elgastat Option 2 water purification unit, Elga Ltd., UK) at -20°C or -70°C (Maniatis *et al.*, 1982).

Cultures of *M. methylotrophus* were routinely maintained on solid methanol-minimal salts (SE II) agar medium containing either acetamide or ammonia as nitrogen source. SE II medium was prepared by dissolving the following in deionized water (1l): K$_2$HPO$_4$, 1.9g; Na$_2$HPO$_4$, 1.56g; MgSO$_4$$\cdot$7H$_2$O, 20mg; FeCl$_3$$\cdot$6H$_2$O, 9.7mg; Fisons Trace Elements Solution (Cu, 5ppm; Mn, 24-25 ppm; Zn, 22-23 ppm; Ca, 720ppm), 1ml; Oxoid purified agar, 15g; and (NH$_4$)$_2$SO$_4$, 1.85g l$^{-1}$ (omitted if acetamide was supplied as nitrogen source). After adjusting the pH to 7.2 with KOH the medium was sterilized by autoclaving (121°C, 15 min) and subsequently cooled (to minimize loss of methanol through vaporization) to approximately 48°C, prior to the aseptic addition of methanol, 5ml, and acetamide solution (1.18g dissolved in a small quantity of deionized water). Both methanol and the acetamide solution had been sterilized previously by filtration through disposable Acrodisc syringe filter units (0.2μm pore size, Gelman) into sterile plastic universal tubes. The medium was mixed thoroughly to ensure homogeneity prior to pouring.

*E. coli* JM109 was routinely propagated on glucose-M9 mineral salts-agar plates (Maniatis *et al.*, 1982) containing in 1l: Na$_2$HPO$_4$, 6g; KH$_2$PO$_4$, 3g; NaCl, 0.5g; and NH$_4$Cl, 1g. The medium pH was adjusted to 7.4 with KOH before the addition of Oxoid purified agar, 15g, and was subsequently autoclaved (121°C, 15 min) and cooled as above. The following filter-sterilized solutions in deionized water (sterilized by Acrodisc treatment as above) were added to the cooled medium: 1M-MgSO$_4$, 2ml; 20% (w/v) D-glucose, 10ml; and thiamine-HCl (10mg ml$^{-1}$), 1ml. Strains of *E. coli* JM109 transformed with plasmid pUC19 or recombinant pUC19 derivatives (section 2.20) were grown on glucose-M9 mineral salts-agar plates supplemented with ampicillin (amp) (50μg ml$^{-1}$, added after the medium was cooled). On certain occasions, as indicated in the text, acetamide was included in the cooled medium after
autoclaving (filter-sterilized 10M-acetamide solution in deionized water, 2ml) either in addition to, or as replacement for NH$_4$Cl, as potential nitrogen source. Similarly, when required, IPTG (1M-IPTG solution in DMF, 300μl) was incorporated into cooled M9 medium in conjunction with the overlaying of each solidified plate with X-gal (3% [w/v] solution prepared fresh in DMF; 50μl dried onto the surface of each plate). This latter procedure facilitated blue/white selection of clones harbouring recombinant plasmids (section 2.20).

Ps. aeruginosa EF2 was grown on solid Luria-Bertani (LB) medium (Maniatis et al., 1982), or on nutrient agar containing in 11: Oxoid nutrient broth, 15g, and Oxoid bacteriological agar (agar No.1), 15g. All bacteria cultured on solid media were incubated at 37°C and subsequently stored at 4°C to prevent further growth. Cultures of M. methylotrophus produced in this way remained viable for approximately 2 weeks only; E. coli JM109 and Ps. aeruginosa EF2 were still viable after approximately 8 weeks.

2.1.3 Growth of bacteria in batch culture

M. methylotrophus was cultured in batch using a two-step procedure: a sterile 5ml aliquot of minimal salts (SE II) medium containing (NH$_4$)$_2$SO$_4$ (3mM), and methanol (0.5% [v/v]), as nitrogen and carbon sources respectively, was aseptically inoculated with a single colony of M. methylotrophus in a 50ml flask and incubated for approximately 8 h at 37°C, with rotary shaking at 150 rpm. This starter culture was then used as an inoculum for a 150ml batch culture, grown overnight in a 500ml baffled flask, of the same medium composition as above except that the methanol and (NH$_4$)$_2$SO$_4$ concentrations were increased to 1% (v/v) and 14mM, respectively.

E. coli JM109, and Ps. aeruginosa EF2 were grown in batch cultures of variable volume and medium composition. Media employed for liquid growth included nutrient broth, LB medium, Terrific Broth (prepared as follows: to 900ml of autoclaved base broth [bacto-tryptone, 12g; bacto-yeast extract, 24g; glycerol, 4ml; made up to 900ml with deionized water] add 100ml of a sterile solution of 0.17M-KH$_2$PO$_4$ and 0.72M-K$_2$HPO$_4$; Tartof & Hobbs, 1987; section 2.16.2) and M9 medium depending on the proposed use for such cells. Growth medium of sufficient volume to yield the required biomass was sterilized in a baffled flask of sufficient capacity to maintain adequate aeration of the culture, which was incubated in all cases at 37°C, with rotary agitation at approximately 150 rpm. Medium constituents refractory to autoclaving were added to the medium prior to sterilization whereas potentially thermolabile constituents such as glucose, amides, ampicillin, thiamine-HCl and IPTG were filter-sterilized as above, and added aseptically to cooled sterilized medium to give the same final concentrations as for solid M9 media. Specific information concerning actual medium composition and volume, for a given experimental batch application, is given under the relevant section of this chapter. All cultures were inoculated with single bacterial colonies and grown to mid-/late log-phase (section 2.2).
2.2 Determination of bacterial cell density

Measurement of bacterial cell density was determined spectrophotometrically using a Pye Unicam SP600 UV spectrophotometer (4ml disposable cuvettes, 1cm pathlength). Silman (1990) showed that for cell suspensions of M. methylotrophus the OD<sub>600</sub> was a linear function of cell density up to an optical density ≥ 1.0. The conversion factor (0.53) derived from this data was used to calculate cell density (mg dry wt ml<sup>-1</sup>) from the relationship OD<sub>600</sub> x 0.53. Similarly, Ps. aeruginosa EF2 and E. coli JM109 cell densities were respectively calculated from OD<sub>600</sub> x 0.7 (Gilbert et al., 1991) and OD<sub>650</sub> x 0.63 (Pye Unicam SP1800 Ultraviolet spectrophotometer; 1ml disposable cuvettes, 1cm pathlength; Sudjadi, 1991).

2.3 Preparation of cell suspensions and sub-cellular extracts

2.3.1 Preparation of cell suspensions

Cells grown in batch were harvested by centrifugation either in an MSE High Speed 18 centrifuge at 10,000 rpm (12,200 x g) for 10-15 min, or in Eppendorf microfuge tubes using a bench-top MSE MicroCentaur microfuge at full speed (13,000 rpm; 13,400 x g) for at least 1 min. Supernatants were carefully decanted from the cell pellet and either discarded or stored on ice, as required. Cell pellets were resuspended and homogenized in an appropriate volume of the correct ice-cold buffer (generally 0.1M-citric acid/0.2M-Na<sub>2</sub>HPO<sub>4</sub>, pH 6.0 or 20mM-bis-Tris, pH 6.8) and re-centrifuged. This washing step was repeated whenever necessary. Finally, the resultant cell pellet was resuspended to the desired cell density in buffer, and the sample either stored on ice or at -20°C until required.

2.3.2 Preparation of cell-free extracts

Cells destined for breakage by ultrasonication were harvested, washed (section 2.3.1) and resuspended to a final cell density of 5mg dry wt ml<sup>-1</sup> in 0.1M-citric acid/0.2M-Na<sub>2</sub>HPO<sub>4</sub>, pH 6.0 buffer. Resuspended cells were disrupted on ice with 4-6 x 30 s sonication periods (10-12 μm amplitude), interspersed with alternate cooling periods, in an MSE Soniprep 150 ultrasonic disintegrator. Alternatively, cells were resuspended to a cell density of 20-50 mg dry wt ml<sup>-1</sup> in 20mM-bis-Tris, pH 6.8 buffer and broken by passage 4-6 times through an Aminco-French pressure cell at 15,000 p.s.i. Unbroken cells and particulate cell matter from the pressure cell were separated from the soluble fraction of the cell extract by centrifugation at 18,000 rpm (40,000 x g) for 20 min. The supernatant from this spin was carefully decanted from the pellet and stored on ice or at -20°C until required.
2.3.3 Preparation of a high-speed supernatant fraction

Cell-free extracts (section 2.3.2) in 20mM-bis-Tris, pH 6.8 buffer were further clarified by differential centrifugation at 44,000 rpm (175,200 × g) for 75 min in a Sorvall ultracentrifuge OTD 65B (DuPont Instruments) to remove cell membranes and other fine particulate cell debris. The resultant high-speed supernatant was carefully drawn off from the pellet with either a Pasteur or Gilson pipette, exercising caution not to disrupt the pellet surface, and either stored at 4°C or at -20°C until required.

2.4 Measurement of the kinetic properties of amidases

2.4.1 Amidohydrolase activity

Amidohydrolase activities were calculated by measuring the initial rate of release of ammonia from amide catalyzed by acetamidase or formamidase at 37°C. This rate was shown to be linear for at least the first 20 min of the reaction with high-activity acetamidase and formamidase. In contrast to the high-activity form of the enzyme low-activity acetamidase was found to exhibit apparently hysteretic kinetics over the first 0.5-2.0 min of the reaction (N.R. Wyborn, unpublished; Carver & Jones, 1993; Chapter 4) after which time the rate of ammonia release became linear as described above. Amidase specific activities were calculated using the linear portion of plots of \( A_{630} \) versus time (min) as described below. This reaction can be represented by the following general equation:

\[
R\cdot\text{CO.NH}_2 + H_2O \rightarrow R\cdot\text{COO}^- + \text{NH}_4^+
\]

The ammonia concentration was measured colorimetrically using a phenol/hypochlorite/nitroprusside assay (M.A. Carver, unpublished) modified from that of Muftic (1964).

The actual composition of the reaction mixture varied depending on the nature of the assay, and the source of amidase activity. A typical reaction mixture contained a volume of prewarmed 0.1M-citric acid/0.2M-Na\(_2\)HPO\(_4\), pH 6.0 buffer such that the final volume of the reaction mixture (buffer plus cells or pure protein) after substrate addition was always 1ml. When pure proteins were assayed, approximately 5-10 µg of high-activity acetamidase, or 20-30 µg of low-activity acetamidase were used; formamidase assays used only 4-6 µg of protein, and all pure proteins were generally added to the reaction mixture in ≤ 100µl buffer. When washed whole cells were assayed the reaction mixture typically contained 0.1-0.2 mg dry wt cells (M. methylotrophus) or 0.3mg dry wt cells (E. coli JM109::pNW3; Chapter 6). Washed whole cells were supplied in ≤ 250µl prewarmed 0.1M-citric acid/0.2M-Na\(_2\)HPO\(_4\) buffer, pH 6.0. In all assays the reaction mixture was prewarmed (37°C, 3 min) to equilibrate the mixture prior to substrate addition. The reaction was started by adding 50µl of 1.0M-acetamide or 1.0M-formamide (final reaction concentration, 50mM; Sigma), or 100µl of
1.0M-acrylamide (final reaction concentration, 100mM; x 4 recrystallized, Serva) to the assay tube at 37°C and mixing. Aliquots (100μl) were withdrawn at timed intervals (1-2 min; full assay time course, 5-10 min) into 1ml of sodium phenoxide/acetone reagent (0.675M-NaOH; 0.064M-phenol; 0.136M-acetone in water; Fisons) which immediately stopped the reaction. The colour was developed by adding 1.5ml of 0.01% (w/v) sodium nitroprusside (Fisons, AR grade) in 0.1M-sodium acetate/0.08M-HCl, pH 6.0 buffer, and 1.5ml of 0.5% (v/v) 'available chlorine' sodium hypochlorite solution (diluted in the same buffer from Spectrosol 12% 'available chlorine' sodium hypochlorite solution; BDH) to each tube and mixing. The tubes were stored in the dark for a minimum of 30 min at room temperature, during which time a green/blue coloured complex developed that was stable for at least several hours. The concentration of ammonia in each assay tube was determined spectrophotometrically by measuring $A_{630}$ (Pye Unicam SP600 UV spectrophotometer; 4ml disposable cuvettes, 1cm pathlength) of the coloured complex and extrapolating from a standard curve which was freshly prepared daily. The standard curve was constructed by dispensing 10μl aliquots from each of several stock NH$_4$Cl solutions (made up in 0.1M-citric acid/0.2M-Na$_2$HPO$_4$, pH 6.0 buffer), containing 0-0.15 μmol NH$_4^+$, into tubes containing buffer and sodium phenoxide/acetone reagent. Standards were developed immediately, as above, and the curve was shown to be linear over this range. Supernatant (section 2.3.1) ammonia concentrations were measured using the same procedure.

Suitable controls (buffer plus cells/enzyme, minus substrate; buffer plus substrate, minus cells/enzyme) were used to correct for ammonia carry-over with cells and spontaneous autohydrolysis of certain amides (particularly propionamide, formamide, and acetamide), which was occasionally a problem with older stock solutions. When deemed necessary, further controls were used to correct for the possible interference with the ammonia assay by other chemicals, such as bis-Tris. All samples, test and control, were of the same final volume.

Occasionally, a discontinuous amidase assay was run, essentially as shown above, except only two aliquots were sampled from the reaction mixture; one immediately after substrate addition, and a second 2-30 min later. This assay method was primarily used as a tool to identify samples containing amidase activity; for example, peak-activity FPLC fractions during protein purification (section 2.6).

2.4.2 Acyl transferase activity

Acyl transferase activity of washed whole cells of *M. methylotrophus* and putative *E. coli* JM109 recombinant clones potentially expressing acetamidase (Chapters 5) was measured colorimetrically, essentially according to the method of Brammar and Clarke (1964), as the initial rate of production of acetohydroxamate resulting from the transfer of the acyl moiety of acetamide to hydroxylamine. This reaction can be described by the following general equation:

\[
R.C.O.NH_2 + NH_2OH + H^+ \rightarrow R.C.O.NH.OH + NH_4^+
\]
Assay reagents were prepared freshly each time the assay was performed by mixing equal volumes of solutions of 200mM-acetamide and 500mM-hydroxylamine in 100mM-Tris-Cl, pH 7.0 buffer and adjusting the pH of the mixture to 6.0. The assay mixture (final volume, 1ml) typically contained 900μl of buffered acetamide/hydroxylamine solution, pre-equilibrated at 37°C. The reaction was started by addition of cells to the assay mixture; 50μg dry wt cells (M. methylotrophus) or approximately 100μg dry wt cells (E. coli JM109), supplied in 100μl of buffer (0.1M-citric acid/0.2M-Na₂HPO₄, pH 6.0). In discontinuous assays, the reaction was stopped after an appropriate incubation period (0, 30, 60, or 90 min), by the addition of 2ml of 6% (w/v) ferric chloride in 2% (v/v) HCl in deionized water, which also developed the red/brown-colour of the ferric-hydroxamate complex whose absorbance at 500nm (A₅₀₀) was measured in the assay. Alternatively, 4 identical assay mixtures were incubated, as above, and the reactions stopped sequentially after the time intervals previously indicated. The A₅₀₀ was read immediately (Pye Unicam SP600 spectrophotometer) against a reagent blank (900μl of buffered substrate solution diluted to 1ml with 0.1M-citric acid/0.2M-Na₂HPO₄, pH 6.0 buffer, developed immediately, without incubation at 37°C) and corrected for the spontaneous reaction between acetamide and hydroxylamine at 37°C by developing the colour of a reagent blank incubated under the same conditions as the test samples. Sample A₅₀₀ values were also corrected for absorbance due to bacterial cells in the assay mixture whenever appropriate.

A standard curve was prepared whenever necessary using acetohydroxamic acid (Sigma). Aliquots were taken from standard solutions (made up in 100mM-Tris-Cl, pH 6.0 buffer) containing 0-2 μmol of acetohydroxamate and diluted to a final volume of 1ml. Standards were developed immediately and the curve was shown to be linear over this range.

2.4.3 Measurement of the reactivation of low-activity acetamidase by activator component

Reactivation of low-activity acetamidase was measured specifically as indicated in the text for each reactivation experiment. All reactivation experiments were carried out using one of the general procedures outlined below, modified from the reactivation method described by M.A. Carver and J. Hinton (1987), (M.A. Carver, personal communication). Acetamidase/acrylamide and formamide, for acetamidase and formamidase, respectively, were used as substrates in all assays unless otherwise stated.

Washed whole cells of M. methylotrophus (section 2.3.1) were diluted in 20 mM-bis-Tris, pH 6.8 buffer to approximately 0.5mg dry wt ml⁻¹ and the amidohydrolase activity of an appropriate-sized aliquot was assayed immediately at 37°C (section 2.4.1). The remainder of the diluted cell sample was incubated in a water bath at 60°C for up to 6 h (typically 1 h), and reassayed after mixing and cooling the sample on ice (4°C, 5 min). Pure low-activity acetamidase was mixed with a source of activator component (either a high-speed supernatant [section 2.3.3], or an aliquot of partially-pure activator component [section 2.6.3]), containing a known quantity of protein; a typical reactivation assay contained
a 10-fold excess of activator fraction total protein (within which the concentration of the true activator component was also assumed to be in excess) over acetamidase protein diluted in 20mM-bis-Tris, pH 6.8 buffer to a final volume of 0.5-1.0 ml. This mixture was vortexed and an appropriate amount immediately sampled and assayed for amidohydrolase activity at 37°C (section 2.4.1). The remainder of the reactivation assay mixture was incubated and reassayed, as described above for whole cells.

A second type of reactivation assay was also employed wherein activator component was incubated at 60°C, as described above, in the absence of acetamidase. Activator component was cooled and subsequently added to an aliquot of unheated low-activity acetamidase and the mixture assayed immediately, and again after incubation at 37°C for a suitable time interval (typically, 1 h), during which time reactivation occurred. Each type of reactivation assay employed appropriate controls to correct for 'autoreactivation' (Chapter 4) or thermal denaturation of low-activity acetamidase. Further controls were used to correct for amidase/activator component activity present in certain high-speed supernatant fractions, and for amidase activity potentially present in partially-pure activator fraction samples.

In all cases the degree of reactivation of acetamidase activity (after correction, or as indicated in the text) was expressed either as a percentage increase in activity over initial unheated specific activity, or as an absolute increase in specific activity.

2.4.4 Determination of $K_m$ and $V_{max}$ values for amidases

The kinetic parameters, $K_m$ (Michaelis constant) and $V_{max}$, were determined for high- and low-activity forms of acetamidase with the substrates acetamide and acrylamide, and for formamidase with formamide. This was achieved by measuring the amidohydrolase activity of an appropriate constant amount of pure enzyme or whole cells except that contrary to the standard assay (section 2.4.1), a constant volume (100μl) of substrate of varying concentration was added to the reaction mixture to give a wide range of final amide concentrations (0.1-100 mM). Stock solutions (1-1000 mM-amide in 0.1M-citric acid/0.2M-Na₂HPO₄, pH 6.0 buffer) of different substrate concentrations were made prior to the amidase assays. Kinetic constants were then determined graphically from a plot of substrate concentration divided by reaction velocity (s/v) versus substrate concentration (s) (Hanes plot). From this graph $V_{max}$ was calculated from the reciprocal gradient value, and $-K_m$ from the intercept on the s value-axis.

2.5 Polyacrylamide gel electrophoresis (PAGE)

2.5.1 SDS-PAGE

Large (1.5mm thick) and small (Mini-Protein II dual slab cell, Bio-Rad; minigel, 0.75mm thick) discontinuous denaturing gels were run using a slab-gel system (Hames, 1981). Large resolving gels (28ml) contained 12.5% (w/v) acrylamide (PROTOGEL Protein and
sequencing electrophoresis grade; National Diagnostics) and 0.1% (w/v) SDS (sodium dodecyl sulphate) in 0.375M-Tris-Cl, pH 8.8 buffer. The stacking gel (approximately 10ml) contained 3.75% (w/v) acrylamide and 0.1% (w/v) SDS in 0.125M-Tris-Cl, pH 6.8 buffer. Minigel resolving gels (3.5ml) contained 12% (w/v) acrylamide and 0.1% (w/v) SDS in 0.375M-Tris-Cl, pH 8.8 buffer. Minigel stacking gels (approximately 2ml) contained 4% (w/v) acrylamide and 0.1% (w/v) SDS in 0.125M-Tris-Cl, pH 6.8 buffer. After mixing, and prior to polymerization, each gel solution was de-gassed under vacuum for 5 min to prevent bubble formation during solidification of the gel. Large resolving and stacking gels were polymerized by the addition of 0.075% (w/v) ammonium persulphate and 0.05% (v/v), or 0.075% (v/v) TEMED (Sigma), respectively. Minigel resolving- and stacking gels were polymerized with 0.05% (w/v) and 0.1% (w/v) ammonium persulphate respectively, in addition to 0.05% (v/v) and 0.2% (v/v) TEMED respectively. Reservoir (‘running’) buffer for both types of gel contained 0.192M-glycine and 0.1% (w/v) SDS in 0.025M-Tris-Cl, pH 8.3 buffer.

Samples were prepared as follows: whole cells, partially-purified cell extracts, and pure proteins were mixed with dissociating buffer (2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol), and 0.002% (w/v) bromophenol blue in 63mM-Tris-Cl, pH 6.8 buffer (Hames, 1981) in the ratio 3:1 sample:buffer (final sample volume was kept as small as possible; 10-20 µl). Prior to loading, samples were denatured by boiling in capped Eppendorf tubes for 2 min. Large gels were loaded using a glass Hamilton microsyringe with 40-45 µg dry wt cells (10-15 µg dry wt cells, minigel) or 30µg total protein (approximately 10µg total protein, minigel), maximum per track. When essentially pure proteins were electrophoresed only enough protein to be easily visible in each expected band was loaded, i.e. on a large gel approximately 1µg per band (0.5µg per band, minigel). Electrophoresis was carried out at 40mA constant current (large gels) or 200V constant voltage (minigels). Gels were run until the blue dye-front had migrated to the bottom of the gel (3-4 h, large gel; 40-45 min, minigel) at which point gels were disassembled, clipped in one corner to denote gel-orientation during loading, and washed in deionized water. Gels were subsequently stained overnight (1 h, minigel) in Kenacid blue R (10% [w/v] Kenacid blue R, 45% [v/v] methanol and 10% [v/v] glacial acetic acid in deionized water) and then destained by several rounds of sequential washing in fresh destain solution (7.5% [v/v] glacial acetic acid and 5% [v/v] methanol in deionized water). This process was continued until all the background stain had been removed.

The following standards were loaded in 2 lanes of each gel to calibrate the gel with respect to protein MW: bovine albumin, 66kDa; ovalbumin, 45kDa; glyceraldehyde-3-phosphate dehydrogenase, 36kDa; carbonic anhydrase, 29kDa; trypsinogen, 24kDa; trypsin inhibitor, 20.1kDa; and β-lactalbumin, 14.2kDa (MW-SDS-70L kit, Sigma). Destained gels were photographed and subjected to image analysis by use of a GDS2000 gel documentation system (Ultraviolet Products) interfaced with an IBM-compatible PC. This system allowed the MW of specific protein to be estimated with respect to protein MW markers, and also allowed estimation of sample purity.
2.5.2 Native PAGE (non-dissociating)

Large native discontinuous gels were made in essentially the same way as above (section 2.5.1) except that SDS was omitted from resolving and stacking gels, reservoir buffer, and dissociating buffer. β-mercaptoethanol was also omitted from the latter. Native gels contained only 10% (w/v) acrylamide. Whole cells were broken by sonication (section 2.3.2) prior to loading; otherwise gels were run and treated as above (section 2.5.1).

2.5.3 Electro-elution of proteins from preparative SDS-gels

Preparative 12.5% (w/v) acrylamide SDS-gels (3mm thick) were cast as above (section 2.5.1) except that twice as much gel solution was polymerized (using only 0.025% [v/v] TEMED), at 4°C, to prevent cracking of glass gel plates by the exothermic polymerization reaction. Preparative gels were run at 80mA for 4-6 h.

To locate the position of the required protein a narrow vertical slice of gel was taken from the MW standard track, or the periphery of the gel track of interest, and stained in Kenacid blue R for 5-10 min. Stained and unstained portions of the gel were then carefully aligned on a light-box and the region of the unstained gel corresponding to the required protein band was excised using a scalpel, avoiding contaminating bands as far as possible. This gel piece was cut into thin slivers and introduced into dialysis tubing (low protein-binding capacity, 3.5kDa MW exclusion limit; Spectrapore) (prepared according to Maniatis et al., 1982) containing 0.1% (w/v) SDS in 100mM-ammonium bicarbonate (Fisons, AR grade) solution. The dialysis tubing was sealed at either end and positioned on the shelf of a horizontal electrophoresis tank, filled with the same buffer, with the gel slice positioned inside the tubing closest to the cathode. Protein was eluted overnight at 15mA and then dissociated from the tubing wall by reversing the polarity of the electrodes for 2 min. Eluted protein was recovered by carefully drawing out the contents of the tubing using either a Pasteur or Gilson pipette.

2.5.4 DNA sequencing gels (manual sequencing)

Polyacrylamide gels for manual sequencing (section 2.22.1) conformed to the following gel-slab specifications: height, 38.5cm; width, 31.0cm; volume, 75ml; and thickness, 0.4mm. The gel (80ml) contained 42% (w/v) urea (Ultrapure, USB) and 6% (w/v) acrylamide (Accugel 40 sequencing grade ultrapure; National Diagnostics) in 1 x TBE buffer, pH 8.3 (prepared by dissolving in 11 of deionized water: Tris base, 10.8g; boric acid, 5.5g; and 0.5M-EDTA, pH 8.0, 4ml; Maniatis et al., 1982). Gel components were polymerized with 0.063% (w/v) ammonium persulphate and 0.1% (v/v) TEMED, and mixed in a 250ml side-arm flask (to facilitate ease of pouring). Gel solutions were quickly poured into a tapered glass sequencing plate sandwich, taking great care to remove any bubbles prior to solidification. Inverted sharkstooth combs were clamped in place at the top of the gel sandwich to form a flat gel interface for insertion of sample wells, and the gel was allowed to set for at least 15 min.
Gel combs were carefully removed, and the tape at the bottom of the gel sandwich was split so that buffer could flow through the gel during electrophoresis. The gel sandwich was then prepared for loading according to the manufacturer's instructions (sequencing gel electrophoresis system, Model S2; BRL, Life Technologies Inc.): 1 x TBE buffer (500ml) was poured into the top and bottom reservoirs, and the top of the gel was flushed with buffer to remove residual acrylamide. Sharkstooth combs were inserted into the top of the gel to form wells which were checked for leaks by pipetting 4μl of Sequenase stop solution (Sequenase Version 2.0 DNA sequencing kit, USB Corp., Ohio) into each well. Gels were then pre-run at 65W constant power for 45 min.

DNA sequence samples prepared using the Sequenase Version 2.0 kit (section 2.22.1) were denatured in a 75°C waterbath for 2 min, and gel wells were flushed with buffer to remove the urea that diffused into them, immediately prior to loading. Samples were loaded into labelled wells using a Gilson P10 pipette and electrophoresed at 65W constant power. Gels were run initially for approximately 2.5 h, or until the second dye-front (green) had migrated 75% of the gel length. New samples were then loaded into fresh wells (pre-rinsed) and the gel electrophoresed for a further 1.5 h, or until the first (blue) dye front from the second set of samples had just run off the bottom of the gel. On certain occasions, further samples were loaded on to the same gel, depending on the length of DNA sequence required, the physical distance of the sequence of interest from the primer, and the necessity for producing overlapping sequence between samples loaded at staggered time intervals.

After electrophoresis the sequencing apparatus was disassembled and the gel prepared for autoradiography (still resting on the glass sequencing plate) by soaking (fixing) in 10% (v/v) methanol/acetic acid solution in deionized water (21 total volume) for 30 min. The gel was then carefully blotted dry with a sheet of 3MM paper (Whatman) after aspiration of the methanol/acetic acid solution, exercising caution not to introduce any air bubbles between the gel and the paper. The glass plate was then inverted so that the gel and the 3MM paper (acting as backing paper to the gel) could be removed and covered with Saran wrap (Dow). Excess backing paper/Saran wrap was trimmed from the gel prior to drying (80°C, 90 min) using a model 583 gel dryer (Bio-Rad). Once the gel was completely dried the Saran wrap was removed and the gel placed in an autoradiograph cassette and exposed (gel-side up) overnight at room temperature to X-ray film (medical X-ray film RX, 35 x 43 cm; Fuji). Autoradiographs were developed using a Cronex CX-130 X-ray automatic film processor (DuPont Instruments).

2.6 Fast Protein Liquid Chromatography (FPLC)

2.6.1 FPLC-purification of acetamidase (high- and low-activity forms)

Acetamidase was purified essentially according to the method of Silman (1990) with several modifications, as shown below, from a high-speed supernatant (section 2.3.3) prepared from fresh or frozen cells of M. methylotrophus. Frozen high-speed supernatants were thawed and
centrifuged at 10,000 rpm (12,200 x g) for 10 min (MSE High Spin 18) to pellet the cellular debris that precipitated upon thawing (precipitation was presumably due to denaturation of labile proteins). Supernatants that had been stored on ice were also re-centrifuged to remove any particulate matter that had precipitated further, and occasionally both frozen and unfrozen supernatants were further clarified by passage through an Acrodisc filtration unit (0.2μm, Gelman).

In order to optimize column resolution the protein concentration of clarified high-speed supernatants was measured (section 2.7), so that the recommended maximum protein loading capacity for each column was not exceeded. FPLC buffers were filtered (0.2μm pore size, cellulose nitrate membrane filters; Whatman) and degassed prior to use, and aliquots of supernatant (≤ 150μg total protein) were loaded on to a Mono Q HR 10/10 anion-exchange column (bed volume, 8ml; Pharmacia) pre-equilibrated with 20mM-bis-Tris, pH 6.8 buffer. The majority of proteins present in the high-speed supernatant bound to the column and were eluted sequentially from the column using a linear KCl gradient (0-1 M-KCl in 20 min; flow rate 4ml min⁻¹) generated by differential pumping (pump P-500; Pharmacia) of a high salt buffer, 20mM-bis-Tris + 1M-KCl, pH 6.8 (controlled by an LCC-500, or GP-250 gradient controller; Pharmacia) into the column. The absorbance at 280nm (A₂₈₀) of fractions eluting from the column was continually monitored in a flow cell coupled to a chart recorder (Pharmacia) which recorded the elution profile of A₂₈₀ versus elution volume/fraction number; fractions were subsequently collected (4ml) in an automatic carousel. In crude preparations, acetamidase was found to elute at somewhat variable KCl concentrations ≥ 250mM (presumably as a function of decreasing column resolution with increasing column age and use), but generally within the range 270-290 mM-KCl. Acetamidase-containing fractions were identified using the discontinuous assay (section 2.4.1) and the most active fractions were pooled, taking care to avoid fractions containing significant amounts of either the activator component (section 2.6.3) or formamidase (section 2.6.2) which eluted at approximately 200mM-KCl (Silman, 1990) and approximately 340mM-KCl (D.J. Scherr, unpublished), respectively.

Pooled fractions from the first anion-exchange step were either concentrated using a stirred ultrafiltration cell (Amicon) to 2ml final volume with a 10kDa (MW) exclusion limit membrane (YM-10, Diaflo), and the retentate subsequently desalted by elution from a PD-10 gel-filtration column pre-equilibrated with 20mM-bis-Tris, pH 6.8 buffer according to the manufacturer's instructions (Pharmacia), or successive 2.5ml aliquots of pooled peak-activity fractions were desalted in parallel using two PD-10 columns, as described above. Pooled, desalted peak-activity fractions were then re-loaded on to the same anion-exchange column.

Proteins eluting from the second anion-exchange step (0-350 mM-KCl in 25 min; flow rate 4ml min⁻¹) were collected and the peak-activity fractions identified and pooled, as before. Pooled fractions were then concentrated by ultrafiltration, either as described above (except that the final concentrate volume was approximately 0.5-1.0 ml) or using ultrafiltration spin columns (Microsep microconcentrators, Flowgen; section 2.13) until the final desired
concentrate volume (0.5-1.0 ml) was reached. As the next step in the purification procedure was a gel-filtration step samples were not subsequently desalted.

Samples (0.5-1.0 ml; ≤ 15mg total protein) were loaded on to a preparative grade Superose 6 HR 16/50 column (bed volume, 100ml) pre-equilibrated with 20mM-bis-Tris + 100mM-KCl, pH 6.8 and eluted at a flow rate of 0.25ml min⁻¹. Acetamidase eluted after approximately 280 min which corresponded to an elution volume (Vₑ) of approximately 68.2ml. After this step, peak-activity acetamidase fractions were adjudged to be ≥ 90% pure by SDS-PAGE (section 2.5.1). In contrast to the method of Silman (1990), all acetamidase samples were purified using the two-step anion-exchange method outlined above (unless indicated otherwise in the text), as it was deemed to yield final protein samples of greater purity.

Occasionally, acetamidase was subjected to a third anion-exchange step (see section 2.14), after gel-filtration, with a shallower sequential multi-step gradient (0-240 mM-KCl over 25 min, 240-280 mM-KCl over 10 min, 280mM-KCl constant for 2 min, and finally, 280-340 mM-KCl in 10 min; flow rate 4ml min⁻¹), depending on the necessary degree of purity required for a given acetamidase experiment. Acetamidase was adjudged to be essentially pure after this final step and was stored either at 4°C (high-activity acetamidase) or at -20°C (high- or low-activity acetamidase) until required; during the purification procedure fractions were stored either frozen (-20°C) or refrigerated (4°C). In marked contrast to the work of Silman (1990) there became apparent a tentative correlation between freezing samples and a significant rapid 'switch-off of high-activity acetamidase activity, characterized by a profound decrease in partial- or post-purification specific activity and the ability of these samples to undergo reactivation' (section 2.4.3; Chapters 3 & 4), such that the majority of the original activity of the enzyme was restored. It is suggested that in view of these, as yet unexplained findings, all FPLC fractions should be refrigerated between purification steps, and only frozen when pure.

### 2.6.2 FPLC-purification of formamidase

Formamidase was purified from *M. methylotrophus* and *E. coli* JM109::pNW3 (Chapter 6) using the same modified method as described above (section 2.6.1) with reference to the purification described by Wyborn et al. (1994). The most active fractions eluted from the first anion-exchange step at approximately 340mM-KCl (D.J. Scherr, unpublished) and were pooled, using the same criterion as above, except this purification necessitated the removal of as much 'contaminating' acetamidase activity from subsequent purification steps, as possible. Formamidase eluted from the gel-filtration step after approximately 290 min, corresponding to an approximate $Vₑ$ of 71.0ml, and in accordance with its smaller native MW (123kDa; section 2.6.6) compared with acetamidase (MW 155kDa). The third anion-exchange step (section 2.6.1) was occasionally employed to purify formamidase further (see section 2.14).
2.6.3 FPLC-partial-purification of activator component

Partial-purification of activator component was achieved using the method of Silman (1990)(section 2.6.1). High-speed supernatant was subjected to a single Mono Q HR 10/10 anion-exchange fractionation step (0-1 M-KCl in 20 min; flow rate 4ml min⁻¹), and the fractions were collected. Fractions containing activator component were identified by mixing an aliquot from each fraction that eluted in the range 150-350 mM-KCl with a small aliquot of low-activity acetamidase. The ability of each fraction to reactivate amidohydrolase activity in each mixture was then identified qualitatively after incubation (1 h) at 60°C (section 2.4.3). Activator component was found to elute at somewhat variable KCl concentrations (again, presumably as a function of decreasing column resolution with increasing column age and use) although generally it was found to elute within the range 200-300 mM-KCl. FPLC fractions containing activator component were found to reanimate low-activity acetamidase by at least 100%; appropriate controls were used to correct for any 'autoreactivation' (Chapter 4) of low-activity acetamidase, and also to identify those fractions in which acetamidase and activator component had co-eluted. Fractions of the latter type were discarded when pooling and homogenizing the activator component fractions that exhibited the greatest ability to reanimate low-activity acetamidase, such that the final partially-pure activator component fraction had no intrinsic amidase activity.

2.6.4 Anion-exchange chromatography of purified high- and low-activity forms of acetamidase

Purified samples of desalted (PD-10 gel-filtration column; Pharmacia) high- and low-activity acetamidase in 20mM-bis-Tris, pH 6.8 buffer (0.2mg total protein; 3.5ml) were chromatographed using a pre-equilibrated Mono Q HR 10/10 anion-exchange column. Samples of both forms of acetamidase were chromatographed separately, using a linear KCl gradient (0-0.4 M-KCl in 27 min; flow rate 4ml min⁻¹), and also as a 1:1 mixture of high- and low-activity acetamidase (0.1mg of each; 0.2mg total protein)(0-0.26 M-KCl in 18 min, followed by 0.26-0.3 M-KCl in 5.5 min) generated as above (section 2.6.1). FPLC and sample parameters (sample volume, chart recorder speed, FPLC machine used etc.) were standardized between different runs in order to produce sharp protein peaks of comparable height, width, and overall shape. The KCl concentration corresponding to the full scale deflection of each peak was then determined from the trace of A₂₈₀ versus elution volume (Vₑ), as a potential measure of the nett surface electrical charge of high- and low-activity forms of acetamidase. Fractions (0.2ml) collected across each protein peak were assayed (section 2.4.1) for amidohydrolase activity.
2.6.5 Measurement of isoelectric point (pI) of high- and low-activity forms of acetamidase: FPLC-chromatofocusing

Purified samples of high- and low-activity acetamidase were desalted and buffer exchanged (PD-10 gel-filtration columns; Pharmacia) into 25mM-bis-Tris, pH 5.5 buffer and then chromatofocused, separately and as a mixture, using a Mono P HR 5/20 chromatofocusing column (Pharmacia) pre-equilibrated with 25mM-bis-Tris, pH 5.5 buffer. Acetamidase samples were identical to those described in section 2.6.4. Acetamidase was eluted from the column by the generation of a pH gradient in situ with Polybuffer 74 (Pharmacia), adjusted to pH 3.5 with iminodiacetic acid, using an isocratic procedure (flow rate 1ml min⁻¹). The pH at which each protein peak eluted was measured by an on-line pH meter, and was also determined from the full scale deflection of each peak recorded on the trace of A280 versus elution volume (Ve). The elution pH value was taken as a measure of the isoelectric point (pI) of each form of acetamidase. Fractions (1ml) were collected and assayed for amidohydrolase activity (section 2.4.1), and also tested for the ability to be reactivated (section 2.4.3). The Mono P facility was kindly provided by Dr. N.J. Silman, University of Warwick.

2.6.6 Determination of formamidase native molecular weight

The native molecular weight of formamidase was determined by chromatographing a pure sample of formamidase using a Superose 12 HR 10/30 gel-filtration column (bed volume, 24ml) pre-equilibrated with 20mM-bis-Tris + 100mM-KCl, pH 6.8 buffer (flow rate 0.3ml min⁻¹), and determining its Ve value (by discontinuous assay). The column was calibrated with the following MW standards: β-amylase, 200kDa; alcohol dehydrogenase, 150kDa; bovine serum albumin (monomer), 66kDa; carbonic anhydrase, 29kDa; and, cytochrome C 12.4kDa (Sigma) which were used to construct a standard curve by plotting log MW protein standard versus elution volume (Ve) (ml). The molecular weight of formamidase was then estimated by extrapolation from the standard curve.

2.7 Determination of protein concentration

The amount of protein in a given sample was determined using a dye reagent kit (Protein assay kit II; Bio-Rad) using a procedure based on the method of Bradford (1976). Sample protein estimations were extrapolated from a standard curve (plotted as optical density at 595nm [OD595] versus protein [µg]), prepared using bovine serum albumin as standard, according to the manufacturer’s instructions for the microassay procedure. OD595 values were measured using a Perkin Elmer Lambda 5 UV/VIS split-beam recording spectrophotometer in disposable plastic 1ml cuvettes.

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2.8 Amino-terminal sequence analysis of pure formamidase

Purified formamidase (2.5μg) (section 2.6.2) from M. methylotrophus and E.coli JM109::pNW3 was subjected to SDS-PAGE (section 2.5.1). Protein samples (< 1nmol) were blotted separately from the gel on to polyvinylidine difluoride (PVDF) membranes and visualized by staining with Kenacid blue R for approximately 5 min; samples were subsequently destained for the same length of time. PVDF membrane samples were then loaded into the sample port of an Applied Biosystems model 470 gas-phase sequencer and the N-terminal sequence of formamidase determined by Miss E. Cavanagh. Amino-terminal amino acids were separated and analysed by HPLC after sequential Edman degradation cycles.

2.9 UV spectrophotometric analysis of high- and low-activity forms of acetamidase

Samples of pure high- and low-activity acetamidase (80μg) were diluted to 1ml final volume with 20mM-bis-Tris, pH 6.8 buffer in back-corrected quartz cuvettes, and absorption spectra were recorded independently over the wavelength (λ) range 250-320 nm against a 20mM-bis-Tris, pH 6.8 buffer blank (1ml). Scans were performed using a Perkin Elmer Lambda 5 UV/VIS split-beam recording spectrophotometer set to a sensitivity level sufficient to detect a single bound nucleotide/nucleoside molecule.

2.10 Sedimentation velocity ultracentrifugation analysis of high- and low-activity forms of acetamidase

Samples of pure high- and low-activity forms of acetamidase (0.4mg ml⁻¹ and 0.08mg ml⁻¹ in 20mM-bis-Tris, pH 6.8 + 100mM-KCl buffer) were subjected to sedimentation velocity ultracentrifugation analysis at 5°C in an Optima XL-A analytical ultracentrifuge (Beckman), or at 20°C in an MSE Centriscan 75 analytical ultracentrifuge. Protein samples were centrifuged with appropriate buffer blanks (containing no protein) at 42,000 rpm (130,250 x g) (XL-A), or 40,000 rpm (118,100 x g) (Centriscan 75), and the rate at which acetamidase molecules migrated towards the outermost boundary of the cell was determined from scanning absorption optics traces of A₂₈₀ versus radial position within the cell. The scan intervals were 20 min, and 10 min, for the XL-A and Centriscan 75 respectively. Sedimentation coefficients, Sₚ,T,b (c = protein concentration [mg ml⁻¹], T = temperature [°C], b = buffer), were generated from absorption traces using an Apple graphics tablet and microcomputer with local software (Centriscan 75), and by on-line dedicated analysis software (XL-A). The values of Sₚ,T,b were corrected to standard conditions (i.e. water at 20.0°C) to give Sₚ,20,w values, after the protein concentration was corrected for radial dilution effects.
2.11 Sedimentation equilibrium ultracentrifugation analysis of high- and low-activity forms of acetamidase

Samples of pure high- and low-activity forms of acetamidase (0.4mg ml⁻¹ and 0.08mg ml⁻¹ in 20mM-bis-Tris, pH 6.8 + 100mM-KCl buffer) were subjected to sedimentation equilibrium ultracentrifugation analysis at 5°C in an MSE Centriscan 75 analytical ultracentrifuge. Protein samples were centrifuged for 20 h, with appropriate buffer blanks (containing no protein) at 7,000 rpm (3,600 x g), to develop steady-state conditions wherein the tendency of acetamidase to sediment in the applied centrifugal force was counterbalanced by its tendency to diffuse against the established concentration gradient (sedimentation equilibrium point). The rotor cells were scanned at 280nm (and 360nm to correct for anomalous protein absorption on cell windows) and data was analysed off-line by digitizing the equilibrium patterns recorded for each equilibrium run using an Apple graphics tablet interfaced with a microcomputer running local software. MW values were calculated from the average slope of a plot of In (absorbance) versus (radial distance)². All ultracentrifugation work was kindly carried out at the National Centre for Macromolecular Hydrodynamics, University of Leicester, by Mrs Nima Mistry.

2.12 Determination of phosphate content of acetamidase

Low-activity acetamidase was assayed for the presence of phosphorylated amino acids under acid and alkaline conditions.

A sample of pure acetamidase (1.4mg), considered to be sufficiently large to release enough phosphate to be within the assay sensitivity range (0-100 nm of phosphate; assuming each acetamidase tetramer was only mono-phosphorylated) was hydrolysed in a nitric acid-washed borosilicate glass stoppered tube (Quickfit, BDH). The hydrolysis was effected following the method of Silman (1990), viz. acetamidase (in 300μl of 20mM-bis-Tris buffer, pH 6.8) was hydrolysed by the addition of 1ml of 4M-HCl and the solution was boiled to dryness. To the tube was then added 2M-HCl (1ml) plus 20μl of hydrogen peroxide solution (30% v/v) and again the mixture was boiled to dryness. A further five sequential additions of hydrogen peroxide solution (100μl), with boiling to dryness between additions, was used to complete the hydrolysis. The residue was then resuspended in 500μl of 1M-HCl and incubated in a covered heating block for 20 h at 100°C using a method modified from that of Martensen (1984). Hydrolysis was allowed to proceed to liberate phosphate from any acid-labile phospho-amino acids present, prior to the sample being boiled to dryness, and resuspension of the residue in 500μl of deionized water. The sample was then cooled on ice and assayed for phosphate as described below.

A similar sample of low-activity acetamidase was also subjected to alkaline hydrolysis in capped polypropylene scintillation vials (7ml), as base tends to leach significant quantities of phosphate from glassware. Low-activity acetamidase was not acid/peroxide-hydrolysed prior to alkali treatment as it has been shown that the phosphoryl groups of phospho-serine and
-threonine in peptide linkage are much more labile to base than the phosphoryl groups of free amino acids (see Martensen, 1984). Thus, alkaline hydrolysis of acetamidase was effected by incubating non-hydrolysed samples, diluted to 0.5ml final volume in 1M-NaOH, in a water bath at 55°C for 2 h, using a method based on that described by Martensen (1984). After incubation samples were cooled on ice, as above, and assayed for phosphate (see below).

Phosphate was assayed colorimetrically according to the method described by Itaya and Ui (1966). This method employed a colour-developing reagent containing 1 volume of 4.2% (w/v) ammonium molybdate in 5M-HCl mixed with 3 volumes of 0.05% (w/v) Malachite Green solution. This mixture was left to stand at room temperature for 30 min, prior to filtration to remove a green precipitate, which gave rise to a pale yellow filtrate. The assay was performed by mixing the test sample (1ml) with 5ml of filtered colour-developing reagent and incubating on ice for 30 min. The assay mixture was then incubated for a further 15 min at room temperature before measuring the absorbance of the solution at 650nm (A_{650}) (Pye Unicam SP600 UV spectrophotometer) against a water blank.

In both types of experiment, appropriate sample controls were processed with and without added protein, under identical experimental conditions, to correct for extraneous phosphate contamination of the assay via potential heat and/or acid/alkali-induced leaching of phosphate from glassware/polypropylene. Samples of authentic phospho-serine, -arginine, and -tyrosine (50nmol of each; Sigma) were also assayed under the same conditions as respective test samples, to give an estimate of the expected yield of phosphate from authentic phospho-amino acids under the experimental conditions employed.

Construction of a standard curve, prepared freshly with each batch of assays using KH_2PO_4 (dried overnight in an oven), allowed quantification of phosphate. Standards were developed that contained 0-100 nmol of inorganic phosphate, made-up in deionized water, over which range the assay was found to be linear.

2.13 Desalting, buffer exchange and concentration using ultrafiltration spin-columns

Amidases were subjected to spin-column (ultrafiltration) (Microsep microconcentrators; Flowgen) concentration, and/or diafiltration (desalting and buffer exchange) with a 10kDa or 30kDa (MW) exclusion limit, essentially according to the manufacturer's instructions, by centrifugation in a fixed-angle rotor at 6,000 rpm (4,300 x g) maximum at 4°C (Sorvall RC-5B refrigerated superspeed centrifuge; DuPont Instruments). Columns were pre-rinsed with 3ml of deionized water, prior to loading with sample, to remove trace contamination of the membrane (low protein binding; Omega series) with glycerine and sodium azide.

Diafiltration was effected, typically, by including 5 cycles of 10-fold sample concentration and reconstitution in an exchange buffer/solvent of choice, such that the final amount of original buffer/salt remaining in the sample after treatment was negligible.
2.14 Preparation of amidase samples for Electrospray Mass Spectrometry (ESMS)

Samples of high- and low-activity acetamidase, and of formamidase, were highly purified for ESMS by FPLC, including the third anion-exchange step (section 2.6.1). As samples of acetamidase or formamidase (typically, 0.5-1.0 mg total protein; approximately 0.5 mg ml⁻¹), purified by FPLC contained bis-Tris buffer and KCl (270-340 mM-KCl), they were diafiltered (section 2.13) using either deionized water or 50% (v/v) methanol in deionized water as the exchange solvent to make them compatible with ESMS sample requirements (both types of exchange solvent presented practical problems during diafiltration/concentration and the relative demerits of each is discussed in Chapter 3). Occasionally, when no spin-columns were available, diafiltration and sample concentration were effected using a stirred ultrafiltration cell (Amicon). Once diafiltration had been completed the samples were concentrated to a final predicted protein concentration of at least 4 μg μl⁻¹. Samples were subsequently tested to determine relative activity and degree of reactivation (section 2.4.3); such tests were of particular importance for high- and low-activity acetamidase samples in order to estimate the degree of 'switch-off' exhibited by each sample (Chapter 3). Samples were stored at -20°C until required.

ESMS spectra were recorded by injecting the protein sample (10-20 μl; 1-5 pmol μl⁻¹), containing 1% (v/v) acetic acid, into a Kratos analytical (model Concept IH) ESMS, via a silica capillary injection port housed in a stainless steel sheath. Data analysis was performed by on-line operating system software (Sun Mach 3; Kratos). Spectra were produced by Dr. G. Eaton, Department of Chemistry, University of Leicester.

2.15 Proteolysis of activator component

An aliquot of partially-purified activator component (section 2.6.3), containing approximately 225 μg of total protein, was frozen on dry-ice in an Eppendorf tube and lyophilized for 2 h (Edwards Freeze Dryer Modulyo) at -44°C and 9.6 mbar. The residue was resuspended in 0.5 ml of proteinase K buffer (0.01 M-Tris-Cl, buffer pH 7.8 containing 0.005 M-EDTA and 0.5% (w/v) SDS in deionized water; Maniatis et al., 1982), except that SDS was replaced by an equivalent volume of deionized water. An excess of self-digested proteinase K (20 mg ml⁻¹) was added to the lyophilized aliquot, to a final concentration of 200 μg ml⁻¹, and the solution homogenized. Proteolytic digestion was then allowed to proceed by incubation of the sample in a water bath at 37°C for 5 days. An identical control sample was also processed under the same conditions, except that no protease was added. After digestion the efficacy of activator component proteolysis was checked by SDS-PAGE with appropriate controls.

Both samples were subsequently diluted to 965 μl total volume with 20 mM-bis-Tris, pH 6.8 buffer and then incubated in a boiling water bath (100°C) for 20 min to deactivate the protease. Control samples containing proteinase K in protease buffer diluted with bis-Tris,
but no activator component, and protease buffer diluted with bis-Tris, but no proteinase K and activator component, were also similarly treated. After boiling, tubes were cooled on ice (5 min) before 35µl (16µg) of purified low-activity acetamidase was added with mixing. Tubes were then assayed before and after heating at 60°C to determine the extent of reactivation of the specific activity of the low-activity acetamidase exhibited by each sample (section 2.4.3).

2.16 Preparation and analysis of DNA samples

2.16.1 Preparation of chromosomal DNA (mini-prep)

Chromosomal DNA from *M. methylotrophus* and *Ps. aeruginosa* EF2 was prepared according to the method of Wilson (1990) as outlined below. *E. coli* C600 chromosomal DNA was a gift from the ICI Joint Laboratory, University of Leicester.

A batch culture of *M. methylotrophus* was grown from a small starter culture to an OD₆₀₀ of 0.97 as described above (section 2.1.3), except that the overnight batch culture (15 h incubation at 37°C, 150 rpm) was only of 30ml volume in a 250ml baffled flask. A 30ml LB-medium batch culture of *Ps. aeruginosa* EF2 was inoculated with a single colony and grown to mid-log phase only (OD₆₀₀ 0.56 cf. *M. methylotrophus*) as it was found that production of viscous polysaccharide was minimal at this stage of growth; this improved the ease of purification and also the yield of chromosomal DNA (E.J. Gilbert, personal communication).

Bacteria were harvested in Eppendorf tubes in an MSE MicroCentaur microfuge (section 2.3.1) and the supernatant discarded. Pellets were resuspended in 567µl TE buffer, pH 8.0 (1mM-EDTA, pH 8.0 in 10mM-Tris-Cl, pH 8.0; Maniatis *et al.*, 1982) and mixed with 30µl of 10% (w/v) SDS in deionized water and 3µl of proteinase K (20µg ml⁻¹), to give a final concentration of 100µg ml⁻¹ proteinase K in 0.5% (w/v) SDS. Samples were incubated at 37°C for 1 h, during which time the solution became viscous through SDS-mediated bacterial cell wall lysis and proteolysis. 100µl of 5M-NaCl was mixed with each sample followed by the addition of 80µl of CTAB/NaCl solution (10% [w/v] CTAB in 0.7M-NaCl solution in deionized water) prewarmed to 65°C and delivered into the sample through a truncated Gilson pipette tip. After mixing, these samples were incubated at 65°C for 10 min (the latter two additions promote the formation of CTAB complexes with cell wall debris, polysaccharides and denatured protein, whilst retaining nucleic acids in solution). An approximately equal volume (0.7-0.8 ml) of 24:1 chloroform/isoamyl alcohol (prepared freshly) was subsequently mixed with each sample and the tubes were spun in a microfuge for 4-5 min. After centrifugation, the aqueous (viscous) supernatant was removed to a fresh Eppendorf tube taking care not to carry over any of the white interface (CTAB-complexed cell debris) apparent between the two solvent phases. Each supernatant sample was extracted with an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol, to remove any remaining CTAB precipitate, and the samples were then centrifuged for 5 min. Supernatants from this spin were transferred to fresh Eppendorf tubes and 0.6 volumes of isopropanol was added to each tube to precipitate the nucleic acids. Tubes were inserted into a horizontal rack and gently agitated.
on a shaker platform (Rotatest shaker model R100; Luckham, UK) for 15 min, or until a white filamentous DNA precipitate became apparent. The DNA precipitate was pelleted by centrifugation for 10 min and then washed with 70% (v/v) ethanol in deionized water to remove residual CTAB. DNA was re-pelleted by centrifugation for 5 min and then the supernatant was decanted and the pellet dried in a vacuum dessicator for approximately 15 min. Approximately 10μl of deionized water was added to each tube and the pellet was gently resuspended using a Gilson P20 pipette, taking care not to shear the high-molecular weight DNA. Resuspended DNA pellets were pooled to form a homogeneous 'master' sample, which was subsequently subjected to RNAse treatment (2μl of 10mg ml⁻¹ RNAse mixed with 100μl of pooled chromosomal DNA) at 37°C for 1 h, to remove the RNA that sometimes contaminated such DNA preparations. RNAse was removed from the DNA sample either by ethanol precipitation (section 2.16.3), or by use of the GeneClean II kit (Bio 101 Inc.), and the pure DNA sample was stored at -20°C, until required. (N.B. Throughout the procedure, samples were mixed gently by hand, or by Gilson pipette, but not vortexed, to minimize shearing of high-molecular weight DNA).

2.16.2 Preparation of plasmid DNA (mini-prep and midi-prep)

Plasmid DNA for routine restriction analysis was prepared from clones of E. coli JM109 harbouring recombinant pUC19 or native pUC19 plasmid according to the mini-prep method of Maniatis et al. (1982).

Nutrient broth batch cultures (30ml) supplemented with amp (50μg ml⁻¹ final concentration; section 2.1.3) were inoculated with single bacterial colonies, grown overnight, and then harvested in Eppendorf tubes (section 2.3.1). Supernatants were removed by aspiration and cell pellets resuspended in 100μl of ice-cold GTE buffer (50mM-glucose, 10mM-EDTA in 25mM-Tris-Cl buffer, pH 8.0), before storing the tubes at room temperature for 5 min. To each tube was added 200μl of a freshly prepared solution of 1% (w/v) SDS in 0.2M-NaOH, and the tubes were mixed by inversion. Samples were then incubated on ice for 5 min prior to the addition of 150μl of an ice-cold solution of potassium acetate (approximately pH 4.8; prepared by mixing 11.5ml of glacial acetic acid and 28.5ml of deionized water with 60ml of 5M-potassium acetate) to each tube, followed by gentle vortexing of the samples in an inverted position for 10 s. Samples were then incubated on ice for 5 min and centrifuged in a microfuge for 5 min at 4°C. Supernatants were transferred to fresh tubes and an equal volume of TE buffer-saturated phenol/chloroform was added, and mixed by vortexing, to remove any remaining protein from the samples. After centrifugation for 2 min in a microfuge the supernatants were again decanted to a fresh set of tubes, mixed (vortexed) with 2 volumes of absolute ethanol and allowed to stand at room temperature for 2 min. Tubes were subsequently centrifuged at room temperature for 10 min, supernatants discarded, and tubes drained on absorbent paper. Nucleic acid pellets were washed with 1ml of 70% (v/v) ethanol in deionized water and then re-centrifuged (10 min), after which the ethanol was discarded and the pellets dried under vacuum for 15 min. The dried pellets were
then re-dissolved in an appropriate volume (generally ≤ 20μl) of either TE buffer, pH 8.0 or deionized water, and freed of contaminating RNA species by incubation with RNase (final concentration of 50μg ml⁻¹) for 0.5-1.0 h at 37°C. This method yielded approximately 1-3 μg plasmid DNA per 28ml of culture. DNA prepared in this way was stored at -20°C until required.

Alternatively, a second mini-prep procedure (modified mini alkaline-lysis/PEG precipitation procedure; Applied Biosystems Taq DyeDeoxy™ Terminator Cycle Sequencing kit, 2 x Taq new protocol product sheet, Appendix A, pp.12-15, 1993), utilizing Terrific Broth as growth medium, and yielding 5-30 μg of DNA per 1.5ml culture, was used to prepare relatively large quantities of pure plasmid template DNA for automated DNA sequencing (section 2.22.2; Chapter 7).

Terrific Broth batch cultures (75ml) supplemented with amp (50μg ml⁻¹ final concentration; section 2.1.3) inoculated with single bacterial colonies were grown overnight in 250ml baffled flasks, and harvested in Eppendorf tubes (4.5ml of culture harvested per tube; section 2.3.1). Supernatants were removed by aspiration and cell pellets were resuspended in 200μl of GTE buffer using a Gilson pipette. 300μl of freshly prepared 1% (w/v) SDS in 0.2M-NaOH was added to each tube and the contents mixed by inversion. Tubes were incubated on ice for 5 min, and then neutralized by mixing with 300μl of 3.0M-potassium acetate, pH 4.8. Tubes were subsequently incubated on ice for 5 min, after which time precipitated cellular debris was pelleted by centrifugation for 10 min. The supernatant from each tube was removed to a fresh Eppendorf tube, RNase was added to a final concentration of 20μg ml⁻¹ and the tubes were incubated at 37°C for 40 min. After RNase treatment, supernatants were extracted twice with 400μl of chloroform (layers were mixed by hand for 30 s after each extraction and tubes were centrifuged for 1 min to separate the aqueous and ethereal phases) and supernatants were then removed to fresh Eppendorf tubes. Total DNA was precipitated by adding an equal volume of 100% isopropanol and immediately centrifuging the tube for 10 min at room temperature. The isopropanol was discarded, the DNA pellets were washed with 500μl of 70% (v/v) ethanol in deionized water and finally dried under vacuum for approximately 15 min. Plasmid DNA was purified from total DNA by dissolving pellets in 32μl of deionized water, and selectively precipitating plasmid DNA by first adding 8μl of 4M-NaCl, and then 40μl of autoclaved PEG8000. After mixing the samples were incubated on ice for 20 min, and then centrifuged in a microfuge for 15 min at 4°C to precipitate plasmid DNA. The orientation of each Eppendorf tube in the microfuge was marked such that the position of the transparent DNA pellet at the bottom of the tube after centrifugation could be estimated. Supernatants were removed by aspiration, and the pellets rinsed with 500μl of 70% (v/v) ethanol in deionized water. Finally, the pellets were dried under vacuum for approximately 15 min, and then resuspended (using a Gilson pipette) in 20μl of deionized water. Resuspended samples were pooled to form an homogeneous 'master' sample, and stored at -20°C.

Plasmid DNA for manual sequencing (section 2.21.1; Chapter 5) was isolated according to the alkaline-lysis/PEG precipitation procedure of Kreig and Melton (1991) from which a yield of up to 500μg of pure plasmid DNA could routinely be attained. This method is
essentially identical to the Terrific Broth mini-prep procedure, except that larger overnight LB medium batch cultures were harvested and processed using scaled-up volumes of reagents.

Plasmid pUC19, purified using caesium chloride/ethidium bromide equilibrium density band gradient centrifugation (Maniatis et al., 1982) was a gift from Dr. S.G. Williams.

2.16.3 Concentration and buffer exchange of DNA by ethanol precipitation

Samples of DNA were subjected to ethanol precipitation using a method modified from that described by Maniatis et al. (1982). The volume of the DNA sample was estimated and to it was added sterile 3M-sodium acetate, pH 5.2, such that the final sodium acetate concentration was 0.3M. The solution was mixed and exactly 2 volumes of ice-cold absolute ethanol were added, the sample re-mixed and then incubated for 1-2 h at -20°C. DNA samples were then precipitated (4°C) by centrifugation in an MSE MicroCentaur microfuge at full speed for 10 min. The supernatant was carefully discarded and the tube drained on absorbent paper. Each DNA pellet was then washed with 1ml of 70% (v/v) ethanol in deionized water, and after discarding the ethanol, dried under vacuum for approximately 15 min. Dried pellets were resuspended in an appropriate volume of TE buffer, pH 8.0 or deionized water. Concentration and buffer exchange of DNA samples was also effected by use of the GeneClean II kit (Bio 101 Inc.) according to the manufacturer's instructions.

2.16.4 Spectrophotometric estimation of DNA concentration

DNA concentration was estimated spectrophotometrically using a Perkin Elmer Lambda 5 UV/VIS split-beam recording spectrophotometer. Suitable DNA sample dilutions were made (to a final sample volume of 1ml) in either deionized water or TE buffer, depending on which was used as the original DNA solvent, in back-corrected quartz cuvettes. The absorbance of the sample was read against the correct buffer blank (deionized water or TE buffer) at 260nm and 280nm (A$_{260}$/A$_{280}$) and the DNA concentration estimated from the relationship A$_{260}$ 1.0 = 50µg ml$^{-1}$ (double stranded DNA) or 20µg ml$^{-1}$ (oligonucleotide DNA). The purity of the sample was estimated from the ratio of A$_{260}$/A$_{280}$ (A$_{260}$/A$_{280}$ of 1.8 is indicative of pure DNA; Maniatis et al., 1982). DNA concentration was also estimated by eye using agarose gel electrophoresis (section 2.16.6).

2.16.5 Restriction (digestion) of DNA

Samples of DNA were routinely digested with restriction endonucleases using commercially available enzymes and buffers (supplied by Pharmacia and GibcoBRL), according to the manufacturer's instructions. Typical diagnostic plasmid digests contained 0.25-0.5 µg plasmid DNA in a 20µl buffered reaction mixture with 5-15 units (U) of restriction enzyme; chromosomal digests contained 10-30 µg genomic DNA in a 30-50 µl
buffered reaction mixture with 10-15 U of restriction enzyme. Digests were incubated in a waterbath at 37°C for 1-4 h (plasmids) or overnight (chromosomal samples).

2.16.6 Agarose gel electrophoresis of DNA samples

Agarose gels were prepared by diluting 20ml of 50 x TAE buffer (Tris base, 242g; glacial acetic acid, 57.1ml; and 0.5M-EDTA solution, pH 8.0, 100ml, made up to 1l with deionized water) to 1l with deionized water. 100ml of diluted TAE buffer containing 0.6g of agarose (0.6% [w/v] gel) was autoclaved (121°C for 9 min), and the gel solution was subsequently allowed to cool on the bench for 20-25 min until ‘hand-hot’. To the cooled gel solution was added 5μl of ethidium bromide (10mg ml⁻¹), and the mixture homogenized and poured into a perspex gel-tray taped at either end. Sample wells were formed by the insertion of a perspex comb into the molten gel solution, and all bubbles were removed before solidification using a Gilson pipette tip. After 20 min the solidified gel was immersed in the remaining 900ml of diluted TAE buffer (containing 45μl of ethidium bromide) in a horizontal gel electrophoresis tank. Occasionally 1-3% (w/v) gels were used to estimate the total DNA concentration of a given sample by retarding the relative rates of migration of a population of DNA fragments of different sizes in a relatively thicker gel matrix. This allowed the fragments to be visualized as a single band whose fluorescent intensity could be compared to a DNA standard sample of known concentration; the concentration of size-selected Sau3AI fragments (Chapter 5) was estimated in this way, using a 3% (w/v) gel electrophoresed at 100V for 20 min.

DNA samples were prepared for electrophoresis by mixing 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, and 30% [v/v] glycerol in deionized water; Maniatis et al., 1982) with the DNA in a 1:5 ratio. Samples were loaded into gel wells as quickly as possible to avoid sample diffusion into the gel, and electrophoresed at 60-100 V until the necessary separation, or otherwise, of DNA fragments had been achieved. Typically, plasmid digests were electrophoresed for 1-2.5 h at 75V, and chromosomal digests for 2-5 h at 65V. The DNA samples in the gel were visualized on an ultraviolet (UV) transilluminator (for the minimum practicable amount of time to avoid physical damage to DNA induced by UV-irradiation), from the fluorescence of ethidium bromide that had become intercalated with the DNA helical structure. Estimation of DNA fragment sizes was achieved by co-electrophoresing an aliquot of BsrEII-digested wild-type bacteriophage Lambda (λ) genomic DNA (pre-heated to 60°C for 2 min prior to loading, to dissociate the cohesive ends of the 8.45kb and 5.69kb fragments), and comparing the migration of standard λ fragments with that of the fragment of interest. The size of useful DNA fragments present in BsrEII-digested λ standards were as follows: 8.45kb, 7.24kb, 6.37kb, 5.69kb, 4.82kb, 4.32kb, 3.68kb, 2.32kb, 1.93kb, 1.37kb, 1.26kb, and 0.70kb.

Gels were photographed using either Kodak Tmax 100 professional film, or after image analysis by use of a GDS2000 gel documentation system (Ultraviolet Products) interfaced with an IBM-compatible PC. The latter system was also used to size specific DNA fragments, with respect to DNA standards, with a high degree of accuracy.
2.17 Recovery of DNA samples from agarose gels

DNA samples were electrophoresed and visualized, as described above (section 2.16.6), and the region of the gel containing the DNA fragment(s) of interest excised using a sharp scalpel. DNA was recovered from the gel piece(s) using the GeneClean kit according to the manufacturer's instructions (Bio 101 Inc.).

2.18 Manipulation of DNA samples for Southern hybridization

2.18.1 Southern transfer (blotting) of DNA from agarose gels to hybridization membranes

Southern transfer of DNA to hybridization membranes was effected essentially using the method described by Southern (1979). Electrophoresis was carried out, as described above, (section 2.16.6) and the gel was photographed with a ruler focussed in the same focal plane as the DNA samples. This allowed measurements from the photograph to be extrapolated to the hybridization membrane/autoradiograph for identification of the position of bands of interest. The gel was subsequently rinsed in deionized water, and then washed gently in 250ml of 0.25M-HCl on a shaker platform for exactly 7 min to hydrolyse partially the DNA by acid depurination, a treatment that improves the efficiency of transfer of large DNA fragments. The gel was then rinsed in deionized water and washed in 250ml of 0.5M-NaOH/1.5M-NaCl solution for 30 min to denature the DNA, before being rinsed in deionized water and neutralized in 250ml of 0.5M-Tris-Cl/3M-NaCl solution for 30 min. The gel was then transferred, face-(wells)-down, on to a long piece of 3MM paper (Whatman), soaked in sterile 20 x SSC buffer (NaCl, 175.3g, and sodium citrate, 88.2g, made-up to 11 final volume with water) acting as a wick, folded over a clean glass plate resting across an open plastic box, with either end of the 3MM paper dipping into a reservoir of the same buffer contained below in the plastic box (care was taken to avoid the formation of air bubbles between the gel and the 3MM paper). On to the uppermost surface of the gel was placed a piece of Hybond-N nylon hybridization transfer membrane (0.45μm size; Amersham), cut to the size of the gel and soaked in 3 x SSC buffer (prepared by diluting 90ml of 20 x SSC buffer, pH 7.4 to 600ml final volume with deionized water), taking care to remove any air bubbles caught between the membrane and the gel surface. Overlaying the membrane was placed a sheet of Saran wrap which also completely covered the gel/plastic box, but with a window cut out corresponding to the area of the gel covered by the Hybond-N. Rectangles of 3MM paper (x 2) soaked in 3 x SSC buffer, pH 7.4 slightly bigger than the Saran wrap window, were placed on top of the Hybond-N membrane, and on top of this was placed a 5-10 cm stack of dry paper towels. A glass plate was used to form a platform on top of the towels upon which was rested a weight (e.g. 500ml of water in a glass bottle). This assembly was left on the bench overnight to allow DNA transfer from the gel to the membrane via capillary action.
After overnight transfer the gel assembly was dismantled, and the position of each gel well and the orientation of the gel during DNA transfer, was marked on the uppermost side of the membrane, which was subsequently trimmed to fit the dimensions of the hybridization chamber. The membrane was briefly rinsed in 3 x SSC buffer, pH 7.4 and air-dried. The membrane was then enveloped in a single layer of Saran wrap and DNA was cross-linked to the membrane (DNA-side down) by UV-irradiation for 10 s, using a longwave UV transilluminator.

2.18.2 Radio-labelling of hybridization oligonucleotide probe

A mixed 17-mer oligonucleotide probe, exhibiting 48 degeneracies (ATG AT[ACT] CA[CT] GO[ACTG] GA[CT] AT), was designed by Silman (1990) on the basis of the first six amino-terminal amino acids of the wild-type high-activity acetamidase. The concentration of the probe solution was determined spectrophotometrically (section 2.16.4), and the amount of probe required (ng) was calculated assuming an average base MW of 333 Da and 20pmol 5'-ends per hybridization. The probe solution was diluted (in deionized water) such that 20pmol of 5'-ends were added to the labelling reaction in 5µl. The labelling reaction contained 20pmol diluted probe, 5µl; deionized water, 5µl; 0.1M-DTT, 1.25µl; T4 polynucleotide kinase, 3µl (10U µl⁻¹; Cambio, UK); and 10 x kinase buffer, 2.5µl (Cambio) which were mixed and pre-warmed in a lead pig half-filled with water at 37°C in a water bath. The pre-warmed probe mix was then added to 10μl of [γ-³²P] ATP (3.7MBq; Amersham International) and mixed using a Gilson pipette. This mixture was subsequently replaced inside the lead pig and incubated at 37°C for 1 h to facilitate radio-labelling of the probe, prior to a second 5 min incubation at 70°C to inactivate the kinase. After these incubations the smallest possible volume (<1µl) of radio-labelled probe solution was spotted on to a strip of DE81 paper (Whatman) and chromatographed (using 0.3M-ammonium formate) until the solvent front had migrated at least 7cm from the origin. The DE81 strip was then enveloped in Saran wrap and autoradiographed at -70°C for 10 min (medical X-ray film NIF RX100 [13 x 18 cm]; Fuji) in an autoradiograph cassette. The film was then developed (section 2.5.4) and the efficiency of probe labelling was estimated by the extent of smearing on the autoradiograph; an intense dark spot at the origin, with little or no smearing, was taken as indicative of a well-labelled probe.

2.18.3 Southern hybridization

Hybond-N membranes, to which DNA had been UV-cross-linked, were pre-hybridized in a sealed perspex chamber containing approximately 20ml of hybridization buffer (2ml of 10% [w/v] SDS; 2ml of 15 x SSPE buffer, pH 7.4 [prepared as follows: NaCl, 13.05g; NaH₂PO₄, 2.07g; EDTA, 0.55g; made-up to 100ml final volume with deionized water; pH adjusted to 7.4 with NaOH]; 'Marvel' dried milk powder, 0.1g; PEG₆₀₀₀ or PEG₈₀₀₀, 1.2g; and deionized water, 15ml), pre-warmed to the calculated hybridization temperature (30-38°C) for 1 h in a
gently shaking water bath. This procedure was used to 'block' the membrane, thereby
minimizing the degree of non-specific probe-binding to the membrane. After pre-
hybridization, the radio-labelled probe mix was added to the hybridization buffer, and the
chamber resealed, and incubated overnight in a shaking water bath at the hybridization
temperature.

After overnight hybridization the membrane was rinsed with 100ml of 3 x SSC buffer, pH
7.4, and then washed sequentially (x 3) in fresh 200ml aliquots of 3 x SSC buffer, pH 7.4,
containing 0.1% (w/v) SDS for 10 min. Further washes were carried out, as before, by
replacing the original wash buffer with 1.0 or 0.5 x SSC buffer, pH 7.4 containing 0.1%
(w/v) SDS, depending on the degree of stringency required. All washes employed buffers
pre-warmed to the hybridization temperature (temperatures in the range 30-38 °C were tested
empirically, with all other hybridization variables held constant; hybridization conditions for a
given experiment were as indicated in the relevant text) and were carried out at the same
temperature. The washed membrane was blotted with 3MM paper and left to dry at room
temperature. It was then enveloped in Saran wrap and placed in an autoradiograph cassette,
between two intensifier screens, with a sheet of X-ray film, and exposed at -70°C for 1 h-7
days (duration of exposure time was also determined empirically, and was governed by the
overall stringency of hybridization and washing conditions; increased stringency resulted in
increased exposure time, and vice versa). Autoradiograph film, and development of
autoradiographs was as described above (section 2.18.2).

2.19 Preparation and manipulation of DNA for transformation
of bacterial cells

2.19.1 Phosphatase treatment of plasmid DNA

Plasmid DNA (5µg, pUC19) purified by caesium chloride/ethidium bromide equilibrium
density band gradient centrifugation was linearised by restriction digestion with an
appropriate restriction endonuclease (known to cut within the polylinker region of pUC19;
section 2.16.5) and a small amount of the sample was checked on an agarose gel (section
2.16.6) to ensure complete digestion had occurred. The remainder of the sample was treated
with the GeneClean kit (Bio 101 Inc.) reagents to remove the restriction enzyme, and the
purified linear DNA was treated with calf intestinal phosphatase (CIP, 1.7U µl-1; Pharmacia)
to remove the 5'- phosphoryl groups from the DNA. This treatment prevents self-ligation of
the linearised plasmid during ligation reactions, since ligases require 5'- phosphorylated
terminal, and thereby reduces the background native vector level in transformation experiments.
A typical CIP reaction contained linearised pUC19, 5µg in 40µl of deionized water; CIP, 5µl;
and One Phor All buffer (10 x OPA; Pharmacia), 5µl. The reaction mixture was mixed and
incubated at 37°C for 30 min, prior to the sample again being treated with the GeneClean kit
(Bio 101 Inc.) reagents to remove the CIP.
2.19.2 Ligation of plasmid and insert DNA samples: formation of recombinant DNA molecules

Chromosomal DNA was digested (section 2.16.5) with an appropriate restriction enzyme to form 'insert' fragments with 5'- and 3'- termini compatible with those of the digested plasmid vector into which the chromosomal inserts were to be ligated. Digested chromosomal DNA was electrophoresed (section 2.16.6) to separate fragments sufficiently such that insert size-selection could be achieved by excision of that part of the digest/gel containing only those DNA fragments in a given size range. DNA was then isolated from the gel matrix (section 2.17) and the total DNA concentration in the resultant sample determined (sections 2.16.4, 2.16.6).

Ligation reactions were performed using a range of molar vector:insert ratios (1.0 : 0.1, 1.0 : 0.5, 1.0 : 1.0, 1.0 : 2.0, 1.0 : 3.0, and 1.0 : 5.0); to maximize the chance of favourable ligation conditions occurring in at least one of the samples. Ligation reactions contained approximately 60-360 ng of total DNA (vector plus insert DNAs) mixed with T<sub>4</sub> ligase (1U µl<sup>-1</sup>; GibcoBRL), 1.0µl; T<sub>4</sub> ligase buffer (5 x ; GibcoBRL), 2.0µl; and made-up to 10µl final volume with deionized water. Ligation mixtures were mixed and incubated for 1-7 days at 4°C. (N.B. In all ligation reactions described above, 'vector' refers to linear dephosphorylated pUC19, and 'insert' refers to a population of chromosomal fragments of variable size).

Control samples were also set up to test the efficiency of T<sub>4</sub> ligase- and CIP-catalyzed reactions, viz. linearised pUC19, untreated by CIP, incubated in the presence and absence of ligase at 4°C; dephosphorylated linearised pUC19 incubated in the presence and absence of ligase at 4°C; and undigested pUC19 incubated in the presence of ligase at 4°C.

2.20 Transformation of bacteria and direct selection of clones expressing active amidase

Cells of *E. coli* JM109 were made competent and transformed with plasmid DNA by the method of Kushner (1978). An overnight 5ml nutrient broth batch culture, inoculated with a single bacterial colony (harbouring no plasmid), was itself used to inoculate (0.75ml inoculum) 20ml of pre-warmed nutrient broth. This batch culture was incubated at 37°C for approximately 2.5 h, by which time bacteria were in mid-logarithmic growth phase (OD<sub>600</sub> = 0.4-0.5; section 2.2). Cells were harvested in Eppendorf tubes (1.8ml culture per tube) in a microfuge (centrifuged for 30 s) and the supernatant discarded. Cell pellets were gently resuspended in 500µl of sterile 10mM-MOPS buffer, pH 7.0 containing 10mM-rubidium chloride and 50mM-calcium chloride (prepared freshly). Samples were then subjected to centrifugation for 20 s, the supernatant was discarded and the cell pellets were resuspended in 500µl of sterile 10mM-MOPS buffer, pH 6.5 containing 10mM-rubidium chloride and 50mM-calcium chloride (prepared freshly). Tubes were subsequently incubated on ice for 1 h and then pelleted by centrifugation for 20 s. A 350µl aliquot of supernatant was aseptically removed from each tube and 3µl of DMSO was mixed with the remaining 150µl of sample.
Transforming DNA, 50-250 ng, was added to each tube and the cells gently resuspended and incubated on ice for 1 h. Cells were then heat-shocked at 55°C for 30 s, and immediately placed in an ice bath for 1 min. To each tube was then added 1ml of pre-warmed (37°C) nutrient broth, and the tubes were mixed gently by inversion, and incubated at 37°C for 1 h to allow for phenotypic lag. Putative transformants (100μl) were then aseptically plated on to pre-warmed solid media and incubated at 37°C for 16 h-7 days.

On certain occasions (as indicated in the text), putative transformants were plated directly on to high-stringency selective media, as described below, without first being plated on to complete (rich) media (nutrient broth or glucose-M9 mineral salts-agar plates containing ammonia as N-source). In such experiments, cells were plated on to M9 mineral salts-agar plates containing amide (generally acetamide) supplied either as sole N-source (with glucose as C-source), or sole C/N-source (no glucose) to directly select clones expressing active amidase. Prior to plating, these cells were aseptically washed (x 2), after incubation in nutrient broth for 1 h, in pre-warmed M9 medium containing no C/N-source. It was envisaged that this washing step would avoid conditions under which selection pressure for N-source or C/N-source was potentially alleviated by carry-over of rich medium. When putative transformants were plated directly on to complete medium this washing step was omitted. All solid and liquid media for the selection of transformants were supplemented with amp and, in the majority of transformation experiments, solid media were also supplemented with IPTG/X-gal (section 2.1.2).

Streaks of actively-growing putative transformants (derived from single bacterial colonies) on solid media (complete or selective) were used to inoculate 20ml M9 mineral salts batch cultures (section 2.1.3) to test for the ability of these clones to grow in liquid medium containing 20mM-amide (generally acetamide) as either sole N-source or sole C/N-source (direct selection for active amidase phenotype) as described above for solid media. These cultures were also supplemented with IPTG unless otherwise stated. The batch growth characteristic of each putative transformant after incubation at 37°C for several days was correlated with the analogous growth characteristic on solid selective medium, and then used in conjunction with restriction analysis of the plasmid harboured by each clone (section 2.16.5) and the measured whole cell specific activity (sections 2.4.1, 2.4.2) to determine the best potential candidate clones for further study.

Aliquots of competent cells were also transformed with similar quantities of control DNA samples (section 2.19.2) in the same experiment, and the cells plated as described above.

2.21 Investigation of the effect of IPTG on heterologously-expressed formamidase activity

The effect of IPTG on heterologously-expressed formamidase activity in E. coli JM109::pNW3 was determined according to the method described by Stark (1987).

A 20ml nutrient broth batch culture containing 0.5% (w/v) D-glucose (to ensure maximal repression of the lac promoter of pUC19) and amp at a final concentration of 0.1mg ml⁻¹ was
inoculated with *E. coli* JM109::pNW3 and incubated overnight (17 h) as described above (section 2.1.3). Cells (0.83ml) were then sub-cultured into an identical volume of fresh medium and the incubation continued (approximately 2.5 h) until the culture had reached mid-log phase growth (OD600 = 0.5; section 2.2). The sub-culture was then harvested aseptically by centrifugation at 6,000 rpm (4,300 x g) for 3 min at 4°C (Sorvall RC-5B refrigerated superspeed centrifuge; DuPont Instruments). After the supernatant was decanted the pellet was resuspended aseptically in 20ml pre-warmed glucose-M9 mineral salts medium containing 20mM-acetamide as N-source and supplemented with amp (0.1mg ml⁻¹ final concentration); 5ml aliquots were then transferred to 4 sterile 50ml flasks, two of which were supplemented with 0.5mM-IPTG. All flasks were then re-incubated, with shaking, for approximately 4.5 h, after which time cells were again in mid-log phase growth. Cells were harvested in a microfuge and washed, before finally being resuspended in pre-warmed 0.1M-citric acid/0.2M-Na₂HPO₄, buffer pH 6.0 (section 2.3.1) and assayed for formamidase activity (section 2.4.1) with formamide as substrate. The original overnight nutrient broth culture was similarly assayed.

Samples of whole cells from each culture were then subjected to SDS-PAGE (section 2.5.1) to visualize cellular proteins and to compare the relative intensities of formamidase. The specific formamidase activity of each culture was correlated with the amount of formamidase expressed by that culture (Chapter 6).

### 2.22 DNA sequencing

#### 2.22.1 Manual DNA sequencing

Double-stranded supercoiled template plasmid DNA for manual sequencing was isolated and quantitated as described above (sections 2.16.2, 2.16.4). Approximately 4μg of plasmid DNA was alkali-denatured (Promega Biological Research Products, Applications and Protocols Guide, Nucleic Acid Sequencing and Mutagenesis section, part VIII, p.107, Second Edition, 1991: Promega Corporation, WI, USA) by diluting the DNA sample to 18μl total volume with deionized water, and mixing with 2fxl of 2M-NaOH solution containing 2mM-EDTA for 5 min at room temperature. The reaction was neutralized by adding 2μl of 2M-ammonium acetate, pH 4.6 to the sample and vortexing the mixture. Absolute ethanol, 75μl, was then added, and the mixture vortexed and incubated on dry-ice for 10 min. The sample was then centrifuged at full speed for 10 min in a microfuge. The supernatant was then decanted, the sample washed with 200μl of cold 70% (v/v) ethanol in deionized water and re-centrifuged for 1 min. The supernatant was then discarded and the DNA pellet dried under vacuum for 10 min, prior to the pellet being re-dissolved in 5μl of deionized water.

Chain-termination sequencing reactions were performed using the Sequenase T7 DNA polymerase kit (Sequenase Version 2.0 DNA sequencing kit, USB Corp., Ohio) according to the manufacturer's instructions. Annealing of primer to single stranded plasmid template was achieved by mixing in an Eppendorf tube denatured template DNA, 5μl (4μg); Sequenase
reaction buffer (5 x), 2μl; oligonucleotide primer (0.5pmol), 1μl; and deionized water, 2μl.
The mixture was heated to 65°C in a water bath for 2 min, and then cooled slowly to < 35°C
on the bench in a beaker of water from the same water bath, over approximately 30 min.
Annealing was complete at temperatures < 30°C and the tube was then stored on ice.
Sequenase labelling mix (5 x) was diluted 5-fold with deionized water to a final volume of
5μl, and Sequenase Version 2.0 enzyme, 0.5μl, was diluted to 4μl final volume with 3.5μl of
ice-cold Sequenase enzyme dilution buffer immediately prior to use. Both samples were
stored on ice until required. Aliquots (2.5μl) of each of the 4 different Sequenase termination
mixtures (containing 2', 3'-dideoxynucleoside 5'-triphosphates (ddNTPs)) were dispensed
into separate Eppendorf tubes (labelled A, T, C, and G) which were capped and pre-warmed in
a water bath at 37°C. The labelling reaction was initiated by adding to the annealed template-
primer mixture (10μl) on ice 0.1M-DTT, 1μl; diluted labelling mix, 2μl; [α-35S] dATP
(Amersham International), 0.5μl (0.185MBq); and diluted Sequenase Version 2.0 enzyme,
2μl (added last). The sample was immediately mixed using a Gilson P20 pipette, and the tube
incubated for 3 min at room temperature. When the labelling incubation was complete, 3.5μl
of labelling reaction mixture was transferred to the first termination mixture tube (A); this
tube was mixed and centrifuged briefly, before being incubated at 37°C in a water bath for 5
min. A further 3.5μl of labelling reaction mixture was sequentially transferred to each of the
remaining termination mixture tubes (T, C, and G) in turn, and each was processed as for (A).
After this final incubation, samples were sequentially removed from the water bath and the
chain extension reactions ended by adding 4μl of Sequenase stop solution to each of the
tubes and mixing. Samples were either stored on ice (for short periods of time) or at -20°C
(for up to 1 week) prior to loading on to the sequencing gel (section 2.5.4).
DNA, and derived amino acid sequences determined using manual sequencing techniques
were compiled and analysed using the DNA Strider 1.0/1.2 software package on an Apple
Macintosh LC 10 microcomputer. Homology comparisons between these sequences and
those already published were achieved by using the BLAST (Altschul et al., 1990) and
FASTA (Pearson & Lipman, 1988) protein and nucleotide database search facilities (Irix 1
operating system).

2.22.2 Automated DNA sequencing

Double-stranded supercoiled template plasmid DNA for automated sequencing was isolated
and quantitated as described above (sections 2.16.2, 2.16.4). Approximately 1.0-1.5 μg (0.20-
0.25 μg μl⁻¹) of template DNA and 160pmol of primer (3.2pmol μl⁻¹) was submitted for
automated sequencing on an Applied Biosystems Model 373A automated DNA sequencer.
Thermal-cycling (PCR) of fluorescence-based DNA sequencing reactions was performed
using either the Taq DyeDeoxy™ Terminator cycle sequencing kit chemistry (ABI) for
custom-designed primers, or Taq dye-primer chemistry (ABI) for commercially available dye-
labelled universal primers compatible with the M13mp19 sequences of pUC19. These
reactions generate a population of sequencing extension products, of variable length, each of
which is labelled with a fluorescent 'tag' (either a primer, or a dideoxynucleotide terminator, each of which has been labelled with one of four different fluorescent dyes, each in turn corresponding to one of A, T, C, or G DNA bases). The tagged DNA molecules are then subjected to a spin column purification procedure to remove unincorporated label and then loaded on to an automated sequencing gel. DNA molecules are then separated electrophoretically, such that a laser scanning the gel can detect the nature of each DNA molecule passing through the scan, by the type of fluorescence that it exhibits; hence the sequential passage of labelled sequencing products of increasing size through the scan allows the sequence of that DNA molecule to be read. Typical sequencing runs of this nature yielded high-quality reproducible data of up to 400 nucleotides per primer. Automated sequencing data was produced independently by Mrs J. Bartley, University of Durham and Dr K. Lilley/Mr T. Seddon, University of Leicester, and DNA and derived amino acid sequences were manipulated and analysed as described above (section 2.22.1), with reference to hard-copies of the data.

2.22.3 Synthesis of oligonucleotide sequencing primers

Custom-designed oligonucleotide primers (18-20-mers), based on DNA sequence produced both manually and automatically, were synthesized by Mrs D. Langton using standard chemical procedures on an Applied Biosystems 380B synthesizer. Primer samples (approximately 1mg ml⁻¹) were diluted to an appropriate concentration and annealed with templates, as described above.

Presentation of results

Results are presented as the mean ± SEM (Y), where SEM is the standard error of the mean and Y is the number of independent determinations.
CHAPTER 3

PHYSICO-CHEMICAL CHARACTERIZATION OF ACETAMIDASE
CHAPTER 3

PHYSICO-CHEMICAL CHARACTERIZATION OF ACETAMIDASE

3.1 Introduction

3.2 Purification of acetamidase from \textit{M. methylotrophus}

3.3 Physico-chemical analysis of acetamidase

   3.3.1 \textit{In vitro} switch-off of acetamidase activity
   3.3.1.1 Switch-off of acetamidase activity during purification
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   3.3.9 The effect of ammonia on high- and low-activity acetamidases at 37°C

3.4 Discussion
3.1 Introduction

Many enzymes have been purified from pro- and eukaryotic sources that are able to hydrolyse the amide function of various substrates. A few examples of the diverse range of amidases catalysing this reaction include acylamide amidohydrolases, nicotinamide amidohydrolases, formamide amidohydrolases, urea amidohydrolases and aminopeptidases (for an extended list of microbial amidases see Clarke, 1970; Maestracci et al., 1988). Consideration of amidase properties in this section will be primarily confined to prokaryotic amidases although there are numerous more esoteric amidase activities associated with certain eukaryotic proteins, such as human α-thrombin (De Cristofaro et al., 1990; Di Cera et al., 1991) and bovine des-I-41 light chain-activated protein C (Hill & Castellino, 1986), amongst others.

The physico-chemical properties and regulatory modes of different groups of amidases are exceptionally varied if viewed on a global scale, but less so if comparison is confined to the members of a particular group. For example, human α-thrombin is a eukaryotic serine protease that selectively cleaves four specific Arg-Gly peptide bonds of fibrinogen to form fibrin as part of the blood clotting cascade (see Stryer, 1981), whereas the Ps. aeruginosa amidase is a prokaryotic acylamide amidohydrolase that specifically hydrolyses a limited range of short chain aliphatic amides, such as acetamide and propionamide, with the concomitant formation of ammonia and the organic acid. The former amidase is subject to a complex regulatory system involving pH- and temperature-dependent interactions with a number of different low-MW allosteric effectors (Di Cera et al., 1991) and is synthesized as an inactive precursor (prothrombin) from which the mature catalytically-active form of the enzyme is liberated by proteolytic cleavage; conversely, the latter amidase is apparently regulated at the level of transcription only (Brammar & Clarke, 1964; see below) and is synthesized in the catalytically-active form which undergoes no further structural modification. Whilst this particular example is somewhat extreme in that it compares a highly specialized eukaryotic enzyme with a less-specialized prokaryotic one, it does highlight the potential diversity of properties that can exist between different amidases. In contrast to this, functionally similar amidases, for example the acylamidases, exhibit general similarities with respect to substrate specificity, pH optima, regulation of activity etc. The acylamidase (acetamidase) of M. methylotrophus has been previously purified to homogeneity and partially characterized (Silman, 1990; Silman et al., 1989, 1991) and it exhibits various similarities to the amidases of Ps. aeruginosa (Clarke, 1970; Brown et al., 1973), Brevibacterium sp. R312 (Thiéry et al., 1986b; Maestracci et al., 1988), and Arthrobacter sp. J1 (Asano et al., 1982a,b). Purification of the amidases from these organisms showed that they constituted 2-5 % of total cellular protein, and were all homo-oligomers of similar MW...
monomers (M. methylotrophus 4 x 38kDa, 155kDa; Arthrobacter sp. J1 8 x 39kDa, 300-320 kDa; Ps. aeruginosa 6 x 38-40 kDa, 200kDa; and Brevibacterium sp. R312 4 x 43kDa, 180kDa). More recently, sub-unit MW values have been more accurately determined and revised by molecular cloning of the structural genes for certain of these enzymes (Brammar et al., 1987; Soubrier et al., 1992). All four amidases hydrolyse acetamide, acrylamide and propionamide, with activity towards these substrates decreasing in the order propionamide > acrylamide > acetamide, except for the Arthrobacter sp. J1 enzyme for which propionamide and acrylamide are reversed. The Arthrobacter sp. J1 amidase also exhibits the narrowest substrate specificity range, being active only towards the three amides mentioned above, whilst activity of the Ps. aeruginosa amidase extends to glycollamide and butyramide (trace activity). The Brevibacterium sp. R312 enzyme conversely shows activity with a much broader range of amides, including aromatic amides such as benzamide. The K_m for acetamide for each enzyme is in the range 0.8-2.4 mM and in all cases is less than that for acrylamide or propionamide. Thermostability studies show that the Ps. aeruginosa (stable at 60°C for ≥ 15 min) and M. methylotrophus (t_{1/2} = 3.2 h; 60°C) amidases are much more thermostable than the Arthrobacter sp. J1 (t_{1/2} = 10 min; 50°C) and Brevibacterium sp. R312 (t_{1/2} = 10 min; 60°C) amidases. Isoelectric focusing shows that all are acidic proteins with pI values in the range 3.5-4.1 pH units (the pI of the Ps. aeruginosa enzyme has not been reported); all amidases are also inhibited by the thiol reagents DTNB and p-mercurichlorobenzoate, which indicates that cysteine residues are structurally important for activity (7-9 thiol groups per monomer). The amidase activities from all four organisms are maximal in a narrow essentially neutral pH range (pH 6.0-8.0), and all exhibit acetyl transferase activity, but this latter property will not be considered further in this Chapter.

The amidase of Ps. aeruginosa has been extensively studied (see Clarke, 1970, 1984; Clarke & Drew, 1988) and various mutant strains have been generated exhibiting novel regulatory and/or physico-chemical properties (see Chapter 1). Mutants of particular note exhibited altered substrate specificities that allowed growth on butyramide, valeramide and phenylacetamide, none of which could support growth of the wild-type. These mutants appeared to exhibit generally similar physico-chemical properties to the wild-type, except that the amidase that allowed growth on butyramide showed anomalous electrophoretic mobility, and certain of these mutant enzymes were significantly more labile at 60°C and -30°C than the wild-type. It was concluded that in the majority of cases these deleterious properties resulted from disruption of the native enzyme conformation by single or double amino acid substitutions altering amino acid bonding potentials, but that these changes did not necessarily occur at residues associated with the active site of the enzyme. Thus, it was envisaged that the altered substrate specificities of the mutant enzymes resulted from subtle conformational changes that allowed access of the more bulky amide substrates (butyramide, valeramide and phenylacetamide) to the active site. Mutant amidase (acetamidase) strains exhibiting altered physico-chemical properties have also been described for M. methylotrophus (Silman, 1990; Silman et al., 1989, 1991; see Chapter 1).
Regulation of amidase activity can occur at both the genetic and post-translational levels. The amidases of *Brevibacterium* sp. R312, *Ps. aeruginosa* and *Arthrobacter* sp. J1 appear to be regulated at the genetic level only. All are induced by amides (although inducer specificities vary) and repressed by acetate (but not ammonia); *Ps. aeruginosa* is also subject to strong catabolite repression by certain TCA cycle intermediates and *Brevibacterium* sp. R312 is repressed by other organic acids, but not glucose. Comparison of the N-terminal amino acid sequence of the *M. methylotrophus* acetamidase (Silman, 1990; Silman et al., 1991) with the amidases of *Ps. aeruginosa* (Ambler et al., 1987; Brammar et al., 1987) and *Brevibacterium* sp. R312 (Soubrier et al., 1992) shows that these sequences exhibit substantial homology. The latter two organisms show absolute identity over the first 19 amino acid residues (and > 80% strict identity across the complete primary sequence) whilst the sequence from the former organism shows two changes only in this region. The complete primary sequence of the *M. methylotrophus* acetamidase is unknown, although it is likely that it will be highly homologous to the *Ps. aeruginosa* and *Brevibacterium* sp. R312 proteins given the N-terminal homology. In view of this supposition, it is interesting to note that the acetamidase of *M. methylotrophus* exhibits certain regulatory differences to the *Ps. aeruginosa* and *Brevibacterium* sp. R312 amidases in that it is repressed by ammonia (but not acetate), and its activity is further reversibly regulated *in vivo* by some cryptic post-transcriptional/post-translational mechanism in response to ill-defined growth conditions. It is known that the culture ammonia concentration is critical and that fluctuating acetate levels are probably also implicated in the control of enzyme activity (see Carver & Jones, 1993). No other microbial acylamidase has been reported to exhibit an analogous regulatory system, and the modulation of *M. methylotrophus* acetamidase activity therefore appears to be unique within this particular group of amidases. The activity of certain other microbial amidases are also reversibly regulated at the protein level by non-covalent mechanisms involving ionic effects and/or other low-MW effector molecules. These enzymes include the *N*-acetylmuramoyl-L-alanine amidases of *Staphylococcus simulans* 22 (Bierbaum & Sahl, 1987), *Bacillus subtilis* 168 trpC2 (Foster, 1991) and *Streptococcus pneumoniae* (Briese & Hakenbeck, 1985), all of which are bacterial autolysins exhibiting the ability to hydrolyse peptidoglycan.

The cryptic reversible modulation of acetamidase activity in *M. methylotrophus in vivo* has been termed 'switch-on/switch-off' by analogy with the reversible regulation of nitrogenase activity in *Rhodospirillum rubrum* (see Ludden & Roberts, 1989). This organism is capable of fixing atmospheric N₂ by virtue of its nitrogenase enzyme complex, but will preferentially utilize other available nitrogen sources, such as ammonia and glutamine, by switching-off the more energetically-expensive nitrogenase. Switch-off of nitrogenase *in vivo* occurs in response to elevated ammonia concentrations (and under conditions of darkness and uncoupled respiration) and continues until the ammonia has been depleted from the culture, at which time nitrogenase activity is reactivated. The *M. methylotrophus* acetamidase shows a phenotypically-similar response to excess ammonia (see Chapter 1) and it is probably significant that both of these enzymes are involved with nitrogen assimilation. Primary
regulation of nitrogenase activity involves reversible covalent post-translational modification of dinitrogenase reductase by ADP-ribosylation (catalysed by dinitrogenase reductase ADP-ribosyltransferase [DRAT]) in response to ammonia, which renders the nitrogenase inactive. Reactivation occurs by removal of the ADP-ribosyl group (catalysed by dinitrogenase reductase-activating glycohydrolase [DRAG]), which can be mimicked in vitro by heating the enzyme at 60°C (Pope et al., 1985); a process which is also superficially very similar to the heat-reactivation of low-activity acetamidase in the presence of activator fraction (see Chapter 4). However, the regulation of nitrogenase activity is complex, and the metabolic signals eliciting switch-off have not been fully defined, although it is known to be part of a regulatory cascade which appears to be further subject to the effects of certain cations and nucleotides. Conversely, the nitrogenase activity of *Methylosinus trichosporium* and *Rhodobacter sphaeroides* is also reversibly switched-off in response to excess ammonia, but there is no evidence for covalent modification of nitrogenase in these two organisms (Yoch et al., 1988). These observations suggest that either the attachment of the modifying group in *M. trichosporium* and *R. sphaeroides* is labile and does not survive in vitro manipulation or that the switch-off mechanism in these organisms is different to that of *R. rubrum*. Investigation of ammonia-mediated switch-off of various enzymic activities in different organisms therefore suggests that superficial similarities exist between these regulatory systems. However, the regulatory mechanism(s) appears to involve multiple control elements and is complex, and it is likely that phenotypically-similar switch-off systems might be characterized by diverse underlying regulatory mechanisms.

The molecular basis of certain of the switch-off systems described above appears to contain elements of covalent post-translational regulation in coupling with allosteric effectors, but currently remains elusive in other organisms. The possibility that switch-off of acetamidase activity in *M. methylotrophus* occurs by ADP-ribosylation has been previously discounted, as has the covalent modification of this enzyme by various other chemical groups (Silman, 1990). However, the putative covalent modification of acetamidase has not been exhaustively studied and there exist certain other candidate modifying groups that cannot currently be discounted. These include the post-translational incorporation of D-amino acids into gene-encoded peptides (see Mor et al., 1992), amino acid side chain methylation (see Paik et al., 1980) and post-translational deamidation of glutamine residues to yield methyl-accepting glutamate residues which has been implicated in the control of bacterial chemotactic efficiency (Park et al., 1990). Finally, carboxyl-terminus modification remains to be investigated with possible alterations including amidation of certain amino acids, and limited C-terminal removal of amino acids by proteolysis.

The technique of Electrospray Mass Spectrometry (ESMS) is a recent technological advancement that allows the accurate measurement of proteins of MW in the approximate range 0.04-200 kDa (Geisow, 1992). The prime characteristics of this technique are its mass accuracy, which is approximately 0.01% of the mass calculated from the primary sequence for a protein in the mass range 40-150 kDa, and its theoretical resolution which is approximately 0.1% of the protein mass. ESMS does not require proteins to be fragmented.
for analysis, and the sensitivity is such that miniscule quantities of protein (≥ 1pmol) can be successfully analysed. ESMS therefore potentially constitutes a very powerful analytical tool which has already proved invaluable in the characterization of a number of different covalent post-translational modifications.

This Chapter describes the purification of acetamidases from cultures of *M. methylotrophus* exhibiting high- and low whole cell specific activities, and describes their properties in relation both to the acetamidases purified previously from wild-type and mutant strains of the same organism, and also to the properties of various other amidases. These enzymes were primarily purified so that a systematic comparison of the physico-chemical properties of high- and low-activity enzymes (switched-on and switched-off enzymes) could be undertaken in order to identify differences between the different enzymic forms which might intimate the nature of the putative modifying group(s). The switch-off process, and its apparent attributes *in vitro*, are discussed in relation to physico-chemical differences observed between high- and low-activity acetamidase samples purified in this study and others.
3.2 Purification of acetamidase from *M. methylotrophus*

Acetamidase was purified approximately twenty-fold from wild-type cells of *M. methylotrophus* (Materials & Methods) from cell samples kindly donated by ZENECA Bio Products (Billingham, UK) and J. Mills (author's laboratory). Cultures were grown either at high cell density (10-12 mg dry wt ml\(^{-1}\)) or low cell density (0.5-1.0 mg dry wt ml\(^{-1}\)) under industrial- or laboratory-scale continuous culture conditions at low-dilution rate (\(D = 0.1\) hr\(^{-1}\)). Industrial cell samples, supplied as frozen biomass (-20°C) and grown under acetamide-limitation with an excess of methanol or under pseudo-dual methanol-acetamide limitation, exhibited relatively high- and low (switched-on and switched-off) whole cell specific acetamidase activities respectively. Conversely, cultures grown at low cell density under true dual methanol-acetamide limitation exhibited only high (switched-on) whole cell specific acetamidase activity, as was also the case for similar acetamide-limited cultures, and these samples were harvested overnight on ice without freezing (see Table 3.1).

Routine purifications of low-activity acetamidase from the same frozen cell paste sample (cultured under pseudo-dual methanol-acetamide limited and high cell density conditions) consistently yielded high quality > 95% pure protein preparations of reproducibly low specific activity in the range 6-15 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\)) (Fig. 3.1). These results were in accordance with the purification and specific activity (15.0 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\))) described by Silman (1990). Further identical purifications carried out in this study and others (ZENECA Bio Products; M.A. Carver, personal communication), using similar starting material, yielded acetamidase preparations that exhibited significantly lower activities and which appeared to represent acetamidase samples that were more highly switched-off. All low-activity preparations exhibited the characteristic approximately > 200% reactivation of specific activity to a level commensurate with, or approaching that of the high-activity enzyme (49.5 \(\mu\)mol min\(^{-1}\) (mg protein\(^{-1}\))), as described by Silman (1990) and Silman *et al.* (1991). Other characteristics of the purification, such as the loss of the ability to reactivate fully low-activity acetamidase after the first anion-exchange step by heat in the absence of the activator fraction, were also as indicated by Silman (1990). Thus, the specific activities of purified low-activity acetamidase samples were generally as predicted from the low whole cell specific activities of the cultures from which they were purified.

Initial attempts to purify high-activity acetamidase from crude partially-pure samples (gifted by Dr. N.J. Silman) stored at \(-20°C\) yielded unexpected results. Homogeneous preparations (Fig. 3.1) were isolated exhibiting specific activities much higher than that reported by Silman (1990) and Silman *et al.* (1991). These very high-activity acetamidase samples also showed a pronounced loss of activity upon heating at 60°C in the presence of activator fraction (Table 3.1) rather than the stimulation observed with low-activity acetamidase under identical conditions. Interestingly, yet frustratingly so, additional attempts to purify high-activity acetamidase from high-activity whole cells grown either under acetamide-limitation (irrespective of the source of starting material) or true dual methanol-acetamide limitation (low cell density culture starting material) using an identical purification protocol and the same
Table 3.1 Comparison of specific activity and reactivation properties exhibited by different samples of acetamidase

The specific activities of representative acetamidase samples purified from cell samples grown under the culture conditions indicated were measured before and after heating (60°C, 1 h) in the presence of a standard amount of partially-pure activator fraction. Assays were performed with acetamide as substrate at 37°C (Materials & Methods). The changes in activities after attempted heat-reactivation are expressed as a % of the unheated activity. The intracellular acetamidase concentration was up to 4.3% of total cell protein*. * Data taken from Silman (1990) and J. Mills (unpublished) respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole cell activity (μmol min⁻¹ [mg dry wt]⁻¹)</th>
<th>Pure acetamidase activity (μmol min⁻¹ [mg protein]⁻¹)</th>
<th>Δ Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetamide-limited</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(low cell density)</td>
<td>—</td>
<td>105.5</td>
<td>-40.5</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>87.7</td>
<td>-16.8</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>26.9</td>
<td>+34.2*</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>49.5</td>
<td>-</td>
</tr>
<tr>
<td>Acetamide-limited</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(high cell density)</td>
<td>2.7</td>
<td>19.9</td>
<td>+83.9</td>
</tr>
<tr>
<td>True dual methanol-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acetamide limited (low cell density)</td>
<td>1.7</td>
<td>9.6</td>
<td>+291.7*</td>
</tr>
<tr>
<td>Pseudo-dual methanol-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acetamide limited (high cell density)</td>
<td>0.6</td>
<td>14.8</td>
<td>+218.2</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>15.0</td>
<td>+198.7*</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>8.1</td>
<td>+311.1</td>
</tr>
</tbody>
</table>

* Data taken from Silman (1990) and J. Mills (unpublished) respectively.
Wild-type cells of *M. methylotrophus* grown under the appropriate nutrient limitation in continuous culture (D = 0.1 h⁻¹) were harvested, washed, disrupted and then subjected to differential centrifugation to produce a high-speed supernatant from which acetamidase was purified by anion-exchange and gel-filtration FPLC. Purification and SDS-PAGE of samples were as described in Materials and Methods. Tracks: 1, MW standards; 2, Superose 6 peak-activity high-activity acetamidase; 3, Superose 6 peak-activity low-activity acetamidase; 4, MW standards. The numbers underneath the tracks indicate representative specific activities (μmol min⁻¹ [mg protein]⁻¹) of pure high- and low-activity acetamidases.
FPLC equipment as before, all yielded further unexpected results. These purifications were notable for an inability to isolate protein preparations exhibiting the expected kinetic properties of pure high-activity acetamidase, as reported by Silman (1990). All attempts to achieve this aim ultimately yielded pure samples exhibiting a wide range of lower specific activities, viz. 10-27 μmol min⁻¹ (mg protein)⁻¹, and these samples were also characterized by the ability to be heat-reactivated (Table 3.1). *N.B.* the effect of heat-reactivation on the high-activity acetamidase purified by Silman (1990) has not been reported; see Discussion.

A comparison of the diverse specific activity and reactivation properties exhibited by different preparations of purified acetamidase is shown above (Table 3.1), and analysis of the data contained therein clearly shows the very high- and anomalously low specific activities, viz. 88-106 μmol min⁻¹ (mg protein)⁻¹ and 10-27 μmol min⁻¹ (mg protein)⁻¹ respectively, of samples that were expected to show specific activities similar to that of the original high-activity sample (Silman, 1990). These data also highlighted the unexpected heat-reactivation properties of the latter samples by virtue of their significant reactivation when heated with the activator fraction, in contrast to the 20-40% loss of activity shown by very high-activity samples under the same conditions. A relationship was apparent such that a decrease in the specific activity of a purified acetamidase sample correlated with a relatively more positive Δ activity value after heat-treatment. The samples with anomalously low specific activities therefore appeared qualitatively more similar to low-activity samples and these results are reminiscent of those described for different purifications of RubisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) from R. sphaeroides (Wang & Tabita, 1992a) in which inactivated RubisCO was found to reactivate during purification, although the change of activity during RubisCO purification was reversed in direction (switch-on *cf.* switch-off) to that exhibited by high-activity acetamidase samples. These workers showed that certain preparations of RubisCO purified from starting material containing inactivated RubisCO were found to exhibit variable elevated specific activities depending on the amount of inactivated enzyme that became reactivated during purification, despite the precautions taken to prevent this.

The attempted purification of high-activity acetamidase from different samples of starting material that exhibited genuinely high whole cell acetamidase activities unexpectedly yielded both very high-activity samples (on two occasions only; 87-106 μmol min⁻¹ [mg protein]⁻¹) and a wide range of lower activity samples (exhibiting specific activities in the range 10-27 μmol min⁻¹ [mg protein]⁻¹); the reduced specific activity of these latter samples, in comparison to samples of higher activity, was attributed to the elevated levels of switch-off of activity that occurred during purification (see section 3.3.1.1), as these samples underwent significant heat-reactivation of activity as described above. In general, the purification of acetamidase was characterized by the isolation of pure samples exhibiting a very wide range of specific activities (8-106 μmol min⁻¹ [mg protein]⁻¹). It was therefore concluded that the reproducible isolation of very high- and high-activity acetamidases of a given specific activity was not possible under the conditions employed due to the variable extents of switch-off which occurred in different purifications (and which was exacerbated by further activity loss.
through post-purification switch-off; see section 3.3.1.2). Thus, purified acetamidase samples from high-activity whole cells showed highly-variable specific activities which were generally significantly lower than those predicted from the specific activity of the original high-activity starting material. However, in marked contrast to this, low-activity acetamidase could be isolated reproducibly from whole cell samples exhibiting low specific activities and the specific activity of the purified samples was generally as predicted from the low specific activity of the purification starting material.

In the results presented below the terms 'high-activity' and 'low-activity' are used arbitrarily to define samples respectively exhibiting specific activities ≥ 35μmol min⁻¹ (mg protein)⁻¹ and variable inactivation after attempted heat-reactivation, and samples with specific activities ≤ 20μmol min⁻¹ (mg protein)⁻¹, some of which also exhibited a tendency towards further reduction of specific activity ('switch-off'). In each case, the loss of activity resulting from 'switch-off' was wholly recoverable (to an activity level approaching that of high-activity acetamidase) by heat-reactivation. Samples with activities intermediate between high- and low-activities, which exhibited similar 'switch-off' and heat-reactivation properties to the low-activity enzyme were also purified. The term 'switch-off' is used to describe the process by which acetamidase samples purified from cultures exhibiting high whole cell specific activities subsequently showed a very wide range of significantly diminished specific activities in comparison to high-activity acetamidase, and the (unexpected) ability to be quantitatively heat-reactivated in the presence of activator fraction, in a similar fashion to that elucidated above for the low-activity enzyme.

3.3 Physico-chemical analysis of acetamidase

3.3.1 In vitro switch-off of acetamidase activity

3.3.1.1 Switch-off of acetamidase activity during purification

A note on the nature and complexity of the switch-off event, and the difficulties of its detection, is deemed prudent at this point in order to clarify the discussion of results presented below (see also Discussion). As is shown below (section 3.3.3) high- and low-activity acetamidases exhibit significantly different thermostabilities at 60°C (the temperature at which samples are incubated during the reactivation assay) with the low-activity form exhibiting much higher stability at this temperature than the high-activity form. This study has shown (see Table 3.1) that high-activity acetamidase rapidly loses activity (presumably due to thermal denaturation) and exhibits no reactivation under conditions (incubation for 1 h at 60°C in the presence of activator fraction) which exerted a significant stimulatory effect on the low-activity form of the enzyme. Since the switch-off event is apparently characterized by the conversion of a proportion of high- to low-activity acetamidase molecules, the overall change in the specific activity of a population undergoing switch-off after heat-reactivation will presumably be governed by a balance between a loss of activity (primarily due to the
greater thermolability of high-activity molecules) and an increase in activity from heat-reactivation (low-activity molecules only). Thus, observed specific activities of reactivated samples shown in this study represent a 'snapshot' of the activity balance attained between differential thermolability and reactivation of high- and low-activity acetamidase molecules at the time of assay.

The occurrence and magnitude of switch-off of high-activity acetamidase during purification attempts was manifest in an intrinsically variable fashion. The potential effect of nutrient selection pressure, culture cell density and past nutrient status history of the culture on the propensity of high-activity acetamidase to undergo switch-off during purification was addressed by purifying batches of acetamidase from cells grown in continuous culture under acetamide-limitation (high or low cell density culture) and true dual acetamide-methanol limitation (low cell density culture). All different high-activity samples investigated in this study exhibited the switch-off phenomenon, and no clear correlation was found between the aforementioned factors and the conditions under which switch-off was observed during the subsequent purifications. Switch-off was therefore observed variably at different stages in separate purifications in a manner that appeared superficially to be independent of the nutrient limitation and the source of the starting material. However, since only one switch-off profile has been characterized in detail (Table 3.2) the observed variations may be a true reflection of the effects of particular growth conditions that are currently obscure. These results are therefore potentially similar to those of Hutchinson and Goodwin (1993b) who reported that the stability of methanol- and methylamine dehydrogenases \textit{in vitro} varied depending on the growth conditions in \textit{M. methylotrophus}, as did the stabilities of certain thermolabile mutant versions of these enzymes.

Attempts to correlate the incidence of switch-off of partially-pure or pure high-activity acetamidase samples with a particular characteristic of the purification protocol were largely unsuccessful. The extent of switch-off in fractions of a representative high-activity acetamidase purification from starting material grown under acetamide-limitation was investigated (Table 3.2). Two clear-cut trends arose from this purification and others. The first concerned the absolute requirement for cell disruption to initiate switch-off, \textit{i.e.} the apparent absence of switch-off in any of the high-activity acetamidase whole cell samples used as the starting material for purifications, as adjudged from unheated and heated whole cell specific activities (high-activity whole cells generally showed 30-70 % loss of activity as a result of heating at 60°C for 1 h, but also occasionally showed little or no loss of activity under identical conditions; N.R. Wyborn, J. Mills & C.W. Jones, unpublished). This was tested by dividing a high-activity culture (low cell density, grown under acetamide-limitation) and storing one half of the culture at 4°C whilst acetamidase was purified rapidly from the other half. Results showed that the purified acetamidase showed a 57% increase in total sample activity after heat-reactivation compared to an 80% decrease in the whole cell sample activity assayed at the same time. It was therefore concluded that maintenance of acetamidase in the high-activity form was adversely affected by cell breakage. The second obvious trend highlighted the general decrease in the loss of total sample activity remaining after heat-
Table 3.2 Switch-off of high-activity acetamidase during FPLC-purification

Wild-type cells of *M. methylotrophus* grown under acetamide-limitation in continuous culture (D = 0.1 h⁻¹) were harvested, washed, disrupted and then subjected to differential centrifugation to produce a high-speed supernatant from which acetamidase was purified by anion-exchange and gel-filtration FPLC. Samples at each stage of the purification (stored at 4°C) were mixed with a standard amount of partially-pure activator fraction (except for whole cells) and heated at 60°C for 1 h to detect any switch-off of high-activity acetamidase that had occurred. All assays were performed at 37°C with acetamide as substrate, and activities are expressed as the difference between unheated and heated total sample activities as a % of the unheated total sample activity. Prior to gel-filtration chromatography, the final anion-exchange peak-activity sample was divided into two; one half was subjected to concentration and ultrafiltration by spin column centrifugation (a), and the other half by stirred cell ultrafiltration (b). The whole cell specific activity was 2.5 μmol min⁻¹ (mg dry wt)⁻¹ and pure sample specific activities were (a) 26.9 μmol min⁻¹ (mg protein)⁻¹ and (b) 39.3 μmol min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetamidase activity +Heat (μmol min⁻¹ [ml sample]⁻¹)</th>
<th>Δ Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>146.6</td>
<td>-96</td>
</tr>
<tr>
<td>Broken cells</td>
<td>98.8</td>
<td>-41</td>
</tr>
<tr>
<td>High-speed supernatant</td>
<td>123.8</td>
<td>-32</td>
</tr>
<tr>
<td>Desalted anion-exchange peak-activity sample</td>
<td>14.8</td>
<td>-16</td>
</tr>
<tr>
<td>Final anion-exchange peak-activity sample</td>
<td>8.6</td>
<td>-40</td>
</tr>
<tr>
<td>Gel-filtration peak-activity (pure) sample (a)</td>
<td>0.5</td>
<td>+20</td>
</tr>
<tr>
<td></td>
<td>(b) 10.8</td>
<td>-4</td>
</tr>
</tbody>
</table>
reactivation as the purification proceeded. These patterns are evident in Table 3.2 which shows the initial substantial loss of activity (96%) in the whole cell sample (specific activity 2.5μmol min⁻¹ [mg dry wt]⁻¹) after attempted reactivation, thereby confirming the presence of high-activity acetamidase in these cells. Further analysis showed that after cell-breakage there was an immediate and significant decrease in the Δ activity value, i.e. the Δ activity value became less negative, which was also generally true throughout the purification, and which was presumed to be indicative of partial switch-off of the high-activity acetamidase. Desalting of acetamidase FPLC samples (by PD-10 gel-filtration chromatography) elicited a further smaller decrease in the loss of activity after attempted reactivation, but this effect was apparently reversed after the final anion-exchange step, as the measured loss of total sample activity after heat-reactivation returned to the pre-desalting level. This was suggestive of an ionic effect in which the level of switch-off was enhanced by removal of salt (KCl) from the enzyme sample, and subsequently diminished by re-introduction of salt during elution from the final anion-exchange step. High ionic strength has also been reported previously (Shukuya & Schwert, 1960) to decrease the cold-inactivation of glutamate decarboxylase from E. coli, and higher concentrations of KCl have been shown to protect against loss of activity from the unrelated enzyme pyruvate carboxylase from chicken liver mitochondria (Irias et al., 1969). Alternatively, since this putative ionic effect has not been repeated it may be interpreted as an artefact, but this seems unlikely because certain partially-pure or pure high-activity acetamidase samples also appeared to exhibit enhanced levels of switch-off after diafiltration (section 3.3.6) and/or ultrafiltration spin column treatment (see below). The putative effect of the latter treatment on acetamidase samples is also highlighted in Table 3.2 wherein after the final anion-exchange step, but prior to gel-filtration chromatography, the pooled peak-activity acetamidase fractions were divided equally and concentrated rapidly by two different ultrafiltration methods (but not diafiltered) - one half by stirred cell ultrafiltration and the other by spin column centrifugation - to determine the effect of the different treatments on the level of switch-off in these samples. Results showed that when the level of switch-off of pure samples was subsequently assayed approximately 3-4 days later an increase in total activity was measured after reactivation for the spin column sample cf. a small loss of total activity in the stirred cell sample after attempted reactivation. Thus, it appeared possible that spin column ultrafiltration elicited an increase in the level of switch-off of desalted samples it is tempting to speculate on the involvement of a small molecule(s) in the regulation of acetamidase activity in view of results described above, and those presented below (see Discussion; Chapter 4).

3.3.1.2 Post-purification switch-off of acetamidase activity

Data presented below (Fig. 3.2) contrasts the rate and degree of post-purification switch-off of representative samples of high- and low-activity acetamidases from M. methylotrophus over time, and highlights the switch-off of activity in high-activity samples in comparison to
low-activity enzyme preparations. The stability of the low-activity enzyme over storage, as evinced by the negligible variation in its specific activity, shows that this form of the enzyme generally did not exhibit post-purification switch-off. Further to the switch-off observed during purification of high-activity acetamidase it also became apparent that switch-off continued to occur in various pure preparations. Pure samples of high-activity acetamidase stored at -20°C were found to undergo a very slow spontaneous switch-off of specific activity over a period of months/years of storage at this temperature, which was possibly related to freezing/thawing of these enzyme preparations (see section 3.3.3). Similarly, intermediate- and some low-activity acetamidase samples also exhibited post-purification switch-off, although the rate at which it occurred was much faster than that exhibited by higher activity samples (data not shown). All such samples became heat-reactivatable in an identical fashion to low-activity acetamidase samples. Moreover, the level of switch-off in these samples appeared to increase in a time-dependent fashion over the duration of storage. A similar situation appears to exist for pyruvate carboxylase from chicken liver mitochondria (Irias et al., 1969), although the inactivation time-scale is much more rapid than that observed for the switch-off of acetamidase activity. Pyruvate carboxylase is rapidly inactivated by incubation at low temperature, but can be almost completely reactivated by rewarming under appropriate conditions. The abolition of enzyme activity was shown to be due to rapid and reversible dissociation of the tetrameric enzyme into four protomers involving the intermediate formation of an inactive tetramer. Pure preparations of this enzyme also showed a multiplicity of different catalytic activities which were attributed to activity losses during storage and handling and which were significantly less than the activities measured for the same samples immediately after purification. Various acetamidase samples showed qualitatively similar traits as described above.

3.3.2 Kinetic properties of high- and low-activity acetamidas

The kinetic properties of pure preparations of high- and low-activity acetamidas were investigated to determine the effect of the level of switch-off on the affinity of the enzyme for acetamide and acrylamide (Table 3.3). Comparison of high- and low-activity acetamidase $K_m$ values for acetamide (0.7-1.1 mM, and 0.1-0.5 mM respectively) showed a general decrease in the $K_m$ for more-highly switched-off samples; i.e. an increase in the affinity of each enzyme for the substrate (acetamide) generally correlated with a diminution in $V_{\text{max}}$. Similarly, a decrease in $K_m$ values for acrylamide also appeared to correlate with enhanced switch-off. Thus, it appeared that in general an increase in the level of switch-off in a given sample was accompanied by a decrease in the $K_m$, although no strong correlation was apparent between the magnitude of switch-off (diminution in $V_{\text{max}}$) and the magnitude of the reduction in the $K_m$. $K_{\text{cat}}$ values for all enzyme samples were significantly lower than that reported for the original high-activity enzyme (Silman, 1990) with both acetamide and acrylamide. Specificity constants ($K_{\text{cat}}/K_m$) were also calculated to provide an indication of the catalytic efficiency of each enzyme preparation and it was found that greater efficiency
Figure 3.2 Post-purification switch-off of acetamidase activity

The specific activities of pure high- and low-activity acetamidase samples stored at -20°C for variable time periods were measured at 37°C with acetamide as substrate (Materials & Methods). (a) high-activity acetamidase (— O — ); (b) low-activity acetamidase (— — ).
was associated with acetamide as substrate for both high- and low-activity forms of the enzyme. Comparison of the specificity constants of high-activity acetamidases with acetamide as substrate showed only a minor variation in these values, which was found to be due to a reduction in $K_m$ offsetting the reduction in $K_{cat}$. Measurement of the analogous specificity constants of the low-activity samples showed much greater variation (over a four-fold range), which was mainly due to non-uniform decreases in the $K_m$ and $K_{cat}$ values for a given sample. Calculation of specificity constants for all samples with acrylamide as substrate showed greater variation (over an eleven-fold range) than with acetamide. Most samples exhibited specificity constants that were similar to that of the original high-activity enzyme (9 s$^{-1}$ mM$^{-1}$) described by Silman (1990), again because reductions in $K_m$ were offset by reductions in $K_{cat}$. In contrast, the highest specificity constant (45 s$^{-1}$ mM$^{-1}$) for acrylamide resulted from a substantially reduced $K_m$ and an essentially unaltered $K_{cat}$. It was therefore concluded that high- and low-activity acetamidases exhibit significant differences in their kinetic properties. Since the physico-chemical basis of these kinetic differences are not known it is possible that the physiological basis of the observed differences between high-activity samples that switched-off rapidly during purification, and those that switched-off relatively slowly during storage at -20°C (see section 3.3.1.2) might have been the selection of mutant acetamidases in continuous culture with altered properties, as has been described previously for this organism by Silman (1990) and Silman et al. (1989, 1991). The kinetic properties of the purified mutant enzymes from these latter studies showed that whilst they are not identical to those of the samples described above, specificity constants are similar which indicates that the efficacy in vivo of all the acetamidase samples purified in this study, and the mutant acetamidases, might plausibly be considered to have been roughly equivalent. This supports the hypothesis that the high-activity enzymes that subsequently exhibited switch-off in this study may have resulted from mutation and selection. It is also noteworthy that the kinetic properties of one of the high-activity acetamidase samples (see above) appeared to resemble those of the *M. methylotrophus* mutant MM15 enzyme (similar $K_{cat}$ and eight-fold lower $K_m$ for acrylamide cf. original high-activity enzyme; Silman, 1990; Silman et al., 1991; Chapter 1), which suggests that potentially similar physico-chemical attributes exist between these two forms of acetamidase. Kinetic aspects of these samples and the possibility that purified acetamidases from this study that exhibited switch-off may have originated from mutant strains is considered further in the Discussion.

### 3.3.3 Investigation of acetamidase thermostability

The thermostability profile and extrapolated $t_{1/2}$ value (3.2 h) at 60°C of the original high-activity acetamidase (in addition to certain mutant versions of this enzyme) has been reported previously (Silman, 1990; Silman et al., 1991). In this study, high- and low-activity acetamidases were incubated under similar conditions to those of Silman (1990), but for a greater length of time (3.5 h cf. 2 h) and with a longer sampling period (approximately every 60 min cf. 20 min) to characterize more-fully the effect on acetamidase of prolonged
Table 3.3 Comparison of the kinetic properties of pure high- and low-activity acetamidases

The $K_m$, $K_{cat}$ and $V_{max}$ values were determined for certain of the high- and low-activity acetamidase samples purified in this study with acetamide and acrylamide as substrates at 37°C (Materials & Methods). $K_{cat}$ values were calculated from $V_{max}$ values (Hanes plot) using a native MW of 155kDa. $V_{max} = \mu$mol min$^{-1}$ (mg protein)$^{-1}$; $K_m = \mu$M; $K_{cat} = s^{-1}$; $K_{cat}/K_m = s^{-1}$ mM$^{-1}$; — = not available. * Data taken from Silman (1990) (the specific activity of this enzyme with acetamide as substrate was 49.5\mu$mol min$^{-1}$ (mg protein)$^{-1}$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetamide</th>
<th></th>
<th>Acetamide</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}$</td>
<td>$K_m$</td>
<td>$K_{cat}$</td>
<td>$K_{cat}/K_m$</td>
</tr>
<tr>
<td>High-activity</td>
<td>35.1</td>
<td>0.7</td>
<td>91</td>
<td>130</td>
</tr>
<tr>
<td>Low-activity</td>
<td>18.0</td>
<td>0.3</td>
<td>47</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>0.4</td>
<td>23</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>0.5</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>6.4</td>
<td>0.1</td>
<td>17</td>
<td>170</td>
</tr>
</tbody>
</table>
exposure to this temperature (Fig. 3.3). This property is of particular importance as it has a
direct effect on the heat-reactivation of low-activity acetamidase (Chapter 4). These data
clearly showed the initial extreme thermolability of the high-activity form (70% loss of
activity after 1 h incubation) compared to the low-activity form which exhibited a small
increase in activity after 1 h; this increase in activity has been termed 'autoreactivation' and is a
reproducible phenomenon representing a small, but significant, reactivation of acetamidase
activity in the absence of activator fraction (see Chapter 4). After the first hour of incubation,
the profiles exhibited essentially the same features, and it was interesting that although in
general terms the high-activity form was found to be much more thermolabile than the low-
activity form (losing 91% of its total activity in 3.5 h cf. 51% loss of activity in the low-
activity form), the majority of the activity-decay which occurred in this sample was completed
after 1 h incubation at this temperature. After this time the further loss of activity was
relatively minimal and appeared to mimic the pattern of specific activity loss exhibited by the
low-activity form. Conversely, after the initial small increase in activity the low-activity form
exhibited no sudden decreases in activity, but rather only a gradual loss of activity, and can be
considered to be highly stable at this temperature. Thermostability data from this study
therefore conflicts with the extrapolated $t_{1/2}$ value (3.2 h) reported by Silman (1990) and
Silman et al. (1991) for high-activity acetamidase, and since the high-activity sample in this
study exhibited a 70% loss of activity in 1 h and an elevated specific activity (87 μmol min$^{-1}$
[mg protein]$^{-1}$) it is tentatively suggested that it was probably more similar to the mutant
MM8 acetamidase studied by Silman (1990) which exhibited a $t_{1/2}$ value of 1.5 h at 60°C and
an unheated specific activity of 11 μmol min$^{-1}$ (mg protein)$^{-1}$.

In addition, the stability of acetamidase at lower temperatures has also been investigated.
Silman (1990) reported that a single freeze-thaw cycle (-20°C) inhibited the original high-
activity acetamidase by < 1% compared with 85% for the mutant MM15 enzyme
(unfortunately, it is not known whether any of the activity lost in these experiments was
recoverable by heat-reactivation). Extending this work, results from this study also indicated
that multiple short-term (≤ 5) rapid freeze-thaw cycles (-20°C), over a period of several days,
had little effect on the specific activity and reactivation properties of either form of
acetamidase. Multiple cycles of freeze-thaw over the longer-term (months/years), however,
suggested a possible correlation with switch-off in high-activity acetamidase (Fig. 3.2),
although it has not been determined whether switch-off in these samples was a consequence
of freeze-thaw or whether it was occurring independently of freeze-thaw, i.e. spontaneously at
-20°C, as the level of switch-off could only be ascertained by reactivation assay which
obviously necessitated thawing of the sample. Similarly, increases in the level of switch-off of
certain intermediate- and low-activity acetamidase samples have been observed and tentatively
ascribed to the effects of short-term storage at -20°C/freeze-thaw (cf. mutant MM15 enzyme
above), although the rate of any switch-off event in these instances appeared to be accelerated
in comparison to high-activity acetamidase samples. In contrast to these findings, there
appeared to be no deleterious effect on the specific activity and reactivation properties of very
low-activity acetamidase (specific activity < 12 μmol min$^{-1}$ [mg protein]$^{-1}$) over long-term
Figure 3.3 Differential thermostability of high- and low-activity forms of acetamidase at 60°C

Pure samples of high- and low-activity acetamidase (approximately 40μg of each) were diluted to 900μl final volume with 20mM-bis-Tris buffer, pH 6.8 in duplicate and incubated at 60°C for 0-3.5 h. Aliquots (200μl) were removed from homogenized heated samples at timed intervals and assayed for amidohydrolase activity at 37°C with acetamide as substrate (Materials & Methods). High-activity acetamidase (–—–); low-activity acetamidase (–––).
storage at -20°C coupled with the effects of multiple freeze-thaw cycles (Fig. 3.2). In addition, the effect of multiple (≤ 5) short-term 'snap' freeze-thaw cycles on dry-ice was also investigated using samples of high- and low-activity acetamidases. Results showed that this treatment caused a 10-20 % decrease in specific activity in both samples which was presumably caused by denaturation (rather than switch-off) as the activity losses were not recoverable by heat-reactivation. This was qualitatively similar to the effect of repeated freeze-thaw at -30°C on the amidase of Ps. aeruginosa which caused gradual loss of activity and concomitant protein precipitation (Brown et al., 1973). Thus, the fact that significant differences existed in the rate and pattern of change of specific activity in response to incubation at different temperatures (particularly 60°C) suggests that the different forms of acetamidase exhibit different physical and/or chemical properties.

3.3.4 Amidohydrolase substrate profiles of high- and low-activity forms of acetamidase

Amidohydrolase substrate profiles of high- and low-activity forms of acetamidase were carried out (Table 3.4), and consideration of the data contained therein shows that the substrate profiles of the two different forms of acetamidase are very similar. Acetamidase activity decreased in the order propionamide > acrylamide > acetamide > formamide > butyramide/urea except that the relative positions of propionamide and acrylamide were reversed for the low-activity form in comparison to the high-activity form. The relative activities of the high- and low-activity enzymes towards propionamide and acrylamide appeared to differ significantly, with the low-activity enzyme exhibiting reduced activity towards both of these substrates. A possible explanation of this difference may reside in a consideration of the relative sizes of the substrate molecules and their interaction with the active site of the enzyme. The best substrates (propionamide, acrylamide and acetamide) have carbon chains either 2 or 3 C atoms in length which appears to be the optimal length to promote maximal activity. Reduction of chain length to 1 C atom (formamide) significantly reduces activity and addition of a fourth C atom (butyramide) drastically reduces activity, as does replacement of the -CH₃ group with an -NH₂ group (urea cf. acetamide). These data may indicate that some structural or conformational difference exists between high- and low-activity forms of acetamidase such that the active site is made relatively more inaccessible to amides larger than acetamide in the low-activity enzyme in comparison to the high-activity enzyme. Thus, restricted access of certain substrates to the catalytic site potentially accounts for the reduced activity of the low-activity form of acetamidase towards propionamide and acrylamide. Alternatively, physical and/or chemical modification to catalytic/substrate binding residue(s) might generate differential affinities for particular substrates which could explain the observed discrepancies in activity towards propionamide and acrylamide shown by the two different enzymic forms of acetamidase.
The substrate profiles of highly-purified high- and low-activity acetamidase were measured at 37°C with a range of different amides (50mM final concentration) as described in Materials and Methods. Amides tested included: propionamide, acrylamide, acetamide, butyramide, formamide and urea. Activities are expressed as a % of the activity with acetamide as substrate for each separate profile.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amidohydrolase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-activity</td>
</tr>
<tr>
<td>Propionamide</td>
<td>202</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>146</td>
</tr>
<tr>
<td>Acetamide</td>
<td>100</td>
</tr>
<tr>
<td>Formamide</td>
<td>21</td>
</tr>
<tr>
<td>Butyramide</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Urea</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
3.3.5 Sedimentation velocity and equilibrium ultracentrifugation analysis of high- and low-activity forms of acetamidase

Pure samples of high- and low-activity acetamidase were submitted for sedimentation velocity ultracentrifugation analysis (Materials & Methods) to identify any conformational differences between the two forms of acetamidase by comparison of corrected sedimentation coefficients. Three independent S-value determinations were made for paired samples of low- and high-activity enzymes respectively, co-centrifuged under identical conditions. The resultant S-values of 9.59 S and 9.04 S, 10.14 S and 9.46 S, and 10.30 S and 9.98 S were substantially higher than the values obtained previously (Silman, 1990; Silman et al., 1991) for the wild-type high-activity acetamidase (6.63 S) and the mutant MM15 acetamidase (5.26 S). These data suggested that both forms of acetamidase had aggregated to form higher MW species that sedimented significantly faster than the acetamidase samples (presumably mono-tetramers) of Silman (1990), and that this aggregation probably occurred as a result of prolonged storage at -20°C. The high-activity sample exhibited a greater propensity to form aggregates as is evident from its consistently higher S-values in comparison to those of the low-activity form. A similar situation, involving the formation of multiple higher MW species after prolonged storage at -30°C or repeated freeze-thaw cycles has been described previously by Brown et al. (1973) for the Ps. aeruginosa amidase. These aggregation phenomena may be related to the reversible self-association reactions that have been described for a number of hysteretic enzymes which are important in the regulation of certain metabolic pathways (see Frieden, 1970). The rate of polymerization/depolymerization in such systems has not been extensively studied, but the rate-controlling step is quite slow in some cases and it has been postulated that it might involve a protein conformational change leading to a MW change. Considerable variation in S-values was apparent between independent experiments in this study (see above), which can probably be attributed to the formation of random aggregates of acetamidase of variable MW on different occasions in the two samples. Thus, even after sample dilution with buffer to a protein concentration estimated to be sufficient to shift any quaternary equilibrium in a direction favouring 95% disaggregation, measured S-values were still significantly higher than those expected for mono-tetramers. Identification of conformational differences using this method was therefore abandoned.

Identical acetamidase samples were also subjected to sedimentation equilibrium ultracentrifugation (Materials & Methods) to determine any difference in native MW between high- and low-activity forms of the enzyme. Two independent determinations of paired low- and high-activity samples co-centrifuged under identical conditions yielded MW values of 156kDa and 160kDa, and 218kDa and 175kDa respectively, compared with a MW of 155kDa reported previously by Silman (1990) for the high-activity enzyme. The variation in values between different experiments precluded meaningful conclusions being drawn concerning MW differences between the different enzymic forms of acetamidase. Further to this, observed MW values appeared to be inconsistent with S-values recorded for the same samples, i.e. the MW values were much lower than the predicted values for putative
aggregates of di- or tri-tetramers (approximately ≥ 310kDa) which was advanced above as an explanation for the elevated S-values. It therefore appears more likely that monomers were associating into oligomers (pentamers, hexamers etc.) which could possibly account for the increase in relative S-values. The exact specific activity and reactivation properties of the high-activity sample used in these experiments were not known; however, it was assumed by analogy with other high-activity samples undergoing switch-off during storage at -20°C that the specific activity would have been approximately 40μmol min⁻¹ (mg protein)⁻¹. In view of the probability that both samples used in these experiments exhibited variable levels of switch-off it is interesting to speculate on the involvement of the (dis)association of acetamidase sub-units with respect to the specific activity and reactivation properties of acetamidase.

3.3.6 Electrospray Mass Spectrometry (ESMS) of high- and low-activity forms of acetamidase

A direct attempt to elucidate the nature of the putative physico-chemical inequality between high- and low-activity forms of acetamidase was made through use of Laser Desorption Mass Spectrometry (LDMS; Matrix-Assisted Laser Desorption Ionization-Time Of Flight [MALDI-TOF]) and ESMS to measure accurately the MW of the acetamidase monomer. The prime concern for this work was the production of highly-purified desalted high- and low-activity acetamidase samples exhibiting switched-on and switched-off kinetic properties, so that any differences in measured MW could be interpreted in terms of the authentic properties of high- and low-activity samples. This proved particularly troublesome as many of the manipulative techniques either gave very poor protein yields, or appeared to adversely alter the acetamidase properties. Various different preparative protocols and techniques were tested, but without success, including reversed-phase HPLC and PD-10 desalting, and electro-elution from preparative SDS-PAGE. Lyophilization (-44°C, 9.6 mbar, 24 h; Edwards Freeze Dryer Modulyo) could not be utilized as it was found to selectively denature high-activity samples (causing an approximately 85% loss of specific activity which was not recoverable by heat-reactivation), and to effect a 77% reduction in the extent of heat-reactivation of the low-activity acetamidase. These results suggest that lyophilization variably perturbs the high- and low-activity enzyme properties, and reduces the efficacy of the heat-reactivation of the low-activity enzyme.

Further preparative problems were encountered with spin column diafiltration exchange solvents as 50% (v/v) methanol in deionized water significantly diminished membrane flow rates which necessitated excessively long spin times. Replacement of methanol by deionized water allowed diafiltration to proceed much faster, but unfortunately deionized water caused selective precipitation of high-, but not low-activity samples (suggesting that either the high-activity enzyme is more hydrophobic than the low-activity form or more prone to denaturation in water). Both forms of the enzyme appeared equally soluble in 50% (v/v) methanol in deionized water; and this was used as the solvent of choice; however, on occasions samples
appeared to undergo variable enhancement of switch-off after spin column treatment
(although the possibility that this was an effect of the altered pH, or some other property of
the solvent, rather than simply desalting the sample could not be discounted). Unfortunately,
these results were variable and not always reproducible, although heat-reactivation values of
900% and 150% were common for low- and high-activity samples respectively, after this
treatment. Dialysis of samples at 4°C against deionized water also proved problematic and
showed that the low-activity form of the enzyme was much more stable to this treatment than
the high-activity form (losing only 29% of its activity after 48 h dialysis cf. > 85% loss of
activity for the high-activity sample); activity losses were irreversible by heat-reactivation
suggesting that they probably resulted from denaturation of acetamidase rather than switch-
off.

A single MALDI-TOF determination was carried out in addition to multiple independent
ESMS mass determinations effected at three separate ESMS facilities with variable success.
On certain occasions, ESMS spectra were poor and exhibited high background non-specific
signals (‘noise’) which were sufficiently great to interfere with spectral analysis; these spectra
were discarded. The causative agent(s) of noisy spectra remains obscure, but was unlikely to
be related to salt-contamination or low sample protein concentration as care was taken to
optimize these factors prior to analysis. On other occasions, samples prepared in an identical
fashion to those giving poor results, yielded clear spectral peak progressions with minimal
background noise (Figure 3.4.a,b). All results (Table 3.5) were in good agreement with the
acetamidase sub-unit MW of 38kDa estimated using SDS-PAGE (Silman, 1990). However,
ESMS was more successful in producing MW data to the level of accuracy required than was
MALDI-TOF spectrometry and data from the latter technique was therefore discarded. The
mass accuracy of ESMS (0.01%) sets the theoretical mass accuracy limit for the acetamidase
monomer (38kDa) at approximately 4Da. Thus, taking this limit and the range of MW values
obtained from independent ESMS determinations into account, it was cautiously concluded
that the high- and low-activity forms of acetamidase differed in mass by ≤ 52Da, and
although the data do not unequivocally identify the modifying group to acetamidase they have
significantly narrowed the range of candidate groups. This eliminates larger modifications
resulting from ADP-ribosylation, adenylylation and glycosylation amongst other commonly-
found high-MW modifying groups, and suggests that progress might be made by specifically
targeting further protein chemistry work to the candidates listed in Table 3.7 (see
Discussion).

3.3.7 UV spectrophotometric analysis of high- and
low-activity forms of acetamidase

Ultraviolet spectral analysis of acetamidase was undertaken to compare the absorption
properties of high- and low-activity forms of acetamidase. This technique has previously been
employed to infer the covalent attachment of a nucleotide-containing modifying group (ADP-
ribose) to dinitrogenase reductase in *R. rubrum* (see Ludden & Roberts, 1989) through the
Highly-purified and desalted high- and low-activity acetamidase samples were submitted for analysis by LDMS (MALDI-TOF) and ESMS. The best available paired (average) monomer MW values from independent determinations for each technique are shown below (standard deviations [SD] are given in parentheses; MW difference was defined as the maximum difference between adjusted paired MW values with one SD taken from absolute MW determinations in either direction).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Monomer MW (Da)</th>
<th>MW difference (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low-activity</td>
<td>High-activity</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>37,423 ± [188]</td>
<td>37,603 ± [182]</td>
</tr>
<tr>
<td>ESMS</td>
<td>37,748 ± [17]</td>
<td>37,756 ± [27]</td>
</tr>
</tbody>
</table>
mass/charge ratios of each of the significant ions of the spectrum. From this immediately adjacent peaks by a single positive charge (proton). The average molecular weight of high-activity acamidine was calculated from the separated on the basis of their mass/charge ratios and are shown above at ion spectrum. Each separated peak corresponds to an ion of the given charge. The sets of multiply charged ions thus separated were 3' phospho-ATP into a mass spectrometer fitted with an electrospray element (VG Bio Logic). The sets of multiply charged ions were sufficient (102)

An extensively desalted high-activity acamidine sample in 50% (v/v) acetonitrile in deionized water containing 0.5% (v/v) formic acid was infused (102)
mass/charge ratios of each of the significant ions of the spectrum. From this immediately adjacent peaks by a single positive charge (proton). The average molecular weight of low-activity acetylcholinesterase was calculated from the separated on the basis of their mass/charge ratios and are shown above the ion spectrum. Each separate peak corresponds to an ion of the protein diferent separated in 3.2 pmol/μL into a mass spectrometer fitted with an electrospray ionization (ESI-TOF) inlet. The series of multiply charged ions thus generated were an extensively desalted low-activity acetylcholinesterase sample in 50% (v/v) acetonitrile in deionized water containing 0.5% (v/v) formic acid, was injected (10 μL). The spectrum is shown in Figure 3.4b. ESMS spectrum of low-activity acetylcholinesterase.
Figure 3.5 UV spectrophotometric analysis of high- and low-activity forms of acetamidase

Samples of pure high- and low-activity acetamidase in 20mM-bis-Tris, pH 6.8 buffer were scanned independently in back-corrected quartz cuvettes against a buffer blank. Absorption spectra were determined over the wavelength range 250-320 nm in a Perkin Elmer Lambda 5 UV/VIS split-beam recording spectrophotometer (Materials & Methods). A) high-activity acetamidase. B) low-activity acetamidase.
characteristic appearance of a shoulder at 265nm in the absorption spectrum of the modified enzyme. The absorption spectra of high- and low-activity acetamidase were essentially identical exhibiting absorption maxima at 280nm, but no inflection at 265nm (Fig 3.5). These results strongly suggest that neither form of acetamidase undergoes covalent nucleotidylation which therefore precludes the involvement of adenylylation, uridylylation and ADP-ribosylation in the reversible regulation of acetamidase activity.

3.3.8 Investigation of the isoelectric point (pI) of acetamidase by FPLC

Anion-exchange and chromatofocusing FPLC were undertaken to investigate any nett surface charge differences between high- and low-activity forms of acetamidase. Preliminary data generated by Silman (1990) using slab-gel isoelectric focusing suggested that a pI differential of 0.1 pH unit existed between high- and low-activity forms of the enzyme with the low-activity form exhibiting the lower pI (4.0 cf. 4.1). Initial confirmation of this result was sought by subjecting pure samples of high- and low-activity acetamidase to anion-exchange FPLC under standard experimental conditions and comparing the KCl concentration at which the two forms of the enzyme eluted. The high-activity enzyme eluted at a slightly lower KCl concentration than the low-activity enzyme (272.3 ± 0.5 (4) cf. 274.2 ± 0.9 (6) mM-KCl) suggesting that its affinity for the positively-charged quaternary amine groups of the column matrix was slightly lower than that of the low-activity form, i.e. the high-activity form of the enzyme appeared slightly more positively-charged than the low-activity form as indicated by Silman (1990). These data were viewed cautiously, however, due to the magnitude of the standard errors, which were possibly elevated by decreased sample resolution resulting from an ageing column. Unequivocal confirmation of the putative acetamidase pI difference was therefore sought using chromatofocusing FPLC. Although results showed some variability between separate experiments in which a single pure sample was chromatofocused, chromatofocusing of an equimolar mixture of pure high- and low-activity acetamidases clearly showed a doublet peak corresponding to pI values of 3.80 and 3.68 (Fig. 3.6). The identities of the separate peaks were confirmed by standard and heat-reactivation amidase assays which showed that the highest unheated specific activities were associated with fractions from the leading edge of the doublet peak (higher pI) and that the extent of heat-reactivation of fractions approximately doubled towards the trailing edge of the doublet peak (lower pI). These results implied that the high-activity acetamidase had eluted first at higher pH (fractions 1 and 2) and that low-activity acetamidase had eluted slightly later at lower pH (fractions 3 and 4). This result confirms that high-activity acetamidase has a higher pI than low-activity acetamidase (3.80 cf. 3.68) and reaffirms the 0.1 pH unit difference between the high-and low-activity forms of acetamidase reported by Silman (1990), even though the absolute values from this study are slightly lower. It can therefore be concluded that acetamidase is an acidic protein, and that high- and low-activity acetamidases exhibit different nett surface electrical charges.
Figure 3.6 FPLC-chromatofocusing of high- and low-activity forms of acetamidase

Samples of purified high- and low-activity acetamidase (0.1mg of each) in 25mM-bis-Tris buffer, pH 5.5 were mixed and chromatofocused as described in Materials and Methods. Samples from fractions, numbered 1-4 across the doublet peak (over which range the fraction pH decreased), were assayed in the presence of activator fraction, with and without heat (60°C for 1 h), to produce unheated (■) and heat-reactivated (□) specific activity profiles with acetamide as substrate at 37°C. Activities are shown as a % of the total summed activity from each unheated or heat-reactivated profile. (—) $A_{280}$ elution profile.
The effect of ammonia on high-activity acetamidase was investigated in view of its ability to repress acetamidase synthesis in *M. methylotrophus* *in vivo* at concentrations greater than approximately 0.1mM (Silman *et al.*, 1989, 1991; Silman, 1990) and to promote switch-off of acetamidase activity when pulsed into steady-state dual C/N-limited cultures (Carver & Jones, 1993; J. Mills & C.W. Jones, unpublished).

It is feasible that ammonia exerts a direct effect on acetamidase activity *in vivo* by acting as an allosteric effector, *e.g.* by inducing a conformational change that modulates specific activity (allosteric modulation of acetamidase activity could also be effected by acetate alone, or by acetate in concert with ammonia as part of a feedback inhibition loop). Table 3.6.a,b shows the effect on the specific activity and reactivation properties of acetamidase after incubation at 37°C in the presence or absence of ammonia. Ammonia had no effect on low-activity acetamidase over 4 h incubation at 37°C and this form of the enzyme generally exhibited a slight increase in specific activity (in this case, 20%) over this time which can be attributed to autoreactivation (see Chapter 4). Table 3.6.a also highlights the elevated thermostability of low- over high-activity acetamidase at this temperature (in accordance with thermostability results at 60°C; section 3.3.3).

Comparison of high-activity acetamidase samples incubated with and without ammonia at 37°C (Table 3.6.b) strongly suggested that whilst both samples lost activity at this temperature (presumably due to thermal denaturation) ammonia apparently accelerated the loss of specific activity. Identical high-activity samples incubated with or without ammonia exhibited 49% and 35% activity losses respectively over the 4 h incubation *cf.* average activity losses of 46% (2) and 26% (2) in samples with and without ammonia respectively, found in earlier identical experiments. These activity losses can be attributed either to enhanced denaturation of the enzyme or to switch-off of high-activity acetamidase, and the data apparently confirms that the former hypothesis is correct. Results tentatively suggested that the increased loss of unheated specific activity in ammonia-incubated high-activity samples over that evident in ammonia-free samples (approximately 47% *cf.* 25% respectively) was not heat-reactivatable, *i.e.* the loss of activity was due to enhanced denaturation of acetamidase rather than switch-off, as evinced by heat-reactivated ammonia-sample activities which decreased over time in an approximately proportional manner to the unheated activities of the same samples (26% *cf.* 47% activity loss), in contrast to the smaller decrease in unheated ammonia-free activities and the approximately constant level of heated-reactivated ammonia-free activities. Thus, incubation of ammonia with acetamidase appears to selectively enhance the rate at which high-activity acetamidase denatures at 37°C (the optimal growth temperature for *M. methylotrophus*), but has no effect on the integrity of low-activity acetamidase structure. Moreover, this effect was shown to be unrelated to [H+] as the pH of samples with and without added ammonia remained constant throughout the incubation. The differential effect of ammonia on stability of high- and low-activity forms of acetamidase therefore
Samples of pure high- and low-activity acetamidase (25µg and 100µg respectively) were incubated at 37°C in 20mM-bis-Tris buffer, pH 6.8. Ammonium sulphate (2mM final concentration) or an equal volume of buffer was added at zero time and activities were measured at intervals up to 4 h with acetamide as substrate at 37°C (Materials & Methods) (Table 3.6.a). In an essentially identical experiment two aliquots were withdrawn at each time point; one was assayed immediately for acetamidase activity, whilst the other was mixed with a standard amount of activator fraction and heated at 60°C for 1 h, before being cooled (5 min, 4°C) and re-assayed (Materials & Methods) (Table 3.6.b). Results are presented as % activity (unheated specific activity = 100%). High = high-activity acetamidase; Low = low-activity acetamidase; +/- indicates presence or absence of ammonia.
### Table 3.6.a

<table>
<thead>
<tr>
<th>Sample time (h)</th>
<th>Sample activity (%)</th>
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<tr>
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<td>High(-)</td>
<td>Low(+)</td>
<td>Low(-)</td>
<td></td>
</tr>
<tr>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>1</td>
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<td>92</td>
<td>125</td>
<td>—</td>
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<td>2</td>
<td>79</td>
<td>87</td>
<td>110</td>
<td>—</td>
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<tr>
<td>3</td>
<td>79</td>
<td>81</td>
<td>97</td>
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<td>51</td>
<td>65</td>
<td>100</td>
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</tbody>
</table>

Overall change
-49   -35   0   +20

### Table 3.6.b

<table>
<thead>
<tr>
<th>Sample time (h)</th>
<th>Sample activity (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td>4</td>
<td>53</td>
<td>114</td>
<td>75</td>
<td>134</td>
</tr>
</tbody>
</table>

Overall change
-47   -26   -25   -3
highlights a further physico-chemical difference, and challenges the significance of these findings with respect to the reversible down-regulation of acetamidase activity in response to the delivery of excess exogenous ammonia to a dual C/N-limited culture.

3.4 Discussion

The occurrence of the variable switch-off of high-activity acetamidase during purification by an unknown mechanism was discovered at a very late stage in the course of this research, and it constitutes the first recorded instance of the significant and rapid switch-off of acetamidase activity in vitro. Reproducible purification of samples of high-activity acetamidase with a given specific activity therefore proved impossible for reasons that are obscure. Thus, the existence of the multiplicity of different activity forms of acetamidase was not apparent until a late stage in this research. The results presented above, and the discussion below, can therefore only give a superficial description of the switch-off phenomenon and provide speculation concerning its significance and physico-chemical basis in the absence of further work. The relevance of many of the observations is currently unclear, but an attempt has been made to integrate them into a plausible working hypothesis concerning the possible nature of switch-off and its modulation of acetamidase activity.

The significant variation between the observed specific activities (10-106 μmol min⁻¹ [μg protein]⁻¹) of acetamidase samples purified from cells grown under conditions expected to yield consistently high acetamidase activities, and that of low-activity samples (8-15 μmol min⁻¹ [μg protein]⁻¹), strongly suggests that the acetamidase of M. methylotrophus is unlikely to exist solely in discrete switched-off or switched-on states, and implies that this terminology is inherently ambiguous. This view is substantiated by comparison of the properties of the multiplicity of high-activity acetamidase samples (that exhibited specific activities of 35-106 μmol min⁻¹ [μg protein]⁻¹ and variable activity losses after heat-reactivation) purified from high-activity cells with those of the original high-activity enzyme (specific activity 49.5μmol min⁻¹ [μg protein]⁻¹; Silman, 1990; Silman et al., 1991) for which no thermostability and heat-reactivation profiles were carried out. All high-activity samples from this study underwent switch-off and at the extremes of this process certain samples became more switched-off than low-activity (switched-off) acetamidase samples purified from low-activity cells. It would therefore be less ambiguous to describe acetamidase samples in general terms, i.e. high- or low-activity, in conjunction with a definition of its unheated specific activity and reactivation properties. However, this system is not entirely practical or satisfactory as these properties (particularly those of the high-activity samples) were found to change with time and after certain physico-chemical treatments (see below). However, it is particularly noteworthy that low-activity acetamidase samples were generally much more stable to potentially detrimental physical treatments (e.g. freeze-thaw, long-term storage at -20°C, incubation at 60°C) than high-activity samples. These properties of the low-activity form of the enzyme were convenient for use as a reference point to which the properties of higher-activity samples could be compared. Thus, pure high-activity forms of acetamidase
appeared to acquire characteristics more readily associated with low-activity preparations during storage, but the putative high-to-low-activity transition occurred at variable rates in different high-activity preparations. Since the low-activity form of the enzyme appeared to be the more physically robust it is tempting to speculate that it exists in a more energetically stable conformation than the high-activity form, and that the energetically-compromised high-activity conformation is stabilized by either a covalent post-translational modification or a non-covalent allosteric interaction, and that it is this modification that defines the difference in specific activity between enzymic forms of acetamidase. The process of switch-off in vitro might then be mediated by the lability of this modification such that the gradual spontaneous or induced decay of the stabilizing influence allows the high-activity form of the enzyme to convert to the energetically more stable low-activity form.

The origin of the differences between the properties of the high-activity enzyme originally described by Silman (1990) and the acetamidase samples described in this study that exhibited switch-off are currently obscure. A possible explanation of these differences is the putative existence of genetic variation between different cultures of *M. methylotrophus* from which biomass for acetamidase purifications was harvested, i.e. the inadvertent selection of mutants exhibiting altered acetamidase physico-chemical properties in chemostat culture. The technique of directed evolution is based on the appearance and selection of spontaneous mutants resident in steady-state continuous culture which are endowed with a selective growth advantage under the imposed culture conditions. Only those cells that have acquired the new mutation will enjoy increased biological fitness, such that cells without the mutation will be disadvantaged in competition for the available nutrient resources and the new mutant strain will eventually take over the culture. Directed evolution has been used previously to rapidly select mutants exhibiting acetamidases with altered kinetic properties, such as mutant MM15 (Silman, 1990; Silman et al., 1989, 1991), to which high-activity acetamidases exhibited certain similarities (see below). Since certain purified acetamidases exhibited unfavourable characteristics in vitro (structural instability and tendency to switch-off) it must be concluded that the properties of these enzymes were either artefactual or that their deleterious nature in vivo was favourably offset by certain other characteristics of the enzyme. This supposition follows the reasoning of Silman et al. (1991) concerning the selection of mutant MM15 from mutant MM8, and in keeping with this view the observed properties of the MM15 acetamidase (and possibly acetamidase samples subject to switch-off in this study) do support the notion that the putative mutation(s) conferring enhanced catalytic properties on these enzymes also appeared to destabilize their overall structure (at least as determined in vitro), but that the overall biological fitness of organisms carrying these mutations must have been greater than that of the wild-type otherwise these organisms would not have been selected. Presumably, certain *Ps. aeruginosa* mutants that produced considerably more thermostable amidases than the wild-type, yet exhibited growth at 37°C despite this fact, were also able to do so by similar reasoning (see Betz & Clarke, 1972). Since acetamidase is the first enzyme in the pathway for nitrogen assimilation from amides (employed as the growth-limiting substrate during continuous culture and which constitutes a strong selection
pressure), and in view of the reported genetic diversity of culture A (see Chapter 1; Silman, 1990; Silman et al., 1989), it therefore seems plausible that high-activity cultures from which acetamidase was purified may have been characterized by genetically heterogeneous bacterial populations similar to culture A containing mutants expressing 'altered' high-activity acetamidases that were inadvertently selected under amide-limitation. The fact that similarities exist between the properties of certain high-activity acetamidase samples and those of mutant MM15 (increased inactivation at 60°C and by repeated freeze-thaw, decreased $K_m$ for growth amide; Silman et al., 1991) also supports this hypothesis, and in view of this it would also be interesting to compare their respective heat-reactivation properties, but unfortunately no such data are available for the MM15 enzyme.

It should also be borne in mind that the switch-off of acetamidase in *M. methylotrophus* also involves reversible modulation of enzyme activity which is apparently absent from the *P. aeruginosa* system. Consequently, the observed alterations in the acetamidase activity of the former organism may be complicated by the effects of an additional putative chemical/structural modification to acetamidase further to the altered amidase properties of the latter organism, which were generally attributable to simple amino acid substitutions. Thus, the generation of acetamidases exhibiting switch-off in *M. methylotrophus* could feasibly originate not only from simple amino acid substitutions altering activity solely due to perturbations of the native enzyme conformation (not affecting any putative regulatory sites within the primary sequence), but also by mutations additionally affecting activity due to amino acid substitution(s) disrupting the putative modifying group or altering the correct modification pattern. Alternatively, a further variation on the mutant hypothesis postulates the occurrence of a spontaneous mutation in a protein responsible for mediating the correct processing and/or folding/modification of acetamidase, such that these processes were not effected authentically leading to an acetamidase with altered properties. This possibility has been suggested previously to explain the thermolabile properties of multiple enzymes involved in C1-metabolism in *M. methylotrophus*, although in this case the mutation(s) was chemically-induced (Hutchinson & Goodwin, 1993a). It is also of potential interest that the desensitization phenomenon described by Monod et al. (1963) for certain allosteric enzymes exhibited a mutational basis in some cases (see Chapter 1). The only other possible source of acetamidase samples prone to switch-off might be a subtle difference between purification protocols which somehow promoted switch-off during purifications in this study, but not those of Silman (1990). This must be considered unlikely, however, as every effort was made to standardize purifications. The only identifiable difference between the purification protocols of this study and that of Silman (1990) resides in the use of spin column ultrafiltration in the former to concentrate pooled peak-activity acetamidase fractions during FPLC-purification (see below).

A plausible working hypothesis derived from the limited data from reactivation assays carried out in this study is presented below and to which the concepts of the differential thermostability and reactivation potentials of the high- and low-activity forms of acetamidase are central. Since the switch-off event is apparently characterized by the conversion of a
proportion of high- to low-activity acetamidase molecules the change in specific activity of the population undergoing switch-off will be governed by their relative proportions. The actual ratio will fix the observed specific activity of a given sample as the total value of the activity contributions from both high- and low-activity molecules. Thus, if the ratio of high- to low-activity molecules is high, the measured specific activity will be relatively high and vice versa. In addition, intermediate specific activities could presumably be achieved by modulating the relative proportions of molecules of different intrinsic activities. Similarly, the ratio of high- to low-activity molecules will also fix the intrinsic thermolability and reactivation potentials of a sample which will respectively increase and decrease with the proportion of high-activity molecules in that population. Thus, the change in activity of a given acetamidase sample after attempted heat-reactivation can be considered as a composite function whose value is governed by the relative contributions to the overall activity of the diametrically-opposed forces of differential thermolability (high- and low-activity molecules), and the reactivation potential (low-activity enzyme only). Consequently, this hypothesis proposes that a sample exhibiting X% switch-off will only exhibit an overall increase in total activity after attempted reactivation, rather than a reduction in the loss of activity (itself in relative terms equivalent to an increase in the total sample activity remaining after reactivation, when compared to the total activity exhibited by a sample that is less switched-off under identical conditions), when X reaches a critical threshold value. This critical threshold value coincides with the point at which the contribution to the measured heated specific activity from reactivated activity (originating from low-activity molecules) is greater than the loss of activity resulting from thermolability (originating from high-activity molecules). Thus, this hypothesis predicts that the switch-off over time of a high-activity sample would be characterized by a decrease in the specific activity accompanied by a reduction in the magnitude of the loss of specific activity observed after attempted heat-reactivation. This trend would continue until a point at which the specific activity remained the same as the heat-reactivated specific activity, i.e. when there was no loss of activity after attempted reactivation. After this point the specific activity would continue to diminish, but the heat-reactivated specific activity would be greater than the unheated activity. This new trend would continue until maximal switch-off had occurred and would be accompanied by a progressive increase in the magnitude of the difference between heat-reactivated and unheated specific activities. Thus, different populations of acetamidase molecules could conceivably exist which exhibit all intermediate specific activities between the extreme upper and lower limits of activity and it is possible that certain of the samples isolated in this study represent examples of these intermediate activity forms of acetamidase. Furthermore, it is possible that the original high-activity acetamidase sample described by Silman (1990) was mis-identified as the fully switched-on enzyme and was actually similar to the acetamidase samples purified in this study that were subject to switch-off, exhibiting partial switch-off and a specific activity (49.5±mol min⁻¹ [mg protein]⁻¹), which could be considered as an intermediate activity. This possibility is discussed in more detail below.

The purification of high-activity acetamidases presented significant problems concerning the unexpected loss of activity of this enzyme. A number of different variables appear to be
important in determining when switch-off will occur and to what extent, and although this work has not elucidated the biochemical basis for switch-off, it has provided a more thorough description of the process, and highlighted a few key areas that might prove fruitful for further investigation into this phenomenon.

The heat-reactivation assay used to investigate switch-off, is itself a complex multi-factorial system that is ill-defined (see Chapter 4) and therefore constitutes an unsatisfactory means of accurately measuring switch-off. However, heat-reactivation data were generally reproducible to a satisfactory level and if viewed with caution could be used to test various aspects of switch-off. An improvement in the characterization of switch-off could only be achieved realistically by finding an alternative method to the heat-reactivation assay for its quantification, however, this is not currently feasible. Two significant observations were made concerning the correlation of switch-off with particular characteristics of the purification protocol. The first was that switch-off occurred only after whole cell-disruption, which was manifest as a significant decrease in the loss of activity after heat-reactivation in crude cell extracts, and which suggested that high-activity acetamidase was stably maintained in whole cells until the cell wall was ruptured. Possible reasons for this include (i) putative mixing of cell components that are spatially separated in intact cells, but which are capable of effecting switch-off when in intimate association in the presence of acetamidase, (ii) a dilution effect such that upon cell breakage the relative concentration of a stabilizing component maintaining the high-activity form is diminished resulting in conversion to the low-activity form, and (iii) potential oxidation of a compound responsible for maintenance of acetamidase in the high-activity form resulting in the conversion of the high-activity form to the low-activity form due to the change in the redox state of the stabilizing influence. The second key finding involved the putative effect of buffer ionic strength on the level of switch-off. Removal of KCl (by PD-10 gel-filtration or diafiltration) from acetamidase samples appeared to enhance the level of switch-off (and hence heat-reactivation), the effect of which was apparently reversed upon reintroduction of KCl to the sample. Diafiltration-mediated switch-off appears similar to the conversion of the low molecular mass active C-form of the N-acetylmuramoyl-L-alanine amidase of *Streptococcus pneumoniae* to the inactive E-form, as demonstrated by Briese and Hakenbeck (1985) after prolonged dialysis (amidase activity was only 30% of the original activity after 20 days of dialysis). These workers showed that choline could effect the conversion of the E-form of the enzyme to the C-form which was presumed to result from the formation of a choline-amidase complex which was necessary for the establishment and maintenance of the active C-form. It was therefore suggested that the activity of the C-form was dependent on the presence of choline molecules complexed with the enzyme and that it was the decay of this complex which was the rate-limiting step in the 're-conversion' of the C-amidase to the E-amidase during prolonged dialysis. Prolonged dialysis of acetamidase from *M. methylotrophus* against deionized water caused a significant loss of activity (this activity loss was markedly worse in the high-activity sample) that could not be recovered by heat-reactivation. The fact that activity losses were irrecoverable in this instance may have been due to the absence of buffering during dialysis, which may have promoted enzyme denaturation.
and masked any resultant switch-off. It would therefore be interesting to repeat this experiment with buffered samples and to determine the effect of heat-reactivation on dialyzed samples. An apparent discrepancy also existed between switch-off results involving spin column ultrafiltration by centrifugation and pressurized stirred cell ultrafiltration, such that the former unpredictably effected variable levels of switch-off of high-activity samples and the latter did not (see section 3.3.1.1). Certain differences exist between these two ultrafiltration methods that might explain their putative differential ability to effect switch-off: 1) the smaller MW exclusion limit of the stirred ultrafiltration cell membrane (10kDa) cf. that of the spin column (30kDa), which may suggest that a molecule of MW in the range 10-30 kDa is involved with switch-off of the high-activity acetamidase; 2) the exclusion-membranes may exhibit differential binding affinities for a molecule(s) involved with switch-off, thereby altering relative sample concentrations of these molecules which are important for the stable maintenance of the high-activity form; and 3) switch-off could possibly be mediated by the effect of centrifugal force on spin column samples which is absent in the stirred cell ultrafiltration unit. However, the lack of consistent correlation of the various physical pretreatments (-20°C storage/cycled freeze-thaw and spin column diafiltration) that appear to effect switch-off of acetamidase with the actual occurrence of switch-off, in addition to the variation in the extent of switch-off when it did occur, suggests that the simplest explanation is that switch-off is a spontaneous event that spuriously correlates with various physical pretreatments. Thus, it appears likely that it is the time element of the spontaneous decay of acetamidase activity during switch-off that is the most important factor governing the level of switch-off. In addition to this, the rate of decay of the modification that putatively stably maintains the high-activity form might possibly be accelerated by the effect of repeated freeze-thaw or diafiltration, thereby eliciting enhanced switch-off. If this hypothesis is true then the modification that decays during switch-off must be spontaneously labile and under certain obscure circumstances the rate of decay can be significantly enhanced, as evinced by comparison of the rates of switch-off of high-activity samples. It would also be of interest to determine whether this switch-off in vitro occurs by the same mechanism as switch-off in vivo, or whether the observed switch-off in vitro is mediated by a different mechanism and might therefore be artefactual.

That a putative subtle conformational difference exists between high- and low-activity forms of acetamidase is suggested by their slightly different substrate profiles and the increased sensitivity of the low-activity form to the thiol reagent p-chloromercuribenzoate in comparison to the high-activity form (see Carver & Jones, 1993). It would therefore be interesting to determine whether intermediate-activity samples also exist in more relaxed tetrameric conformations by determining the effect of thiol reagents on these samples in comparison to the mutant acetamidase of MM15 (which showed approximately doubled sensitivity to DTNB in comparison to the wild-type high-activity enzyme; Silman, 1990) and higher-activity samples. In addition, the MM15 acetamidase was also shown to exist in a less compact tetrameric conformation (Silman, 1990; Silman et al., 1991) than the wild-type enzyme by sedimentation ultracentrifugation. Similar work from this study suggested that
High- and low-activity acetamidases formed putative high-MW aggregates which effectively prevented an investigation of putative conformational differences between the different enzymic forms using this technique. It is likely, however, that further investigations of this phenomenon would benefit from repetitive S-value determinations from freshly-purified samples at a range of different protein concentrations. Determination of conditions under which aggregation was minimized and a statistical consideration of the results might also clarify the results. Further techniques that might be used to investigate the putative conformational difference between high- and low-activity acetamidases include differential scanning calorimetry and circular dichroism. Conformational disparities might also be investigated using hydrophobic interaction chromatography, and spectrofluorimetric assessment of the surface hydrophobicity of the enzyme by monitoring the equilibrium binding of the apolar fluorescent dye ANS to the two forms of acetamidase using the method of Cardamone and Puri (1992).

The variable effect of incubation at high (60°C) and low (4°C and -20°C) temperatures on the specific activities of acetamidase samples infers structural and/or chemical differences between the different forms of the enzyme. The thermo-profile of high-activity acetamidase at 60°C (Silman, 1990; Silman et al., 1991) was derived from a sample with a specific activity of only 38.8 μmol min⁻¹ (mg protein)⁻¹; this value is lower than the original specific activity reported for the same sample (49.5 μmol min⁻¹ [mg protein]⁻¹) which suggests that it was probably partially switched-off at the time of this work. Consideration of its more stable t½ value, in comparison to that of a less switched-off sample tested in this study (specific activity 87 μmol min⁻¹ [mg protein]⁻¹), which exhibited a significantly increased loss of activity after 1 h, also supports the notion that the original high-activity sample had begun to switch-off (see section 3.3.3). If this hypothesis is correct then the original high-activity (switched-on) sample can be considered to be essentially the same as other acetamidase samples that underwent switch-off in this study, except that it was presumably happenstantially less switched-off. The occurrence of multiple high-activity samples exhibiting variable levels of switch-off infers that the thermo-profile of a given acetamidase sample would change as it became more switched-off due to the increasing proportion of thermostable low-activity molecules in the population. This trend would presumably manifest itself as a decrease in the intercept value on the vertical axis of the graph of specific activity versus duration of incubation and would bring about a flattening of the initial portion of the graph, such that the graph would become much more similar to that of low-activity acetamidase. Obviously, to test this hypothesis further many more thermo-profiles would have to be characterized for a range of acetamidase samples exhibiting increasing levels of switch-off. Conversely, if the original high-activity enzyme genuinely was not partially switched-off it is difficult to explain why the high-activity sample profiled in this study was significantly more thermolabile than both the low-activity acetamidase and the acetamidase sample of Silman et al. (1991).

Investigation of the reversible switch-off of nitrogenase activity in R. rubrum, RubisCO activity in R. sphaeroides (see Chapter 4) and acetamidase activity in M. methylotrophus has implicated low-MW effector molecules (ammonia, organic acids and unidentified 'dialyzable'
factors) in the control of switch-off in these organisms. Ammonia was shown to have essentially no effect on the level of switch-off exhibited by either high- or low-activity acetamidase as measured in vitro. Thus, the presence of a physiological excess of ammonia does not effect a simple high- to low-activity enzyme conversion (switch-off) in vitro, suggesting that the role of ammonia in switch-off in vivo is more complicated. However, results did suggest that high-activity acetamidase is destabilized in the presence of excess ammonia which could possibly form the basis for a second system effecting more rapid down regulation of activity, i.e. switch-off. It would be interesting therefore to repeat experiments of this type with a range of ammonia and/or acetate concentrations to determine the effect of ammonia, acetate, and equimolar concentrations of acetate and ammonia on the enzyme to eliminate the possibility that switch-off could be effected in vitro by the end-products of acetamide hydrolysis acting independently or in concert.

The majority of work described above was concerned primarily with characterizing the physico-chemical properties of high- and low-activity forms of acetamidase in order to identify a significant MW difference which might indicate the nature of the putative modification to acetamidase. Alternatively, if there was no difference in MW this might indicate that the two forms of acetamidase differed by virtue of a conformational change (as has been suggested for a number of hysteretic enzymes; see Frieden, 1970) or that a labile modification (covalent or non-covalent) had been destroyed or dissociated from acetamidase by in vitro manipulation (as has been suggested for the nitrogenases of R. sphaeroides and M. trichosporium; Yoch et al., 1988). Covalent modifications that are labile under mild preparative conditions are unlikely, but the concept of the removal of a non-covalently bound allosteric effector by purification and/or ionization is more feasible. Both covalent and non-covalent modifications could be plausibly invoked to explain stabilization of a putative conformational change, although it would presumably be the latter system that would give rise to an observed mass difference of zero from the reasoning presented above. Further to this, it was hoped that ESMS spectra might elucidate the nature of high- and low-activity acetamidase samples, and the basis for their respective specific activities by determining whether low-activity samples were unimolecular populations in which each molecule was only partially active, or whether samples were bimolecular populations exhibiting disproportionate ratios of relatively high- or low-activity molecules. The latter explanation has been forwarded for the basis of activity in different RubisCO preparations from R. sphaeroides (Wang & Tabita, 1992a). Analysis of spectra for confirmation of the uni- or bimolecular nature of a low-activity acetamidase population was inconclusive (specialist interpretation of spectra, which was unavailable, would probably be required to clarify this point). Capillary electrophoresis (possibly in combination with ESMS) could be utilized to probe this problem further, i.e. if enhanced resolution of the components of an artificial mixture of high- and low-activity acetamidase could be demonstrated, then this technique might also be used to investigate the uni- or bimolecular nature of highly-purified very high-, and very low-activity acetamidase samples (assuming that these could actually be isolated intact).
In contrast to this, ESMS data was successful in further reducing the number of potential candidate acetamidase modifying groups to those effecting a mass change of ≤ 52Da (see Table 3.7). Consideration of Table 3.7 shows that all types of phosphorylation can now be eliminated from the list of candidate modifications to acetamidase (which from initial acid/alkaline hydrolysis results was not possible, as phospho-tyrosine would not have been detectable by the experimental methods employed). In addition, N-terminal acylation can be discounted as the N-termini of both forms of acetamidase have been sequenced (Silman, 1990) and were found to be identical (MIH-) indicating that they were not 'blocked'. Pyroglutamic acid formation from N-terminal glycine can also be discarded by consideration of the N-terminal sequence, as can reversible oxido-reduction of sub-unit thiol groups/disulphide bridges (Silman, 1990). This leaves amino acid side chain acylation or C-terminal acylation (formylation, methylation and acetylation), hydroxylation, carboxylation of Asp and/or Glu residues, proteolysis of a single peptide bond, oxidation of Met, deamidation of Asn and/or Gin and C-terminal amide formation from Gly as likely covalent modifications. Since ESMS data cannot identify which form of the enzyme is modified and despite further information concerning physico-chemical differences between the different enzymic forms of acetamidase further work would be necessary to unequivocally identify the nature and location of the modifying group. Two of the remaining modifications are particularly attractive candidates, viz. acetylation and carboxylation.

Acetate is the potentially toxic end-product of acetamide hydrolysis catalysed by acetamidase (in addition to ammonia), and it is tempting to suggest that intracellular acetate levels may be monitored so that acetamidase activity is switched-off (down-regulated) when toxic acetate levels are approached. Further to this, acetyl groups could themselves constitute the modification to acetamidase either by covalent attachment of an acetyl group(s) (acetylation, which would presumably proceed with acetyl CoA as the acetyl donor) or by non-covalent binding of acetyl CoA to the enzyme which has been implicated in the stabilization (and hence protection from inactivation) of chicken liver mitochondrial pyruvate decarboxylase by induction of a conformational change in this enzyme (Irias et al., 1969). Presumably, any putatively similar system operating in *M. methylotrophus* would differ in that acetate would promote switch-off (inactivation) of acetamidase rather than protecting against it. Carboxylated Asp/Glu residues are intrinsically thermolabile and susceptible to lyophilization, and both heating and lyophilization have been shown to significantly affect the specific activity and heat-reactivation properties of acetamidase. Carboxylation of Glu residues is involved with the complex regulatory system governing the amidase activity of bovine activated protein C which is greatly stimulated by the binding of monovalent and divalent cations at at least two sites within the enzyme (Hill & Castellino, 1986). Cleavage of an amino-terminal peptide rich in γ-carboxyglutamic acid residues from the protein C zymogen was shown to affect the binding of divalent cations which in turn influenced the monovalent cation sites and the mechanism of action of the enzyme. Protein C was also shown to undergo conformational changes as a result of amino-terminal peptide release.
Table 3.7 MW values of potential post-translational modifying groups to acetamidase

A list of post-translational modifications of proteins and the corresponding mass change resulting from protein modification by each of the groups indicated is shown below (taken from Geisow, M.J. & Green, B.N. Rapid detection and identification of post-translational modifications by Electrospray Mass Spectrometry. In BioSpec: Mass spectrometry in analytical biochemistry and biotechnology, No. 2. Distributed by Fisons Instruments/VG BioTech). Each of the listed modifications exhibits a mass change that is compatible with the window of MW values in which the putative acetamidase modifying group is presumed to lie.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Mass change (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyroglutamic acid formed from Gln</td>
<td>-17.03</td>
</tr>
<tr>
<td>Disulphide bond formation</td>
<td>-2.02</td>
</tr>
<tr>
<td>C-terminal amide formed from Gly</td>
<td>-0.98</td>
</tr>
<tr>
<td>Deamidation of Asn and Gln</td>
<td>0.98</td>
</tr>
<tr>
<td>Methylation</td>
<td>14.03</td>
</tr>
<tr>
<td>Hydroxylation</td>
<td>16.00</td>
</tr>
<tr>
<td>Oxidation of Met</td>
<td>16.00</td>
</tr>
<tr>
<td>Proteolysis of a single peptide bond</td>
<td>18.02</td>
</tr>
<tr>
<td>Formylation</td>
<td>28.01</td>
</tr>
<tr>
<td>Acetylation</td>
<td>42.04</td>
</tr>
<tr>
<td>Carboxylation of Asp and Glu</td>
<td>44.01</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>79.98</td>
</tr>
</tbody>
</table>
In summary, it can be seen that there are a significant number of physico-chemical differences between high- and low-activity forms of acetamidase and a marked paucity of similarities. That acetamidase activity is reversibly regulated \textit{in vivo} is unquestionable, and presumably the differences in high- and low-activity properties described above reflect the overall relative effect of the putative modifying group on one form of the enzyme, but not the other. Reversible regulation of enzyme activity can occur by post-translational covalent modification or by non-covalent allosteric interactions with low-MW effectors, or a combination of both. Consideration of the inconclusive results presented above shows that either of these alternative mechanisms could potentially account for the observed differences in acetamidase properties, but in many ways the observed effects of physico-chemical perturbations on the two different forms of the enzyme appear to conform more readily to an allosteric model of activity regulation. Examples of this include the apparent ease with which high-activity acetamidase acquires low-activity acetamidase kinetic characteristics through switch-off, which suggests that the putative modification must be relatively labile, and the multiplicity of pure acetamidase samples that were isolated exhibiting a wide range of specific activities and reactivation properties. These properties appear similar to those of hysteretic enzymes (see Frieden, 1970) and are suggestive of a continuous, more 'fluid' activity regulatory system than the discrete switched-on or switched-off activity states apparently observed by Silman (1990), and which were initially presumed to result from covalent regulation of enzyme activity. The possibility that acetamidase exhibits only one discrete MW, which cannot currently be discounted from ESMS data, and the limited number of candidate covalent modifications which are compatible with the putative MW difference between the two enzymic forms also promotes allosteric regulation as an attractive candidate mechanism for the modulation of acetamidase activity. A further appraisal of the covalent versus allosteric activity regulation models and final conclusions concerning acetamidase regulation are given in Chapter 4, after presentation of results concerning heat-reactivation ('switch-on') \textit{in vitro} of low-activity acetamidase.
CHAPTER 4

IN VITRO STUDIES OF HEAT-REAKTIVATION OF THE LOW-ACTIVITY FORM OF ACETAMIDASE
CHAPTER 4

IN VITRO STUDIES OF HEAT-REACTIVATION OF THE LOW-ACTIVITY FORM OF ACETAMIDASE

4.1 Introduction

4.2 Investigation of the heat-reactivation of low-activity acetamidase by activator fraction
   4.2.1 Preliminary characterization of variables influencing heat-reactivation
   4.2.2 Investigation of the stability of activator component under extreme physico-chemical conditions
   4.2.3 Fractionation of activator fraction by gel-filtration
   4.2.4 Fractionation of activator fraction by ultrafiltration
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4.3 Discussion
IN VITRO STUDIES OF HEAT-REACTIVATION OF THE LOW-
ACTIVITY FORM OF ACETAMIDASE

4.1 Introduction

It has been demonstrated recently that form I RubisCO (ribulose 1,5-bisphosphate
carboxylase/oxygenase) from R. sphaeroides, which catalyses fixation of CO$_2$, undergoes
inactivation (switch-off) upon addition of metabolizable organic acids to certain cultures
which was shown to be reversible upon depletion of the organic acids (Wang & Tabita,
1992a,b). The inactivation process was dependent on both the concentration of the organic
acid and the nitrogen status of the cells, which is reminiscent of the reversible ammonia-
mediated switch-off of nitrogenase and acetamidase activities in R. rubrum (see Ludden &
Roberts, 1989) and M. methylotrophus (Silman, 1990, Carver & Jones, 1993; J. Mills &
C.W. Jones, unpublished) respectively. The purified inactivated RubisCO could be reactivated
in vitro in a time- and temperature-dependent fashion which involved dialyzable and non-
dialyzable factors, as is also the case for low-activity acetamidase from M. methylotrophus.

ATP was also found to reactivate RubisCO irreversibly in a time- and concentration-
dependent fashion (Wang & Tabita, 1992c), but no unequivocal effect on the heat-reactivation
of low-activity acetamidase by ATP or other potential modifiers (ammonia, acetate, ADP,
NAD(P)(H), Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$ or Fe$^{2+}$) has been demonstrated (M.A. Carver,
unpublished).

The effects of various cations and other effectors on the switch-off of nitrogenase and the
regulation of activity of certain amidases have been well-documented in the literature. The
nitrogenase of Azotobacter chroococcum has been shown to be released from ammonia-
mediated switch-off by Mn$^{2+}$ (Ruiz et al., 1990), which is postulated to have resulted from the
physiological inhibition of glutamine synthetase by this cation. Mn$^{2+}$ is also involved with the
regulation of the R. rubrum nitrogenase (see Ludden & Roberts, 1989) as DRAG, a Mn$^{2+}$-
dependent enzyme, catalyses the removal of ADP-ribose from nitrogenase, thereby
reactivating the latter enzyme. The critical role of various cations and low-MW
(approximately 3.5kDa) cationic peptide antibiotics in the reversible activation of the N-
acetylmuramoyl-L-alanine amidase of S. simulans 22 has also been demonstrated. This
amidase exhibits an affinity for polyanionic cell wall constituents at low ionic strength, and
therefore binds to teichoic- and teichuronic acids embedded in the bacterial cell wall which
inhibits amidase activity (Bierbaum & Sahl, 1987). These workers showed that at low ionic
strength amidase activity was stimulated by Pep 5 and nisin (both of which are small cationic
peptide antibiotics) and by mono- and divalent cations, in a concentration-dependent fashion
related to the nature of the cation. The activation was probably due to displacement of amidase
from the inner cell wall surface by cations mediating the disruption of the ionic interaction
between amidase and the cell wall polyanions. At elevated concentration ratios of the larger
peptide antibiotics over the substrate concentration activation of amidase was transformed into
inhibition, which was presumed to result from steric hindrance preventing access of the enzyme to the substrate. The reversible regulation of this amidase is therefore complex and results from the indirect effects of non-covalent interactions with a variety of low-MW effectors.

The purification of a dimeric acetamidase activator 'protein' (sub-unit MW 64kDa; native MW 100kDa) from *M. methylotrophus* was reported by Silman (1990), although no purification table was presented and the estimated yield of activator 'protein' was very low. The purified activator 'protein' was demonstrated to stimulate low-activity acetamidase 2- to 3-fold when mixed and heated with acetamidase at 60°C for 5 h, indicating that this 'protein' was exceptionally thermostable. Calmodulin is a heat-stable Ca^{2+} binding protein which retains its ability to bind Ca^{2+} even when denatured. This protein is found ubiquitously in eukaryotes and putative calmodulin-like proteins have been identified in certain prokaryotes (see Onek & Smith, 1992). Calmodulin has been implicated in the control of many of the essential physiological and metabolic functions of eukaryotic cells, although analogous contemporary evidence establishing the central integrative role of calmodulin-like proteins in prokaryotic metabolic functions is much less-well defined. The binding of Ca^{2+} by calmodulin has been shown to effect a conformational change in this protein which exposes a hydrophobic region, and it is this region of the protein which appears to be critical to its stimulatory or inhibitory influence on the activity of the target proteins to which it binds. Calmodulin-like activity has been demonstrated for a number of different prokaryotic proteins including a protein isolated from sporulating cultures of *Bacillus subtilis*. This protein was heat-stable and in marked contrast to the properties of eukaryotic calmodulins showed a complete and irreversible abolition of its ability to activate a well-characterized target protein after removal of Ca^{2+} by chelation or dialysis. Similarly, exceptionally heat-stable proteins from strains of the cyanobacterial genus *Anabaena* have been shown to exhibit calmodulin-like activity even in boiled cell extracts. Thus, several different heat-stable prokaryotic regulatory proteins have been identified whose activities are sensitive to Ca^{2+} concentration, and Onek and Smith (1992) have postulated that prokaryotic Ca^{2+}-mediated regulation of cellular processes may be related to monitoring of the C/N balance or energy status of the cell. All of these properties (cation-influenced heat-stable regulatory proteins etc.) are of potential importance to the switch-off of acetamidase activity in *M. methylotrophus*.

Activator proteins have also been described for methanol dehydrogenase (MDH) from *M. methylotrophus* and Paracoccus denitrificans (Long & Anthony, 1991), and for the thermotolerant *Bacillus* sp. C1 (Arfman et al., 1991), although only the latter activator protein influences MDH activity at high temperature (optimum activation at 57-59 °C cf. 60°C at which acetamidase from *M. methylotrophus* is routinely heat-reactivated) and all stimulatory activity was lost from this protein after incubation at 90°C for 5 min (cf. the exceptional thermostability of the acetamidase activator component; see section 4.2.2). The stimulatory activity of the MDH activator protein required the presence of Mg^{2+} and activator protein-mediated stimulation of MDH was found to be reversible. Further properties of MDH
activator proteins are considered below in relation to those of the acetamidase activator component.

This chapter describes the investigation of the heat- and activator fraction-mediated reactivation of low-activity acetamidase in vitro. Characterization of the various components involved with heat-reactivation was carried out in order to ascertain their nature and relative effects on the heat-reactivation process, with an eventual view to clarifying the mechanism of heat-reactivation and to identifying the putative post-transcriptional modification to acetamidase responsible for the reversible regulation of its activity.
4.2 Investigation of the heat-reactivation of low-activity acetamidase by activator fraction

4.2.1 Preliminary characterization of variables influencing heat-reactivation

Heat-reactivation of low-activity acetamidase has been partially-characterized previously by Carver and Hinton (1987) and Silman (1990), and a brief synopsis of this phenomenon has been presented by Carver and Jones (1993). Initial characterization of low-activity acetamidase, and partially-purified activator fraction samples (purified as described in Materials & Methods) in this study was undertaken to define the effect of experimental parameters (temperature, duration and mode of incubation) germane to the efficacy of the heat-reactivation process.

In view of the relative thermostability of low-activity acetamidase at 60°C (see Chapter 3), the effect of incubation at 37°C on the enzyme was examined initially. The specific activity increased from 11.5 to 18.6 μmol min⁻¹ (mg protein)⁻¹ (62% reactivation) after 10 min incubation in the absence of the activator fraction, and remained at this elevated level for at least 60 min. These results were reproducible and approximately 60% reactivation was apparently the maximum 'autoreactivation' that could be achieved under these conditions (see Fig. 4.1). This autoreactivation appeared identical to that previously shown at 60°C and it was concluded that it represented a significant activator fraction-independent reactivation of acetamidase activity. It was also noted that unheated low-activity acetamidase exhibited a putative hysteretic response (Fig. 4.1) in a significant number of assays when incubated with acetamide at 37°C, which was characterized by non-linearity of the graph of amount of ammonia formed (A630) versus time of incubation (min) during the time period 0-2 min after substrate addition. After this time, an essentially linear relationship was established between the two variables, and specific activities were always calculated from the linear portion of the plot. The hysteretic response was much less conspicuous in high-activity acetamidase and in low-activity samples heat-reactivated in the presence of the activator fraction, and although its occurrence has been corroborated independently (M.A. Carver, unpublished; see Carver & Jones, 1993), its origin cannot currently be explained. However, it is tempting to speculate that its basis may reside in a protein conformational change resulting from an allosteric effect relating to substrate binding or a temperature effect due to incubation at 37°C, which modulates the catalytic activity of low-activity acetamidase.

Heat-reactivation of low-activity acetamidase in high-speed supernatants prepared from industrial pseudo-dual methanol-acetamide limited continuous cultures had been demonstrated previously (Silman, 1990). It was therefore decided to attempt to dissect the biochemical nature of the heat-reactivation process further by using partially-pure activator component and pure low-activity acetamidase, in order to circumvent the problematic interpretation of results from experiments involving high-speed supernatants which contained both acetamidase and the activator component.
Figure 4.1 Autoreactivation and the putative hysteretic response of low-activity acetamidase

Representative acetamidase activity plots of amount of ammonia formed ($A_{630}$) versus duration of incubation at 37°C (min) after addition of substrate (acetamide) (Materials & Methods) are shown. The acetamidase concentrations of reaction mixtures are shown in parentheses. Hysteretic response of unheated low-activity acetamidase (8.3 µg protein ml$^{-1}$) (—•—); autoreactivated (20 min, 37°C) low-activity acetamidase (8.5 µg protein ml$^{-1}$) (—○—); heat-reactivated low-activity acetamidase (8.5 µg protein ml$^{-1}$) (—△—); unheated high-activity acetamidase (8.8 µg protein ml$^{-1}$) (—□—).
Surprisingly, high-speed supernatants expressing no acetamidase activity prepared from a
culture grown under methanol-limitation with an excess of ammonia and in the absence of
amide inducer, or high acetamidase activity (prepared from a culture grown under acetamide-
limitation) both reactivated pure low-activity acetamidase to an extent comparable with that of
the pseudo-dual C/N-limited supernatant investigated by Silman (1990). This suggested that
the activator component was either a constitutively-expressed gene product, or was a
constituent component ubiquitous in cells of *M. methylotrophus* (e.g. an inorganic ion).
Activator component was subsequently partially-purified from a frozen cell sample grown
under pseudo-dual C/N-limitation and the fractions exhibiting the greatest heat-reactivation
capacity were bulked (hereafter referred to as activator fraction). In all subsequent heat-
reactivation assays a ten-fold excess of activator fraction protein was incubated with low-
activity acetamidase, which yielded satisfactorily reproducible levels of heat-reactivation under
standardized experimental conditions. Appropriate sample controls were employed in all
experiments; these were used to correct for autoreactivation or thermal denaturation of
acetamidase (Materials & Methods) and also confirmed that the activator fraction possessed
no intrinsic acetamidase activity (Silman, 1990).

The heat-reactivation profile of a mixture of low-activity acetamidase and activator fraction
was determined by incubating the mixture at 60°C for 6 h and assaying the specific activity of
cooled (4°C, 5 min) samples. The results showed that full heat-reactivation (approximately
240% cf. only 60% autoreactivation) occurred after 1 h and remained constant for a further 3
h, after which time specific activities decreased (presumably as a result of thermal
denaturation of acetamidase). These conditions were used in all subsequent experiments
involving heat-reactivation of acetamidase/activator fraction mixtures.

The effect of heat on the individual components of an acetamidase/activator fraction mixture
was also addressed. Partially-pure activator fraction was heated (1 h, 60°C) (Fig. 4.2) and
subsequently mixed with unheated low-activity acetamidase and incubated at 37°C for 1 h to
allow heat-reactivation of the low-activity acetamidase to occur (Materials & Methods). The
reciprocal situation was also tested in which unheated activator fraction was mixed with heated
(1 h, 60°C) low-activity acetamidase and incubated at 37°C for 1 h as before. Additionally,
samples in which neither acetamidase nor activator fraction were pre-heated, and the converse
situation where both components had been pre-heated separately, and then mixed and
incubated at 37°C were also tested. Significant heat-reactivation only occurred in those
samples where activator component had been pre-heated; i.e. heat was found to act solely on
the activator component. An absolute requirement for heat pre-treatment of activator fraction
(and not of acetamidase or of an acetamidase/activator component complex) was therefore
demonstrated for the heat-reactivation process, and it was concluded that the action of heat
was to somehow 'activate' the activator component, in such a way that only in its heated state
was it able to reactivate significantly low-activity acetamidase. Furthermore, the extent of heat-
reactivation of acetamidase activity per unit time in an acetamidase/activator fraction mixture
was greater at 60°C than it was at 37°C, suggesting that incubation at elevated temperatures
enhanced the extent of heat-reactivation; it also became apparent that heat-reactivation of this
Figure 4.2 The effect of heat on low-activity acetamidase and activator fraction

Master samples containing activator fraction (300μg) and/or low-activity acetamidase (30μg) were diluted to final volume (0.6ml) with 20mM-bis-Tris, pH 6.8 buffer in duplicate; A) activator fraction (---); B) low-activity acetamidase (---); C) activator fraction + acetamidase (mixed) (---); D) activator fraction + acetamidase (separate) (---). The specific activity of all samples except D) were measured with acetamide as substrate at 37°C (Materials & Methods) before and after incubation at 37°C for 1 h. Each sample was then incubated at 60°C for 1 h, prior to cooling (4°C, 5 min) and re-assay. Unheated low-activity acetamidase (30μg) was then added to A), unheated activator fraction (300μg) to B) and buffer only to C). All mixtures were re-incubated at 37°C for 1 h prior to re-assay. The activator + acetamidase (separate) sample D) was treated similarly except that the master samples were not pre-incubated at 37°C prior to incubation at 60°C, and during this latter incubation activator fraction and acetamidase samples were heated in separate Eppendorf tubes. Aliquots from the separately pre-heated samples were then mixed and incubated in the same tube during the final incubation (1h, 37°C) prior to assay (---). Specific activity was based on acetamidase protein only.
Figure 4.2 The effect of heat on low-activity acetamidase and activator fraction
mixture was probably reversible, as the specific activity of the mixture was significantly greater immediately after incubation at 60°C (1 h) than it was after further (1 h) incubation at 37°C (the final mixture specific activity was very similar to the final specific activity of the activator fraction + acetamidase [separate] sample, which re-affirmed the notion that the efficacy of the heat-reactivation process was reduced at lower temperatures). The apparent loss of heat-reactivated activity was presumed to occur as a function of the decay of the heat-reactivated high-activity state either spontaneously over time or possibly due to the relative cooling effect of the final 1 h incubation at the lower temperature of 37°C. All of these observations confirmed the results of earlier work which used a high-speed supernatant as the source of the activator component (data not shown). Thus, the fact that activator fraction could be heated independently of low-activity acetamidase and then mixed with the latter sample to effect heat-reactivation, in addition to the heat-reactivation that could be effected by heating the two components together, provided a second potential method for the measurement of this process. However, as a direct result of the perceived potential problems arising from the ill-defined putative partial-reversal of heat-reactivated activity, it was decided in general to pursue the acetamidase/activator fraction mixture assay method as a means of investigating this process further, and all experiments utilized this method unless otherwise stated.

4.2.2 Investigation of the stability of activator component under extreme physico-chemical conditions

The heat-reactivation work of Silman (1990) reported the purification and partial-characterization of an acetamidase activator 'protein' from a pseudo-dual C/N-limited high cell density culture (see Introduction) which was capable of stimulating acetamidase activity. Heat-reactivation work was extended in this study, but was confined to the use of partially-pure activator fraction as a source of the unidentified activator component. Silman (1990) tentatively concluded that the activator component was a protein and that it probably effected heat-reactivation of acetamidase catalytically \textit{in vitro}, rather than stoichiometrically. The possibility that the activator component was proteinaceous was re-examined by subjecting the activator fraction to physico-chemical conditions which would be expected to denature or inactivate any macromolecules present (Table 4.1).

Autoclave treatment of activator fraction appeared to reduce its heat-reactivation potential, although the treated sample was still able to effect significant reactivation of acetamidase activity. Acid hydrolysis of activator fraction for variable time periods at 100°C, in conjunction with lyophilization and storage of the hydrolysate for variable time periods yielded conflicting results. Assay of the treated sample heat-reactivation potential showed that acid hydrolysis for 1 h at 100°C, coupled with 49 days storage of the lyophilized hydrolysate essentially abolished its heat-reactivation potential (or at least reduced it to a level commensurate with autoreactivation); conversely, longer acid hydrolysis (2.5 h) under the same conditions coupled with only 2 days storage of the lyophilized hydrolysate had no significant deleterious effect on the heat-reactivation potential of the activator fraction. It was
concluded that there was some cryptic deleterious effect on the heat-reactivation potential of the acid hydrolysate associated with protracted storage in the lyophilized state. It was also found that incubation of the activator fraction at 100°C for 1 h had no adverse effect on its heat-reactivation potential; indeed on certain occasions this treatment apparently enhanced the heat-reactivation potential of the sample (data not shown). It was concluded that the activator component was exceptionally thermostable, as has been shown for the Ca\textsuperscript{2+} binding protein, calmodulin. The efficacy of these treatments concerning the inactivation of enzymic activities was assumed to have been satisfactory in all cases due to substantial precipitation of sample macromolecules, which was presumably due to denaturation. In addition, the efficacy of proteolysis by proteinase K was proven by SDS-PAGE which showed substantial smearing of the digested activator fraction cf. a more discrete banding pattern for the undigested fraction gel track (data not shown). This was taken to be indicative of significant proteolysis of the activator fraction.

Control heat-reactivation values were quite diverse in these experiments, but the test sample heat-reactivation level was significantly greater than that which was achieved through autoreactivation of low-activity acetamidase under identical conditions at the same time (data not shown). It should also be noted that there are limitations associated with expressing the extent of heat-reactivation as the change in activity after reactivation as a % of the unheated sample specific activity, as any minor fluctuations in unheated or reactivated activities can lead to anomalously high or low % heat-reactivation values. This is exemplified by the anomalously high % heat-reactivation exhibited by the proteinase K sample which was due to an exceptionally low unheated acetamidase activity coupled with an abnormally large increase in activity after reactivation. These anomalous results (approximately 1000% reactivation cf. 200% observed commonly; 390% autoreactivation cf. ≤ 60% observed commonly) cannot currently be explained, but may be linked to the presence of EDTA in the protease buffer and the deleterious effect of the protease on low-activity acetamidase, although steps were taken to inactivate the protease prior to incubation with acetamidase. Thus, it was concluded that the true activator component is generally exceptionally stable to extreme conditions of temperature, pH and pressure and was therefore unlikely to be a functionally-active regulatory enzyme, contrary to previous conclusions (Silman, 1990). These results are considered further in the Discussion below.

4.2.3 Fractionation of activator fraction by gel-filtration

The nature of the activator component was further investigated by separating the activator fraction into samples containing relatively large and small MW molecules using PD-10 column gel-filtration chromatography. Fractions were eluted from the column using an elution buffer with the same KCl concentration as that of the activator fraction (approximately 230mM-KCl) to eliminate ionic effects. Eluent fractions were collected and used in standard heat-reactivation experiments (Fig. 4.3). The high-MW fraction (0.9mg protein ml\textsuperscript{-1}) exhibited significant heat-reactivation of acetamidase activity (107%) in comparison to the
Table 4.1 Investigation of the stability of activator component under extreme physico-chemical conditions

Standard aliquots of activator fraction were subjected to the treatments indicated below before being cooled (4°C, 5 min) and then mixed and incubated (60°C, 60 min) with low-activity acetamidase (16µg or 30µg) after which time the extent of heat-reactivation was measured (Materials & Methods). Controls were included in which activator fraction had not been pre-treated and the extent of heat-reactivation of low-activity acetamidase activity in these samples is indicated in each case in parentheses. The extent of heat-reactivation is expressed as the change in activity after heat-reactivation as a % of the unheated sample specific activity. After proteinase K digestion of activator fraction (Materials & Methods) samples were lyophilized and left at (a) room temperature for 49 days and (b) 2 days prior to resuspension in 20mM-bis-Tris buffer, pH 6.8 and assay for heat-reactivation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclave treatment (121°C, 30 min)</td>
<td>115 (202)</td>
</tr>
<tr>
<td>Formic acid hydrolysis (98-100 % [v/v]) (a) 100°C, 60 min</td>
<td>25 (286)</td>
</tr>
<tr>
<td>(b) 100°C, 150 min</td>
<td>143 (118)</td>
</tr>
<tr>
<td>Proteinase K digestion</td>
<td>1135 (1054)</td>
</tr>
</tbody>
</table>
control sample, which on this occasion exhibited no autoreactivation (as was observed sporadically), whereas the low-MW fraction (containing essentially no protein) exerted no stimulatory effect. These results initially suggested that the high-MW fraction contained the majority of the activator component and the low-MW fraction contained essentially no activator component. However, interpretation was complicated by the observation that reconstitution of the high- and low-MW fractions (taking into account the dilution effect on any low-MW components present in this sample, resulting from mixing of the final two PD-10 fractions) reproducibly effected a greater heat-reactivation of activity than that achieved by the high-MW fraction alone. This appeared to suggest that a second component of lower MW was involved which could be separated or dissociated from the higher MW component by PD-10 gel-filtration; the former component possessed a negligible intrinsic heat-reactivation potential, but when mixed or re-associated with the latter component was able to promote an enhancement of the efficacy of the heat-reactivation process.

This experiment was then repeated except that i) each fraction was initially heated separately from acetamidase at 60°C to stimulate any activator component present, prior to being mixed and incubated at 37°C with low-activity acetamidase to effect heat-reactivation, and ii) the fractions that were eluted sequentially from the column, and which were subsequently mixed with acetamidase, were not bulked. Each fraction exhibited only a barely-significant extent of heat-reactivation (after correction for autoreactivation), and the first low-MW fraction that eluted reproducibly showed a marginally greater extent of heat-reactivation than the high-MW fraction. A reconstituted high- and low-MW fraction mixture effected slightly greater stimulation of acetamidase activity than any single fraction alone. For reasons that currently remain obscure the majority of the activator component in this work eluted in the low-MW fraction, rather than in the high-MW fraction (this discrepancy must presumably be a function of the different types of heat-reactivation assay employed in the different experiments, and therefore not related to variable activator component PD-10 column elution profiles, as the pattern of results from both types of heat-reactivation assay were reproducibly different). Thus, in view of the limited resolution of the PD-10 gel-filtration method it was tentatively concluded that activator fraction can be fractionated crudely into samples of different (broad) MW ranges that exhibit significant yet variable intrinsic heat-reactivation potentials (depending on the nature of the heat-reactivation assay employed) and that the activator component was of relatively low-MW which possibly eluted in the region of the overlap between the high- and low-MW fractions. Additionally, it appeared likely that a second lower MW component was involved with heat-reactivation, but which was incapable of effecting heat-reactivation in the absence of the higher MW component. Although interpretation of these results is largely speculative it is likely that the higher MW activator component co-eluted in high- and low-MW fractions (due to a small degree of overlap between these fractions in terms of the size of molecules eluting in them which was generated by the gel-filtration column), although the resultant level of heat-reactivation of these fractions was probably complicated by the relative elution pattern of the second lower MW component putatively involved with the heat-reactivation process. Thus, it is likely that the relative
Activator fraction (3.5mg protein) was fractionated by PD-10 column gel-filtration to yield samples containing relatively high- and low-MW components. The sample was fractionated on a pre-equilibrated (20mM-bis-Tris buffer, pH 6.8 + 230mM-KCl) column according to the manufacturer's instructions (Pharmacia). Fractions were eluted sequentially in 3.5ml volumes of the same buffer and assayed for protein: the first was designated the 'high-MW' fraction and the next two fractions were mixed and designated the 'low-MW' fraction. Aliquots containing known amounts of protein were incubated (60°C, 1h) with low-activity acetamidase (30μg) to effect heat-reactivation. Appropriate controls were also included. A) untreated activator fraction + acetamidase; B) high-MW fraction + acetamidase; C) low-MW fraction + acetamidase; D) high-MW fraction + low-MW fraction + acetamidase; E) acetamidase + buffer. (■) unheated specific activity; (□) heat-reactivated specific activity. Specific activity was based on acetamidase protein only.
distributions (and hence concentrations) of these components in each eluted fraction, which is
determined by the relative MW of the two components, governs the observed extent of heat-
reactivation subsequently effected by these fractions, such that those fractions containing the
optimal ratio of the higher MW component to the lower MW component exhibit the greatest
heat-reactivation potential.

4.2.4 Fractionation of activator fraction by ultrafiltration

To further test the hypothesis that both high- and low-MW components of the activator
fraction were involved with heat-reactivation, molecules with a MW < 1kDa were separated
from higher MW components by ultrafiltration. The high-MW fraction was then subjected to
extended washing to remove any small molecules retained by this fraction. Upon mixing
aliquots of high-, low- and high- plus low-MW fractions with low-activity acetamidase and
attempting to heat-reactivate the latter by incubation at 60°C for 1 h it was unexpectedly
found, in contrast to the PD-10 gel-filtration work (section 4.2.3), that none of the fractions
stimulated acetamidase activity significantly, i.e. ultrafiltration of activator fraction appeared to
abolish its ability to effect heat-reactivation (Fig. 4.4). It was therefore concluded that both
high-MW and low-MW (< 1kDa) components are required for heat-reactivation, and that
these components are probably loosely associated, but cannot be re-associated in an active
form simply by re-mixing high- and low-MW fractions.

Slightly different results were reproducibly observed in similar experiments in which each
fraction was pre-heated at 60°C separately from acetamidase to stimulate any activator
component present, prior to mixing with (unheated) low-activity acetamidase at 37°C to effect
heat-reactivation. The results indicated (data not shown) that all test fractions stimulated
acetamidase activity significantly (after correction for autoreactivation), albeit only to a minor
extent, in the order of decreasing efficacy of sample heat-reactivation (D > C > B; see legend
to Fig. 4.4). Additionally, these results re-affirmed the hypothesis that ultrafiltration disrupted
the putative association between the high- and low-MW activator components, but in contrast
suggested that limited heat-reactivation could be effected by re-mixing high- and low-MW
activator components. Thus, it was demonstrated using a different heat-reactivation assay
method that ultrafiltration of activator fraction significantly reduced, but did not completely
abolish, its heat-reactivation potential.

In conclusion, all ultrafiltration results appear to confirm the PD-10 gel-filtration results
which strongly suggested that heat-reactivation was mediated by relatively high- and low-MW
components. These two components can be separated by gel-filtration and ultrafiltration with
variable degrees of completeness, and the resultant extent of heat-reactivation of low-activity
acetamidase by these fractions appears most likely to be a composite function of the relative
concentrations of the two different components in a given fraction. Separation of these two
components by disruption of the putatively labile association between them appears to abolish
all or the majority of the heat-reactivation potential of a sample which can only be regenerated
partially, if at all, by re-mixing of these components. However, this interpretation assumes that
Figure 4.4 Fractionation of activator fraction by ultrafiltration

Activator fraction (1.6mg protein) was diluted with 20mM-bis-Tris buffer, pH 6.8 + 230mM-KCl to a final volume of 2.5ml. A 2ml aliquot of this sample was fractionated using a pressurized (50 p.s.i) stirred ultrafiltration cell with a 1kDa MW exclusion limit. The ultrafiltrate (low-MW fraction) was collected and the retentate was reconstituted with 2ml of the same buffer and ultrafiltrated as before to wash low-MW molecules from the retentate. This washing step was repeated twice and the ultrafiltrate from each wash was discarded. Finally, the retentate was reconstituted with buffer as above (high-MW fraction). Aliquots of high- and low-MW fractions were mixed and incubated (60°C, 1 h) with buffered low-activity acetamidase (30μg), as indicated below, to effect heat-reactivation. Appropriate controls were also included. A) untreated activator fraction + acetamidase; B) washed high-MW fraction + acetamidase; C) low-MW fraction + acetamidase; D) washed high-MW fraction + low-MW fraction + acetamidase; E) acetamidase + buffer. (■) unheated activity; (□) heat-reactivated activity. Specific activity was based on acetamidase protein only.
all of the lower MW component involved with heat-reactivation would appear immediately in
the ultrafiltrate, which pre-supposes that this component is free in solution and not bound to,
or associated with, larger components that might retard its partitioning from the retentate. An
alternative interpretation is that exit of the low-MW activator component from the retentate
was retarded by its association with molecules of MW > 1kDa, such that only a very small
proportion of the available low-MW component passed quickly into the ultrafiltrate. The
subsequent dilution effect of extended washing of the retentate with buffer may then have
elicited the dissociation of the low-MW component from its putative bound state such that it
was then washed from the retentate and discarded. It is therefore possible that the inability to
achieve full heat-reactivation of acetamidase after re-mixing high- and low-MW components,
was not because simple re-mixing of these components could not regenerate the original
putatively labile association between these two components, but because the reactivation
process was limited by the concentration of the lower MW component, which had been
largely separated from both the high- and low-MW fractions and discarded after dilution by
the washing process. This interpretation precludes the necessity to postulate that a labile
association exists between the relatively high- and low-MW components involved with the
heat-reactivation process and constitutes the simplest hypothesis explaining the observed
results. Further to this, the extent of heat-reactivation effected by a given sample appeared to
be influenced by the type of heat-reactivation assay employed in a similar fashion to the
results found for assay of PD-10 gel-filtration fractions by different heat-reactivation assay
methods. These observations are reminiscent of those concerning the complete and
irreversible loss of activity from the regulatory calmodulin-like protein isolated from
*B. subtilis* upon removal of Ca$^{2+}$ by dialysis, and the involvement of dialyzable and non-
dialyzable factors with reactivation of RubisCO activity in *R. rubrum* (see Introduction).

4.2.5 Investigation of the mechanism of heat-reactivation

The question of whether heat-reactivation of low-activity acetamidase was effected
stoichiometrically or catalytically was addressed by characterizing the heat-reactivation profile
of acetamidase at 60°C (Fig. 4.5). The results showed that the heat-reactivation profiles for
two different concentrations of activator fraction, viz. 150µg and 450µg protein (respectively
representing five- and fifteen-fold excesses over the sample acetamidase protein
concentration) were complex. The initial increases in acetamidase activity for both samples
incubated with activator fraction appeared to be essentially complete after 0.5 h incubation,
after which time respective samples appeared to undergo only a small overall change in
activity. The control sample showed a slight increase in activity between 1-2 h due to
autoreactivation, but its specific activity remained essentially unaltered over the duration of the
incubation (i.e. its activity after 3 h incubation was approximately the same as an unheated
sample). Consideration of possible mechanisms of heat-reactivation suggested that if
acetamidase was heat-reactivated in a simple catalytic fashion by the activator component it
would be expected that the 150µg activator sample would have achieved the same final extent
Figure 4.5 Investigation of the mechanism of heat-reactivation

Two different amounts of activator fraction protein (150µg and 450µg, representing 5- and 15-fold excesses respectively over acetamidase protein) were diluted with 20mM-bis-Tris buffer, pH 6.8 and incubated with a constant standard amount of low-activity acetamidase (30µg; final sample volume 700µl) in triplicate at 60°C for 0-3 h. Aliquots (200µl) were withdrawn at intervals and assayed for acetamidase activity with acetamide as substrate at 37°C (Materials & Methods). The extent of heat-reactivation is defined as the heat-reactivated minus unheated specific activities based on acetamidase protein alone. No added activator fraction (volume replaced by buffer) (–●–); 150µg activator fraction protein (–○–); 450µg activator fraction protein (–△–). The average unheated specific activity was 10.2µmol min⁻¹ (mg protein)⁻¹. The extent of autoreactivation or heat-reactivation after 3 h is shown in parentheses.
of heat-reactivation as the 450μg activator sample, except that this would have occurred at only 33% of the rate. Conversely, if the activator component mediated heat-reactivation stoichiometrically it would be expected that the 150μg activator sample would effect only 33% of the total extent of heat-reactivation shown by the 450μg activator sample, but that the rate of reactivation would be similar in each case. Estimation of the overall rate of sample heat-reactivation from Fig. 4.5 was not possible; however, it can be seen that the maximal extent of heat-reactivation of both samples containing activator fraction had been reached after approximately 0.5 h incubation, and that a greater extent of reactivation was evident for the 450μg activator sample than the 150μg activator sample. The final extents of heat-reactivation for these samples, viz. 113% (150μg activator sample) and 207% (450μg activator sample) suggested that an increase in the amount of activator fraction promoted an increase in the extent of heat-reactivation, and that extent of heat-reactivation was probably limited by the concentration of the activator fraction. In the absence of information concerning the rate at which heat-reactivation occurred in these samples, it was concluded that these results are suggestive of stoichiometric reactivation, albeit imperfect.

Results from similar experiments in which a fifty-fold range of activator fraction concentrations were pre-heated separately at 60°C prior to being incubated with unheated low-activity acetamidase at 37°C to effect heat-reactivation also showed the same general trends (data not shown). However, due to the complex nature of the heat-reactivation assay, with its multitude of ill-defined variables, it must be borne in mind that certain assumptions were made during interpretation of these results, viz. that the observed extents of heat-reactivation were not limited by the concentration of acetamidase, i.e. that the concentration of acetamidase was in excess over the concentration of the activator component, and that the duration of incubation was sufficient to give an accurate measurement of the final total extent of heat-reactivation etc. The limitations of this work are considered further in the Discussion.

The mechanism of the heat-reactivation of low-activity acetamidase was further investigated by characterizing the heat-reactivation profile of two different concentrations of low-activity acetamidase by a constant amount of activator fraction at 60°C (Fig. 4.6). If stoichiometric reactivation was occurring, and acetamidase was in excess over the activator component, then it would be expected that the observed extent and rate of heat-reactivation in the two acetamidase samples (containing 30μg and 60μg) would be identical. Conversely, if simple catalytic reactivation was occurring the 60μg acetamidase sample would be expected to exhibit a doubled extent of heat-reactivation in comparison to the 30μg acetamidase sample, but the heat-reactivation of both samples would be expected to occur at the same rate. Estimation of the overall rates of heat-reactivation from these data was again not possible; however, the results showed that the maximal extent of heat-reactivation had been essentially reached in both the 30μg and 60μg acetamidase samples after 0.5 h incubation, and that respective activities exhibited little overall change after this time. From consideration of the observed final extents of heat-reactivation for 30μg and 60μg samples respectively, viz. 172% and 71%, it was evident that in proportional terms, the amount of acetamidase that had been reactivated in the two different samples (i.e. the extent of heat-reactivation), per unit of activator fraction,
Figure 4.6 Investigation of the mechanism of heat-reactivation

Two different samples of low-activity acetamidase (30μg and 60μg) were mixed with a constant amount of activator fraction (150μg) and diluted in duplicate with 20mM-bis-Tris buffer, pH 6.8 to a final volume of 700μl. Samples were incubated at 60°C for 0-2 h and aliquots (200μl) were removed at intervals and assayed for acetamidase activity with acetamide as substrate at 37°C (Materials & Methods). The extent of heat-reactivation is defined as the heat-reactivated minus unheated specific activities based on acetamidase protein alone. Acetamidase (60μg) + activator fraction (---); acetamidase (30μg) + activator fraction (-----); control acetamidase (60μg) + buffer (----). The average unheated specific activity was 11.7μmol min⁻¹ (mg protein)⁻¹. The extent of autoreactivation or heat-reactivation after 2 h is shown in parentheses.
was approximately the same confirming that the activator fraction was limiting. In contrast, the control sample, containing no activator fraction showed only a small increase in activity, due to autoreactivation as expected. The heat-reactivation profiles therefore appear to confirm that heat-reactivation of acetamidase by the activator fraction is mediated by a mechanism suggestive of a stoichiometric event.

The ability of the activator fraction to heat-reactivate low-activity acetamidase was also addressed by incubating a standard quantity of acetamidase with increasing amounts of activator fraction at 60°C for 1 h (Fig. 4.7) (from previous work it was known that maximal heat-reactivation of this amount of acetamidase occurred after 0.5 h incubation at this temperature). A non-linear relationship was apparent between these two variables, such that the extent of heat-reactivation increased smoothly with an increase in amount of activator fraction protein up to a fourteen-fold excess over acetamidase protein. No data was available after this point, but the rate of increase of the extent of reactivation in relation to increasing activator fraction protein appeared to be diminishing. Thus, it was concluded that the extent of heat-reactivation of low-activity acetamidase was directly-related to the amount of activator fraction, although this relationship was not a simple linear function.

4.2.6 The reversible heat-reactivation in vitro of low-activity acetamidase

Previous heat-reactivation studies had suggested that the specific activity of heat-reactivated low-activity acetamidase diminished upon prolonged incubation at 37°C (see section 4.2.1), i.e. heat-reactivated (high-activity) acetamidase was prone either to enhanced denaturation or to re-conversion to the low-activity form. That heat-reactivation was a reversible process was tested by heat-reactivating a sample of low-activity acetamidase at 60°C and then re-purifying the acetamidase, whilst measuring its specific activity at each step of the purification to detect any diminution in activity (Table 4.2). The specific activity of a control sample of heat-reactivated acetamidase (stored at 4°C and not subjected to further treatment), was also measured, at the same time as that of sample C (6 h after samples had been heat-reactivated); both samples exhibited very similar activities at this time (17.1 cf. 17.9μmol min⁻¹ [mg protein]⁻¹ respectively), suggesting that an equivalent diminution in sample activity occurred independently of the initial purification treatments, i.e. that the diminution in activity probably occurred spontaneously. However, since this supposition was based on a single observation it was viewed with caution. It was therefore of potential importance to the interpretation of results that the activity of the final re-purified sample (D) was not measured until 18 h after that of the control sample, viz. whether sample activity loss (switch-off) occurred spontaneously or resulted directly from re-purification.

In view of the fact that the specific activity of the original heat-reactivated sample decreased substantially during re-purification (from 19.6 to 11.1μmol min⁻¹ [mg protein]⁻¹), and the specific activity of the re-purified enzyme was fully-reactivated by heat in the presence of activator fraction at 60°C (11.1 to 25.6μmol min⁻¹ [mg protein]⁻¹), it was therefore concluded
Figure 4.7 Investigation of the relationship between the amount of activator fraction and the extent of heat-reactivation

A standard amount of low-activity acetamidase (30µg) was mixed with a range of amounts of activator fraction (0-420 µg protein) in a final sample volume of 700µl. Aliquots (200µl) were removed from the sample before and after incubation at 60°C for 1 h, cooled (4°C, 5 min) and assayed for acetamidase activity with acetamide as substrate at 37°C (Materials & Methods). The extent of heat-reactivation is defined as the heat-reactivated minus the unheated specific activities based on acetamidase protein alone. The average unheated specific activity was 11.4µmol min⁻¹ (mg protein)⁻¹.
Table 4.2 The reversible heat-reactivation *in vitro* of low-activity acetamidase

Low-activity acetamidase (160μg) was mixed with a ten-fold excess (1600μg protein) of activator fraction and diluted to 1ml final volume with 20mM-bis-Tris buffer, pH 6.8. This sample was then incubated for 2 h at 60°C and its acetamidase activity was assayed with acetamide as substrate at 37°C (Materials & Methods) immediately before and after heat-reactivation (sample A). The heat-reactivated mixture was microfuged to remove precipitated macromolecules and the supernatant (sample B) was desalted by PD-10 gel-filtration (Pharmacia). The desalted sample (sample C) was re-purified using anion-exchange FPLC chromatography (using the third KCl gradient; see Materials & Methods). Peak-activity acetamidase fractions were identified, pooled (sample D) and shown to be homogeneous with respect to acetamidase by SDS-PAGE (data not shown; Materials & Methods). An aliquot of sample D was then incubated with an excess of activator fraction for 2 h at 60°C and assayed for acetamidase activity before and after heat-reactivation. Δ Activity is defined as the difference between unheated and heated activities as a % of the unheated activity. Specific activity was based on acetamidase protein alone.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetamidase activity (μmol min⁻¹ [mg protein]⁻¹)</th>
<th>Δ Activity (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>-Heat</td>
<td>+Heat</td>
</tr>
<tr>
<td>A</td>
<td>6.4</td>
<td>19.6</td>
</tr>
<tr>
<td>B</td>
<td>18.5</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>17.9</td>
<td>—</td>
</tr>
<tr>
<td>D</td>
<td>11.1</td>
<td>25.6</td>
</tr>
</tbody>
</table>
that heat-reactivation is a reversible stimulatory effect, and that the loss of heat-reactivated activity results from switch-off (not denaturation); however, it could not be ascertained from these data whether switch-off resulted directly from re-purification or whether it occurred spontaneously.

4.3 Discussion

This work was carried out at intervals over the first eighteen months of the available laboratory research time and represents a brief biochemical investigation of the heat-reactivation phenomenon. Certain assumptions have been made (as indicated in the relative texts) concerning practical aspects of the heat-reactivation assay, and the limitations so imposed on this work are acknowledged. It is suggested that further investigation of heat-reactivation would benefit greatly from both biophysical and kinetic studies.

That acetamidase can exist in different relatively high- and low-activity catalytic forms in vivo is unquestionable, and seemingly incontrovertible evidence also exists for the presence of a 'fine-tuning' regulatory mechanism (presumably co- or post-translational), other than a transcriptional 'switch' (induction/repression), for the modulation of acetamidase activity. This evidence is based on the decrease in acetamidase activity (switch-off) that occurs upon pulsing excess ammonia into a dual C/N-limited culture which cannot be accounted for by simple cessation of acetamidase synthesis and its subsequent dilution from the culture. Similarly, the increase in acetamidase activity (switch-on) that occurs upon depletion of ammonia from the same culture cannot be accounted for by a transcriptional mechanism (see Carver & Jones, 1993; J. Mills & C.W. Jones, unpublished). Thus, a secondary regulatory mechanism responsible for cycling switch-on and switch-off processes in response to environmental stimuli has been postulated, in addition to the 'coarse' regulatory mechanism of induction/repression by short-chain aliphatic amides and ammonia respectively, for the modulation of acetamidase activity.

Heat-reactivation of acetamidase in vitro is obviously a non-physiological mechanism which is either artefactual (and therefore happenstantially appears to mimic the switch-on of acetamidase in vivo) or the effect of heat must mimic the physiological function of an acetamidase-associated regulatory protein(s) or another type of regulatory interaction. Artefactual enzyme stimulation has been demonstrated previously (Long & Anthony, 1991), in which the activity in the enzyme MDH from M. methylotrophus and P. denitrificans was altered by partially-purified thermostable non-proteinaceous factors (stable from pH 2.0-11.0; approximate MW, 1-3 kDa) which were able to mimic spuriously one particular function of the MDH modifier protein. These non-proteinaceous factors appear to have certain properties in common (high thermo- and pH stabilities, relatively low-MW) with components involved in the heat-reactivation of acetamidase; cautious interpretation of the physiological-relevance of work described in this Chapter is therefore necessary.

Autoreactivation of low-activity acetamidase, i.e. the activator fraction-independent stimulation of activity that occurs after incubation at 37°C or 60°C can be demonstrated by
incubating acetamidase at 37°C in the absence of substrate. The observed increase in specific activity is apparently complete within 10 min, although the exact rate at which it occurs is not known. Thus, the acetamidase activity of the low-activity enzyme at 37°C in vivo may be up to 60% higher than that expected from the specific activity of the purified enzyme, unless this phenomenon is an artefact generated by manipulation in vitro. The physico-chemical basis for autoreactivation is not known, but it is not unreasonable to suggest that it may result from a conformational change which increases access of the substrate (acetamide) to the active site. Similarly, the putative hysteretic effect exhibited at 37°C by the low-activity enzyme after substrate (acetamide) addition may represent a conformational change resulting from an allosteric effect due to substrate binding (and possibly due to the effect of elevated temperature also). This property of the enzyme may therefore be an example of the slow enzyme response discussed by Frieden (1970; see also Chapter 1) resulting from a rapid change in ligand (acetamide) concentration.

The apparent constitutivity of the activator component was unexpected as it would presumably be energetically less-wasteful for this organism to regulate co-transcription of a putative acetamidase regulatory protein(s) with that of the acetamidase structural gene. The putative constitutivity may therefore reflect either a complex regulatory system or suggest that the regulatory molecule(s) involved with heat-reactivation in vitro is not necessarily a gene product, and may therefore be an organic compound or an ion that is consistently present intracellularly.

The heat-reactivation assay employed to investigate the stimulation of low-activity acetamidase is a complex multi-variable system, the results from which require cautious interpretation. The variables that directly influence measured activities include the differential thermostabilities of acetamidase and the activator fraction components at 60°C (particularly the rate at which molecules denature at this temperature), the effect of autoreactivation, the potential ionic effects of buffer/KCl concentration, the loss of activity from the apparently unstable heat-reactivated (high) activity state, and the apparent involvement of more than one activator component (see below). Thus, the specific activities calculated from heat-reactivation data are potentially highly-variable, and represent the observed activity at a particular point in time as a composite function of all the variables described above. If accurate conclusions are to be drawn from this work they must be formulated from data produced under standardized experimental conditions. Since the heat-reactivation process is currently ill-defined, certain arbitrary assumptions must therefore be made (see below) concerning the relative concentrations of activator fraction and acetamidase routinely utilized in heat-reactivation assays, the duration of incubation of samples during reactivation etc.

The mechanism of heat-reactivation is obscure, but it is interesting to speculate on its possible nature. The requirement for heat pre-treatment of the activator fraction is of prime importance and suggests that heat modifies the activator component in such a way that only in its heated form can it effect reactivation of acetamidase activity. This suggests that the activator component either i) loses a chemical group (as heat is unlikely to promote the addition of a chemical group), as is apparent for the nitrogenase from *R. rubrum* in which
heating the inactivated enzyme in vitro causes release of the ADP-ribosyl modifying group and hence reactivation of nitrogenase activity (Pope et al., 1985), or ii) undergoes a conformational change that allows it to interact with, and thereby reactivation, low-activity acetamidase. This interaction may involve acetamidase either donating a chemical group to the activator component (which in its heat-treated state acts as an acceptor) or accepting a chemical group from the activator component (which in its heat-treated state can act as a donor). Alternatively, the heat-treated activator component may be able to interact directly with acetamidase by the formation of an activator/acetamidase complex (ESMS data favours the latter explanation as the MW-difference between high- and low-activity acetamidases is ≤ 52Da; thus the MW of the chemical group postulated above must be correspondingly low, and the number of putative modifying groups fulfilling this criterion is small). The major heat shock/chaperonin protein (cpn60 or GroEL protein) of *R. sphaeroides* has been shown to associate with form I RubisCO in the presence of ATP (Wang & Tabita, 1992c), and these workers have proposed that the regulation of RubisCO activity may be involved with the formation of a stoichiometric complex of RubisCO and cpn60, in the presence of ATP, such that the unfavourable RubisCO protein conformation is overcome by protein re-folding. It is possible that the activator component-mediated reactivation of acetamidase occurs via a similar type of mechanism, and investigation of the mechanism of heat-reactivation also lends credence to the possibility that heat-reactivation occurs stoichiometrically (see below).

Studies concerning the stability of the component(s) of the activator fraction involved with heat-reactivation showed that it was exceptionally stable to adverse conditions of pH, temperature, pressure and proteolysis suggesting that its heat-reactivation potential is unlikely to be enzyme-mediated. Thus, the activator component must be either an exceptionally-stable macromolecule whose tertiary structure is unaffected by the adverse conditions imposed, or is an unstable macromolecule of which only part (a particular protein domain, perhaps) is of importance to the heat-reactivation process. Alternatively, the activator component may be a non gene-encoded molecule that is stable to the adverse effects of all the experimental conditions tested.

Fractionation studies of the activator fraction suggested that more than one constituent molecule was involved with the heat-reactivation process. PD-10 column gel-filtration results, whilst supporting the data from ultrafiltration work, were subject to complications associated with the limited resolution of this column (which generates an overlap between relatively high- and low-MW fractions) and results might be improved by utilizing a gel-filtration column of greater resolution (e.g. Superose 6 or 12; Pharmacia). Results from ultrafiltration work (1kDa MW exclusion limit) showed that this treatment significantly reduced or abolished the heat-reactivation potential of the activator fraction. This was either due (i) to the disruption of a putatively-labile association between the activator components, the maintenance of which was a pre-requisite for its heat-reactivation potential, and which could not be regenerated by simply re-mixing high- and low-MW fractions, or (ii) to the fact that upon re-mixing high- and low-MW fractions the system was deficient in the lower MW activator component, which had presumably been removed by extended washing of the high-MW fraction. This latter
hypothesis is deemed the simplest and most plausible, and it is tentatively concluded that in addition to heat, the in vitro reactivation process requires the presence of at least two more components which act together. The fact that these two components are of relatively high- and low-MW, and that their relative concentrations appear to be of importance to the heat-reactivation process, suggests a stoichiometric reactivation mechanism, which may involve a macromolecule/ligand interaction.

Direct investigation of the mechanism of heat-reactivation also strongly suggested a stoichiometric, rather than a catalytic basis, although the data did not conform ideally to a stoichiometric model. Experimental design was such that it was difficult to ascertain when the acetylamidase protein concentration would be in excess over the activator fraction concentration and vice versa, and the end-point of the heat-reactivation process was often difficult to identify. This therefore led to difficulties with experimental design and interpretation of results. Clarification of these results could be achieved by carrying out experiments in which a constant amount of low-activity acetylamidase is titrated against a wide range (e.g. 0-1 mg) of activator fraction protein amounts, and vice versa (to extend the work shown in Fig. 4.6 & 4.7). Measurement of the extent of heat-reactivation in each case could then be used to determine critical saturating acetylamidase to activator fraction protein concentration ratios under a given set of experimental conditions. The experiments shown in Fig. 4.5 and Fig. 4.6 could then be repeated under better-defined conditions, and perhaps also with a wider range of concentrations of the activator fraction and acetylamidase respectively, to more fully characterize the relationship between their relative concentrations during heat-reactivation in vitro.

The reversible nature of the heat-reactivation process was demonstrated in an experiment in which heat-reactivated acetylamidase was re-purified using FPLC, apparently as a result of which the enzyme underwent a significant diminution of specific activity and became heat-reactivatable in an identical fashion to other low-activity acetylamidase samples. This observation suggests that the specific activity of acetylamidase can be cycled between relatively switched-on and switched-off states in vitro, although the number of such cycles that can be effected without significant denaturation of the acetylamidase is not known. These results also show that the heat-reactivated high-activity state must be a transient unstable state which appears to decay either spontaneously or as a result of re-purification of acetylamidase. This suggests that the physico-chemical alteration to the low-activity acetylamidase induced by heat-reactivation is labile. Consequently, it is unlikely that this modification has a covalent basis as the disruption and reformation of a covalent bond(s) by the treatments described above must be deemed implausible, and the observed change in activity can be more readily explained in terms of a putative heat-induced acetylamidase conformational change that is somehow stabilized at elevated temperatures, but which is reversed spontaneously or upon cooling. Since the rate of loss of heat-reactivated activity appears to be essentially zero over approximately 4 h incubation at 60°C (section 4.2.1), but is much greater at 37°C (Fig. 4.2) than at 4°C (data not shown), then unless there is some obscure significance to these particular temperatures, the simplest interpretation of the re-purification results is that switch-
off occurs spontaneously and is possibly accelerated by the effects of the various treatments of the purification protocol (desalting, anion-exchange chromatography etc.); otherwise, if switch-off was a general consequence of the effect of cooling on a heat-reactivated sample it would be expected that the rate of switch-off would be greater at 4°C than it would be at 37°C. The spontaneous switch-off hypothesis could be tested in future by repeating the re-purification experiment and including a heat-reactivated control sample that is stored at 4°C and not re-purified. The reactivated control sample could then be assayed for acetamidase activity at the same time as the final re-purified sample, to determine the overall effect of time alone versus the effects of the purification protocol treatments on the specific activity of the heat-reactivated sample. Thus, the probable spontaneous mechanism of switch-off appears to be common to all of the switch-off events that have been observed in vitro, viz. switch-off of heat-reactivated high-activity acetamidase samples (this Chapter) and switch-off of other high-activity samples during purification (Chapter 3).

In many ways, the characteristics of the heat-reactivation of acetamidase are similar to the stimulation of MDH activity from the thermotolerant Bacillus sp. C1 by its activator protein (see Arfman et al., 1991). The stimulation of acetamidase and MDH by their respective activators can be effected reversibly (although heat is not necessary for stimulation of the Bacillus enzyme), and both stimulatory effects are dependent on the presence of a low-MW factor (in the case of MDH this factor is Mg²⁺), in addition to the higher MW activator protein (or possibly a fragment thereof for the acetamidase system). Arfman et al. (1991) concluded that the stimulation of MDH probably occurred by a non-covalent mechanism which was mediated by a loose interaction between MDH and its activator protein, due to the reversible nature of the MDH stimulation, and because the formation of an MDH/activator protein complex could not be demonstrated. It is therefore tempting to speculate that a similar system might mediate the heat-reactivation of acetamidase, as this is also a reversible process which probably occurs stoichiometrically, although no attempts have been made to isolate or identify an acetamidase/activator complex.

Many of the properties of acetamidase (e.g. switch-off of high-activity acetamidase during purification and diafiltration [see Chapter 3]; the differential sensitivities of high- and low-activity forms to the thiol reagent p-chloromercuribenzoate which suggests conformational differences exist between the different enzymic forms; the reversible nature of heat-reactivation of acetamidase etc.) are amenable to interpretation in terms of hysteretic and/or allosteric regulatory modes of acetamidase activity. All such observations form a large body of circumstantial evidence only for the non-covalent regulation of acetamidase activity; however, even though no overwhelming evidence exists to refute the presence of a covalent modification to acetamidase, non-covalent regulation must currently remain the most attractive and plausible hypothesis. It is therefore tentatively concluded that acetamidase may be an allosteric/hysteretic enzyme, the activity of which can be reversibly heat-reactivated in vitro by a stoichiometric process involving at least two stable activator components (of relatively high- and low-MW) working in concert. The high-activity forms of acetamidase (see Chapter 3) are much less stable than the low-activity form (which could be considered to be the activity
'ground state' of this enzyme), and it seems that both heat-reactivated and native high-activity enzymes re-convert (probably spontaneously) to the low-activity form over time. This suggests that high-activity forms are transient states that are presumably stabilized in vitro (and possibly in vivo also) by an allosteric enzyme/ligand interaction (as has been reported for various other amidases). Switch-off of acetamidase activity (native or heat-reactivated) probably involves the decay of this allosteric modification such that the dissociation of the ligand from the enzyme allows the gradual re-conversion to the low-activity enzymic form, which may occur by a conformational change. Thus, acetamidase activity could be cycled between relatively switched-on and switched-off states presumably by the higher MW activator component, in response to the concentration of the unidentified ligand (possibly the lower MW activator component), and possibly as part of a feed-back inhibition loop involving monitoring of the intracellular ammonia and/or acetate concentrations. The reasons underlying the inability to heat-reactivate acetamidase activity to a level commensurate with that of very-high activity acetamidases described previously (specific activity ≥ 87μmol min⁻¹ [mg protein]⁻¹; Chapter 3) remain obscure, although this may involve the destruction of one of the activator components by heat or the enhanced thermolability of high-activity acetamidase at the heat-reactivation temperature (60°C; see also Chapter 3).
CHAPTER 5

ATTEMPTED MOLECULAR CLONING OF ACETAMIDASE:
ALL THAT GLISTERS IS NOT GOLD
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ATTEMPTED MOLECULAR CLONING OF ACETAMIDASE:
ALL THAT GLISTERS IS NOT GOLD

5.1 Introduction

5.2 Attempted molecular cloning of the acetamidase structural gene
\((amiE)\) of \(M. \text{methylotrophus}\)

5.2.1 Utilization of Southern hybridization to identify \(amiE\)
5.2.2 Transformation and attempted direct selection of recombinant clones expressing active amidase
5.2.3 Characterization of putative acetamidase-expressing clones
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5.3 Discussion
ATTEMPTED MOLECULAR CLONING OF ACETAMIDASE:
ALL THAT GLISTERS IS NOT GOLD

5.1 Introduction

The acetamidase of *M. methylotrophus* exhibits similar biochemical properties to the amidases of *Ps. aeruginosa* and *Brevibacterium* sp. R312 (Chapter 3). The structural gene (amiE) of the *Ps. aeruginosa* amidase was cloned previously (Drew *et al.*, 1980) using a bacteriophage λ replacement vector and insert DNA derived from a constitutive mutant *Ps. aeruginosa* strain that was also resistant to catabolite repression. The rationale behind this particular choice of 'donor' DNA was based on the supposition that the subsequently cloned DNA would contain amiE contiguous with amiR, the gene product of which (AmiR) was known to regulate positively amidase activity (Farin & Clarke, 1978); therefore it was deemed likely that AmiR would be available to promote amidase activity in recombinant phage particles, and additionally the mutation abolishing sensitivity to catabolite repression might allow transcription of amiE even if the *Ps. aeruginosa* promoter was not recognized by the *E. coli* cAMP receptor protein (CRP-cAMP). Recombinant amiE phage particles (containing a 7.5-10.0 kbp fragment of *Ps. aeruginosa* DNA) were identified as a result of the enhanced growth of 'haloes' of *E. coli* around phage plaques on solid minimal medium containing glucose and acetamide respectively as C- and N-sources. The enhanced growth of the bacterial cells was attributable to the availability of acetate and ammonia released into the medium from acetamide hydrolysis by the phage-encoded recombinant amidase, which was liberated from amiE+ bacterial cells after lysis.

Clarke *et al.* (1981) located amiE to a 2.4kbp DNA fragment by deletion analysis using λ vectors, and also constructed a recombinant *E. coli* strain expressing low plasmid-encoded amidase activity. Fine structure restriction mapping of this plasmid allowed the alignment of the amiE gene within the cloned fragment by translation of the nucleotide sequences defining the restriction sites into amino acid sequences in all reading frames and comparing them with the N-terminal amino acid sequence of the amidase which was already known. Later work (Cousens *et al.*, 1987) studied the control of amidase activity at the genetic level in *Ps. aeruginosa* and *E. coli* using recombinant broad-host-range plasmids carrying the amidase genes. This work located the position of amiR to a site approximately 2kbp downstream from amiE and showed that the two genes were transcribed in the same direction. These studies also indicated that the amiR promoter was located at an undefined upstream position from amiR and that AmiR was probably a 23kDa protein. Further work (Ambler *et al.*, 1987; Brammar *et al.*, 1987) yielded respectively the full amino acid sequence of the amidase (346 residues) (after amino acid sequencing studies of the fragmented protein) and the complementary DNA sequence of the structural gene amiE (1038 nucleotides in length), the results from which were in complete agreement with each other.
More recent work (Drew & Lowe, 1989) showed that the activity of the amidase was positively-regulated by a transcription anti-termination mechanism mediated by AmiR. Analysis of the DNA sequence upstream from amiE strongly suggested that the structural gene was transcribed from a promoter situated 150bp before its start codon, and that the intervening sequence contained a short open reading frame (35 amino acids), an E. coli-like rho-independent transcription terminator and a ribosome binding site. Furthermore, Lowe et al. (1989) elucidated the amiR gene sequence which was found to encode a protein of 196 amino acids (MW 21,776Da), and sequence analysis also suggested that amiR was probably transcribed as an element of a polycistronic transcript. This supposition was based on the upstream presence of an open reading frame (ORF) whose termination codon overlapped with the translation initiation codon of amiR, and the accessory downstream presence of a third ORF; however, neither the identities or the functions of the gene products encoded by the ORFs flanking amiR were known at this time (the downstream ORF was eventually identified as amiS; see below). The gene product transcribed from the ORF immediately upstream from amiR was eventually identified (after cloning of the wild-type Ps. aeruginosa amidase genes; Wilson & Drew, 1991) as a unique protein of 385 amino acids (MW 42,834Da) which exhibited no significant homology with other published protein sequences. The newly-identified sequence was designated amiC and it was suggested that transcription of amiCR was coordinately regulated as the termination codon of amiC overlapped with the start codon of amiR (as postulated previously; Lowe et al., 1989). Since disruption of the amiC reading frame led to constitutive amidase synthesis it was postulated that AmiC was a negative regulator of amidase activity and this was confirmed by trans-complementation studies in which plasmid-encoded AmiC repressed the amidase activity of certain previously characterized constitutive Ps. aeruginosa amidase mutants. Two potential rpoW-dependent promoters were also identified upstream from the amiC sequence.

The most recent work concerning the Ps. aeruginosa amidase (see Drew & Wilson, 1992; Wilson et al., 1993) has suggested that AmiR-dependent anti-termination of amidase transcription is probably mediated by a site-specific interaction between AmiR and the amiE leader mRNA. It has been proposed that binding of AmiR to this particular site which overlaps with the terminator sequence prevents formation of a hair-pin loop RNA structure and therefore allows RNA polymerase to proceed with transcription. Plasmid trans-complementation studies that completely re-constituted a functional amidase regulatory system in E. coli have also shown that AmiR functions only as an anti-termination factor and that AmiC acts post-transcriptionally as an amide-dependent regulator of AmiR (and therefore fulfils the sensory role in this system) which abolishes the ability of AmiR (acting as the regulator) to function as a transcription anti-termination factor. The negative influence of AmiC on AmiR has been suggested to occur via a stoichiometric protein-protein interaction, rather than by AmiC exhibiting catalytic activity resulting in covalent modification of AmiR, or by AmiC acting as a repressor and binding to an operator-regulatory site upstream from amiR and thereby influencing transcription of the latter gene.
The possible influence of the general nitrogen control system on the regulation of amidase activity was also partially clarified by studies in which cloned amidase genes were activated in *E. coli* after growth under nitrogen-limitation. This finding, taken with others from the same study, suggested that amidase expression might be regulated from the putative *rpoN*-dependent promoters located upstream from *amiC* by the *E. coli* ntr system, and that the *rpoN* protein or an *rpoN*-dependent protein exerted a negative regulatory influence on amidase expression. Preliminary work in *Ps. aeruginosa* confirmed that the *rpoN* system appeared to dampen amidase activity, and further work in this area is continuing.

Two further genes have been identified in the amidase operon (Wilson, 1991). The first, *amiS*, is situated immediately downstream from *amiC*, and its gene product (AmiS) shows a hydrophobicity profile which suggests that it is an integral membrane protein. The second gene, *amiB*, is situated in the *amiEC* intergenic region and its gene product (AmiB) is essentially a hydrophilic protein that contains a consensus nucleotide-binding site. Drew and Wilson (1992) have proposed that these two proteins constitute an energy-dependent aliphatic amide-uptake system, and further studies are under way to clarify this notion.

Thus, the *Ps. aeruginosa* operon has been completely sequenced and characterized and its complexity is evident. The operon contains novel positive and negative regulatory proteins, AmiR and AmiC respectively, that interact such that the latter amide-responsive element inhibits the transcription anti-termination function of the former component upon binding of certain amides. This interaction allows transcriptional read-through of the terminator sequence upstream of the *amiE* structural gene by RNA polymerase leading to the biosynthesis of amidase. In addition, the amidase operon is probably subject to the further regulatory effects of the global nitrogen regulation system (ntr) acting on *amiC*.

The cloning and DNA sequencing of the *Brevibacterium* sp. R312 wide-spectrum amidase has also been reported (Soubrier et al., 1992). This gene was cloned indirectly using two 15-mer oligonucleotide primers (each exhibiting 32 degeneracies) that were complementary to the first (sense primer) and last (anti-sense primer) five amino acids of the N-terminal sequence of the amidase which was determined previously (Thiéry et al., 1986b). Sense and anti-sense primers (carrying respectively EcoRI and HindIII linkers) were utilized in PCR reactions which allowed the amplification of a DNA fragment complementary to the 5'-end of the amidase structural gene which was cloned into M13mp19 after EcoRI/HindIII digestion. From sequence analysis of the amplified fragment a 40-mer non-degenerated oligonucleotide probe was designed and subsequently used in Southern hybridization studies with digested chromosomal DNA isolated from *Brevibacterium* sp. R312. A 5.3kb fragment exhibiting a strong hybridization signal with the probe was readily identified using this method and this fragment was then inserted into pUC19.

The DNA sequence of an internal fragment of the 5.3kbp fragment was subsequently determined which showed an ORF (designated *amiE*) encoding a 345 amino acid polypeptide (MW 38.2kDa) exhibiting very high homology with the *amiE* gene product (> 81% strict identity at the amino acid level) from *Ps. aeruginosa* (346 amino acids). In addition, two more ORFs were identified downstream from *amiE*, the first of which was highly homologous to
the regulatory sub-unit of an *E. coli* ATP-dependent protease, and the second ORF apparently encoded a unique polypeptide. It was concluded that further work would be necessary to determine the significance, if any, of the clustering of these genes with respect to the regulation of amidase activity. Soubrier et al. (1992) postulated that the exceptional degree of homology between amIE-encoded enzymes in *Brevibacterium* sp. R312 and *Ps. aeruginosa* probably resulted from horizontal gene-transfer between Gram-positive and Gram-negative organisms, and the absence of significant homology between these amidases and the previously reported enantiomer-selective amidases from *Brevibacterium* sp. R312 and a *Rhodococcus* strain (Mayaux et al., 1990, 1991) was also highlighted. Furthermore, these workers suggested that the amidases of *Brevibacterium* sp. R312 (wide-spectrum), *Ps. aeruginosa*, *Arthrobacter* sp. J1 and *M. methylotrophus* (acetamidase) might constitute members of a related family of amidases (on the basis of biochemical similarities and limited DNA sequence data) that was distinct from the enantiomer-selective amidases mentioned above.

Analysis of the heterologous-expression of the cloned *Brevibacterium* sp. R312 wide-spectrum amidase in *E. coli* showed that it had been successfully expressed to a high level as adjudged from activity measurements from whole cells and SDS-PAGE analysis of whole cell extracts. The latter showed the presence of a protein corresponding to the known amidase MW (45kDa) in addition to the presence of a second protein (38kDa) that was probably a shortened amidase resulting either from proteolytic-cleavage of the native protein or from putative heterogeneity of the initiation site within the amIE sequence.

In view of the observed similarities between the amidases of *Brevibacterium* sp. R312, *Ps. aeruginosa*, *Arthrobacter* sp. J1 and *M. methylotrophus*, as outlined previously (Chapter 3) it was decided to attempt to clone the acetamidase structural gene of the latter organism for comparative studies (including identification of any putative conserved amino acid motifs) with those sequences already published. It was also envisaged that the MW derived from the primary sequence could be cross-referenced with that determined by ESMS, and also to identify possible regulatory amino acid modification sites and potentially useful protease-cleavage sites for isolation of peptide fragments containing the putative acetamidase modifying group (if indeed the latter was present). This Chapter describes the attempted molecular cloning of acetamidase using Southern hybridization and direct selection techniques and attempts to rationalize why all attempts were ultimately unsuccessful. Initial work leading to the isolation of an *E. coli* clone heterologously-expressing formamidase from *M. methylotrophus* is also described.
5.2 Attempted molecular cloning of the acetamidase structural gene (amiE) of *M. methylotrophus*

5.2.1 Utilization of Southern hybridization to identify amiE

Preliminary restriction analysis of *M. methylotrophus* chromosomal DNA was carried out to determine which of a range of commonly-used restriction endonucleases yielded the best chromosomal digest characteristics, viz. a well-separated ladder of fragments, with the majority of fragments falling within a reasonable size range (approximately 1-8 kbp), to facilitate ease of insertion into the proposed cloning vector (pUC19). Chromosomal DNA from *M. methylotrophus* was digested to completion with four arbitrarily-chosen endonucleases (*EcoRI, BamHI, PstI* and *HindIII*) and restricted DNA samples were electrophoresed (not shown). *BamHI* and *PstI* were deemed to yield the best chromosomal digests, taking the above criteria into account, and these digests were used in separate Southern hybridization studies with two different radio-labelled probes.

Initial attempts to identify amiE from *M. methylotrophus* DNA centred on Southern hybridization of restricted chromosomal DNA with a radio-labelled probe (1.5kbp *SmaI* fragment of *Ps. aeruginosa* DNA carrying the complete amiE sequence plus some flanking sequences) that was isolated and purified from a recombinant *X* replacement vector (*XamiS*; see Drew *et al.*, 1980; Clarke *et al.*, 1981) kindly gifted by Prof. W.J. Brammar (University of Leicester). The rationale underpinning this work was to identify a discrete band within digested chromosomal DNA of *M. methylotrophus* which exhibited a strong hybridization signal with a probe that was complementary to at least part of the *M. methylotrophus amiE* sequence. DNA fragments from this band would then be inserted into a plasmid vector and subsequently transformed into *E. coli* (a genotypically ami*"* host). Putatively ami*"* transformants could subsequently be plated on to selective medium and *E. coli* clones heterologously-expressing acetamidase activity isolated.

*E. coli* JM109 was chosen as the host for transformation experiments, and its growth phenotype at 37°C was consequently tested extensively on different types of solid media including M9 mineral salts-amide ± glucose and M9 mineral salts-ammonia plus acetate, to ensure that these media were not toxic to *E. coli*, and to confirm that zero/poor growth was exhibited by host cells on the former types of selective media in order to minimize the background growth of ami*"* transformants. *E. coli* JM109 exhibited no growth on M9 mineral salts-acetamide medium after 48 h incubation at 37°C, and only poor growth on identical medium supplemented with glucose (see Discussion). This latter medium was therefore chosen as the medium on which to select putative recombinant ami*"* transformants.

Hybridization of test and control DNA samples at 37°C (membrane washed sequentially in 3 x and 1 x SSC + 0.1% [w/v] SDS buffer, pH 7.4) with the 1.5kbp probe indicated that a high degree of undesirable non-specific binding occurred with control DNA (*E. coli*) and demonstrated the complete absence of discrete strongly-hybridizing fragments in *PstI*- and *BamHI*-restricted *M. methylotrophus* chromosomal DNA digests (not shown). It was
concluded that the large size of the probe and the presence of amiE flanking sequences contributed significantly to its chance homology with a multitude of different test DNA sequences. Attempts to decrease non-specific binding whilst retaining test DNA signals by empirically altering the hybridization stringency were unsuccessful. Therefore, in view of its relatively non-specific hybridization with *M. methylotrophus* DNA, and the fact that an alternative probe was available (see below) it was decided, after careful consideration, to abandon this work and to proceed with the alternative probe.

The latter was a mixed oligonucleotide DNA probe (17-mer, 48 degeneracies) complementary to the N-terminal amino acid sequence of wild-type acetamidase designed by Silman (1990; Materials & Methods). BamHI- and PstI-restricted *M. methylotrophus* chromosomal digests were electrophoresed together with restricted control samples of DNA, blotted and subjected to Southern hybridization with the new radio-labelled probe. A number of different autoradiographs of the blot were produced after pre-washing of the membrane (to remove unbound probe), and after further washes at variable hybridization stringency.

Initially, washes were undertaken at relatively low hybridization stringency to promote maximal probe-binding to its putative target sequence(s). In subsequent washes the hybridization stringency was increased, to minimize non-specific spurious hybridization between relatively less-complementary probe and target DNA sequences, by sequentially elevating the temperature of washes in increments of 2°C over the range 30-38 °C, whilst maintaining a constant buffer salt concentration. The most informative autoradiograph (Fig. 5.1) produced empirically using this method showed that the probe exhibited very limited (and hence presumably non-specific) hybridization both with restricted *E. coli* and with *X. size-standard DNA*. The hybridization signal was also unexpectedly weak with the DNA fragment containing the complete *Ps. aeruginosa amiE* gene isolated from λami314 (see Drew et al., 1980), the 5' portion of which was expected to show a strong signal with the 17-mer probe as a result of the highly homologous *Ps. aeruginosa* amidase and *M. methylotrophus* acetamidase N-terminal amino acid sequences (Ambler et al., 1987; Brammar et al., 1987; Silman, 1990). However, this weak signal may have resulted from low sample DNA concentration in comparison to the much stronger signal exhibited by the *Ps. aeruginosa* control DNA sample which contained a much greater amount of DNA. This latter sample also showed a more discrete banding pattern which indicated that the probe had hybridized more specifically to certain of the fragments present in this digest.

Hybridization of the probe with *M. methylotrophus* digests yielded results that were both unexpected and difficult to explain, as two discrete bands (rather than the predicted single band) of approximately 3.5-4.0 kbp in the PstI-digested chromosomal sample gave strong signals above background non-specific signals. Since both of these fragments were large enough to carry the whole of the acetamidase structural gene (predicted to be encoded by approximately 1.0kbp of DNA from the monomer MW [38kDa] of the *M. methylotrophus* acetamidase), and its flanking sequences, it was decided to isolate both of these fragments for further study.
Figure 5.1 Southern hybridization of *M. methylotrophus* DNA

Chromosomal DNA was isolated from wild-type *M. methylotrophus* and *Ps. aeruginosa* EF2, and samples were restricted respectively with *Bam*HI and *Pst*I, and *Pst*I only. *E. coli* C600 DNA was similarly restricted with *Pst*I. These digests were co-electrophoresed with undigested *M. methylotrophus* DNA, DNA size-standards (wild-type λ-*Bst*EII restricted genomic DNA) and a DNA fragment carrying the complete *Ps. aeruginosa amiE* gene purified from λami314 after *Sma*I digestion to separate restriction fragments. Samples were blotted to a nylon hybridization transfer membrane and incubated overnight with a radiolabelled 17-mer mixed probe (exhibiting 48 degeneracies and complementarity to the N-terminus of wild-type acetamidase of *M. methylotrophus*) at the minimum calculated hybridization temperature (30°C). The membrane was then washed in pre-warmed buffer (3 x SSC buffer, pH 7.4 + 0.1% [w/v] SDS) at 34°C to remove unbound probe, and then dried and exposed to X-ray film for 48 h at -70°C (Materials & Methods). Track 1) λ-*Bst*EII standards; 2) *Ps. aeruginosa* DNA + *Pst*I; 3) purified *amiE* fragment; 4) *M. methylotrophus* DNA, undigested; 5) *M. methylotrophus* DNA + *Pst*I; 6) *M. methylotrophus* DNA + *Bam*HI; 7) *E. coli* C600 DNA + *Pst*I. The arrows indicate the positions of the two DNA fragments (approximately 3.5-4.0 kbp) which were isolated for further study.
5.2.2 Transformation and attempted direct selection of recombinant clones expressing acetamidase activity

A large-scale PstI *M. methylotrophus* chromosomal DNA digest was subjected to agarose gel electrophoresis, and fragments in the approximate size range 1.9-4.0 kbp were excised from the gel and purified from the agarose gel matrix. It was decided at this stage not to separate the two fragments corresponding to the strongly-hybridizing bands, but rather to ciliate them with plasmid vector in a single ligation reaction and to transform host cells with the complete population of ligation products, with the eventual aim of attempting to isolate these fragments as inserts carried by different phenotypic types of recombinant transformant. Thus, pooled fragments were ligated with PstI-restricted dephosphorylated plasmid pUC19 and the resultant covalently-closed recombinant plasmid vectors were transformed into competent cells of *E. coli* JM109.

A large number of putative transformants were initially plated on to sixty glucose-M9 mineral salts-ammonia agar plates supplemented with amp and IPTG + X-gal to allow visual blue/white selection of non-recombinant versus recombinant colonies. Control cell samples were also plated on similar media and all plates were incubated at 37°C for approximately 24 h. After this time, control cell samples indicated that a high transformation frequency had been achieved and that the ligation and plasmid dephosphorylation steps had been effected efficiently. Each test plate exhibited 20-100 colonies (a very small number of which were blue) and all white recombinant colonies were aseptically picked from each plate and streaked simultaneously on to solid nutrient broth and glucose-M9 mineral salts-acetamide (glucose and acetamide supplied as C- and N-sources respectively) media; both media were supplemented with amp, and the minimal medium also contained IPTG + X-gal. Plates were incubated at 37°C overnight, after which time the nutrient broth plates exhibited good growth; these plates were therefore stored at 4°C to prevent further growth, and to maintain a 'back-up' inoculum of each clone. Conversely, the glucose-M9 mineral salts-acetamide plates showed very little growth, except in a few cases where streaks appeared to be growing quite well. These plates were re-incubated at 37°C for a further 24 h, after which time approximately 20 streaks had grown very well and a further 80 streaks had grown less well (a few streaks exhibited a disconcertingly heterogeneous blue/white phenotype indicating that mutations may have arisen in these cells; see below). Control streaks of *E. coli* JM109 transformed with intact pUC19 (*E. coli* JM109::pUC19) also exhibited very light growth on identical minimal medium, although this appeared to be less than that of the fastest-growing recombinant transformants. It therefore seemed likely that at least some recombinant clones had been isolated that were genotypically amti+ and had acquired the novel ability to utilize acetamide as a nitrogen (and carbon) source. Consequently, it was deemed safe from a probabilistic standpoint that this method would lead to the isolation of an amti+ clone for sequence analysis and it therefore seemed logical to proceed with this work.
5.2.3 Characterization of putative acetamidase-expressing clones

The eighteen fastest growing streaks (clones) on solid glucose-M9 mineral salts-acetamide plates were chosen for further analysis, and their putative amidase activity was tested by sub-culturing them on solid glucose-M9 mineral salts-acetamide (amp) medium and on the same medium deficient in glucose (acetamide supplied as sole C/N source). After incubation at 37°C for 24 h most of the clones showed good growth on the former medium, but none on the latter. After 48 h all clones supplied with glucose as C-source showed good growth in comparison to the control organism (*E. coli* JM109::pUC19), although certain clones exhibited better growth than others; only a single clone showed significant growth after this time on the glucose-deficient plate. Furthermore, the same clone showed enhanced growth after 72 h, but all of the other clones showed variable lesser degrees of growth on this medium. Clones were subsequently designated sequentially A-R; clone A exhibited the fastest growth on solid M9 mineral salts-acetamide ± glucose (amp) medium.

The growth phenotype of clones A-R were also tested in liquid M9 mineral salts-acetamide batch cultures ± glucose supplemented with amp + IPTG. Plasmid samples were prepared from each culture and digested with *PstI* to determine the size of the insert fragment carried by each clone, and an attempt was then made to correlate the observed growth phenotype of each clone with the size of its plasmid insert. A summary of the results (Table 5.1) showed that the clones were genetically heterogeneous with respect to the *M. methylotrophus* insert DNA which they carried, and that on the basis of plasmid insert size, and observed solid and liquid growth phenotypes, could be assigned to six different phenotypic groups.

Consideration of group 1 clones showed that members within this group were genetically homogeneous (all harboured a 3.2kbp insert fragment) and exhibited significant amidase activity. Group 3 clones were also genetically homogeneous (all harboured a 2.4kbp insert), but expressed lower amidase activity and appeared to exhibit a slightly different growth phenotype to group 1 clones as clones D & J occasionally showed heterogeneous blue/white streaks after approximately 48 h growth on solid acetamide-selective medium at 37°C.

Conversely, analysis of group 2 clones showed that although these organisms exhibited light growth on solid glucose-M9 mineral salts-acetamide (amp) medium it was reproducibly demonstrated that no plasmid could be isolated from any of these putative clones and neither did they exhibit growth in liquid culture with acetamide supplied as N-source. It was therefore concluded that these putative ami*+* clones were probably contaminants expressing both amidase and β-lactamase activities (encoded either chromosomally or by a plasmid which could not be isolated by the mini-prep method employed) which allowed growth on acetamide in the presence of ampicillin. This group was therefore discarded.

Clone groups 4 and 5 showed similar light overnight growth on solid glucose-M9 mineral salts-acetamide (amp) medium; however, these organisms curiously exhibited no growth in minimal liquid medium with acetamide supplied as the N-source, even when supplemented with glucose as a C-source and IPTG as a gratuitous inducer of the lac promoter. The insert DNA fragments carried by these clones (3.7 and 4.0kbp respectively)
Table 5.1 Characterization of putative acetamidase-expressing clones

Small (20ml) liquid M9 mineral salts-acetamide batch cultures ± glucose (G) in 50ml flasks were supplemented with amp + IPTG and inoculated with cells taken from streaks (generated from single colonies) of the putative amidase-expressing clones (A-R). Each clone was also sub-cultured on identical solid media (+ X-gal). Solid and liquid media cultures (+G) were incubated overnight, whereas (-G) cultures were incubated for 72 h. All cultures were incubated at 37°C; liquid cultures with rotary shaking. Plasmid samples were isolated by mini-prep from each batch culture and digested with \textit{PstI} to determine the size of the insert harboured by each clone after electrophoresis. Whole cell amidase activities were measured with acetamide as substrate at 37°C (Materials & Methods). Strong growth or high activity (+++); light growth (++); poor growth or low activity (+); no growth, no insert/anomalous insert or no detectable activity (-). Control = \textit{E. coli} JM109::pUC19.

<table>
<thead>
<tr>
<th>Phenotypic group</th>
<th>Growth medium M9-acetamide + G</th>
<th>Insert size (kbp)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solid Liquid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M9-acetamide - G Solid Liquid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. A, B, E, F, I</td>
<td>+++ +++ + +</td>
<td>3.2 ++</td>
<td></td>
</tr>
<tr>
<td>2. C, L, Q</td>
<td>+ + - - -</td>
<td>- -</td>
<td></td>
</tr>
<tr>
<td>3. D, H, J</td>
<td>+++ +++ - -</td>
<td>2.4 +</td>
<td></td>
</tr>
<tr>
<td>4. G, K, O</td>
<td>+ + - - -</td>
<td>3.7 -</td>
<td></td>
</tr>
<tr>
<td>5. M, P</td>
<td>+ + - - -</td>
<td>4.0 -</td>
<td></td>
</tr>
<tr>
<td>6. N, R</td>
<td>+ - - - -</td>
<td>- -</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>+ - - - -</td>
<td>- -</td>
<td></td>
</tr>
</tbody>
</table>
corresponded to the two strongly-hybridizing bands identified by Southern hybridization (see Fig. 5.1).

The group 6 clones exhibited poor growth on solid glucose-M9 mineral salts-acetamide (amp) medium (equivalent to that of the control) and plasmids isolated from these organisms showed a curious banding pattern after PstI-restriction and electrophoresis. Fragments of equivalent size to pUC19 (2.7kbp) were conspicuously absent, but two bands of approximate size 1.9kbp and 1.3kbp were apparent, which indicated that either these organisms were contaminants (*ami*+, *amp*+) that happenstantially harboured a plasmid with two PstI restriction sites, or that they were genuine *E. coli* transformants that carried recombinant plasmids that had undergone mutational deletion events. The occurrence of the latter must be deemed improbable as *E. coli* JM109 is genotypically *recA1*, but the former possibility is also statistically unlikely; therefore, the origin of these organisms remains obscure, and they were not subjected to further investigation.

In summary, at this stage of the work the analysis and interpretation of results was complicated. Two different phenotypic groups of organisms (1 & 3) exhibited similar putative *ami*+ phenotypes, but contained inserts of different sizes (3.2 cf. 2.4kbp) after plasmid restriction with PstI, neither of which corresponded to either of the strongly hybridizing bands identified by Southern hybridization, at least one of which was predicted to encode the *amiE* sequence, and therefore correspondingly give rise to any acetamidase activity subsequently identified in transformants. These clones also exhibited strong growth on solid and liquid media in which acetamide was supplied as the sole N-source. Conversely, organisms from the remaining two phenotypic groups (4 & 5) only exhibited light growth on solid medium containing acetamide as the sole N-source, but would not grow in identical liquid medium. These clones therefore exhibited a more doubtful *ami*+ phenotype, as adjudged from growth studies, but respectively harboured insert fragments (3.7 & 4.0kbp) that corresponded with the strongly-hybridizing fragments identified earlier. In view of the uniform phenotypes and genotypes of members within each of these four groups of clones it was decided to subject a single candidate clone from each group (I, D, G and M respectively) to more detailed investigation.

### 5.2.4 Further characterization of putative acetamidase-expressing clones

To ensure that the putative *ami*+ clones (I, D, G and M) were genuinely recombinant *E. coli* strains, and not *ami*+ contaminants, a representative clone (I) and a control organism (*E. coli* JM109::pUC19) were subjected to biochemical testing, using the API 20 E kit (identification system for enterobacteriaceae and other Gram-negative rods). Results (not shown) from both samples most-closely matched those associated with *E. coli*, and it was concluded that clone I, and therefore probably also clones D, G and M were genuine recombinant *E. coli* transformants.
The plasmids harboured by each of the clones were designated pNW1-4, in order of decreasing insert size (Fig. 5.2), and the clones were correspondingly re-designated E. coli JM109::pNW1-4. Preliminary restriction maps of the plasmid inserts from pNW1-4 were also generated to ensure that the original PstI chromosomal digest from which inserts were isolated had gone to completion, i.e. to eliminate the possibility that the inserts resulted from partial-digestion of chromosomal DNA and might therefore possess common sequences. Plasmids pNW1-4 were each digested with the majority of the restriction enzymes able to cleave the M13mp19 polylinker multiple cloning site and the digest patterns were characterized by agarose gel electrophoresis. Due to the constraints of time, no attempt was made to order all of the restriction sites unequivocally for each insert, as it could be seen from the data (not shown) that the positions of certain unique restriction sites (in relation to other sites) precluded the possibility that inserts contained overlapping sequences (an accurate restriction map derived for pNW2 is shown below; see Fig. 5.4).

The amidase activities of E. coli JM109::pNW1-4 were initially tested with acetamide as substrate using washed cells harvested from nutrient broth batch cultures supplemented with acetamide and amp due to the inability of E. coli JM109::pNW1 and ::pNW2 (clones M & G respectively) to grow in glucose-M9 mineral salts-acetamide (amp) liquid medium. The results showed that the highest amidase activity was associated with E. coli JM109::pNW3 (clone I; specific activity approximately 0.04µmol min⁻¹ [mg dry wt]⁻¹), and that E. coli JM109::pNW4 (Clone D) also exhibited very low-activity with this substrate. The other two clones and E. coli JM109::pUC19 showed no activity with acetamide even after prolonged exposure to the substrate at 37°C. Unfortunately, these results did not greatly clarify the observed clone phenotypes, so the experiment was repeated using cells grown in glucose-M9 mineral salts-ammonia + acetamide (amp) liquid medium ± IPTG, except that ammonia was omitted from the E. coli JM109::pNW3 and ::pNW4 cultures. The results confirmed those from the initial work as E. coli JM109::pNW1 and ::pNW2 exhibited no amidase activity, even after growth in the presence of acetamide and IPTG, and E. coli JM109::pNW3 activity was significantly higher than that of E. coli JM109::pNW4 (additionally, activity measurements for E. coli JM109::pNW3 suggested that growth of this clone in the presence of IPTG diminished its amidase activity; see Chapter 6).

It was decided to characterize the amidase activities of E. coli JM109::pNW3 and ::pNW4 by profiling their relative amidohydrolase substrate specificities after growth in liquid glucose-M9 mineral salts-acetamide (amp) ± IPTG medium. Activity profiles were compiled by incubating washed whole cells with propionamide, acrylamide, acetamide, formamide, butyramide and urea, which showed unexpectedly that neither clone exhibited the substrate specificity expected from amidase (see Chapter 3). E. coli JM109::pNW3 hydrolysed the substrates in the order formamide >> propionamide/acetamide > butyramide/acrylamide (no activity with urea), and in view of the close correlation of this profile with that of the M. methylotrophus formamidase (which had been previously purified and partially-characterized; D.J. Scherr, N.J. Silman & C.W. Jones unpublished) these data suggested that this recombinant strain was probably expressing M. methylotrophus formamidase. More
Figure 5.2 Characterization of plasmid inserts harboured by pNW1-4

Plasmid samples were isolated from nutrient broth batch cultures (20ml) supplemented with amp and inoculated with single colonies of *E. coli* JM109::pNW1-4. All cultures were incubated overnight at 37°C with rotary shaking, and purified plasmids were digested with *PstI* and electrophoresed (Materials & Methods). Clone M = pNW1 (4.0kbp insert); clone G = pNW2 (3.7kbp insert); clone I = pNW3 (3.2kbp insert); clone D = pNW4 (2.4kbp insert). Track 1) pNW1 + *PstI*; 2) pNW2 + *PstI*; 3) pNW3 + *PstI*; 4) pNW4 + *PstI*. Relative sample acetamidase activities are indicated below each track.
convincing evidence that this clone heterologously-expressed formamidase was obtained from SDS-PAGE which showed that *E. coli* JM109::pNW3 expressed a protein that co-migrated with pure formamidase and was present at twice the intensity of a similar protein found in control untransformed host cells. It was therefore concluded that the *M. methylotrophus* formamidase had been cloned and expressed in *E. coli* JM109::pNW3 strain and this was later confirmed (see Chapters 6 & 7; Wyborn et al., 1994).

An analogous substrate profile for *E. coli* JM109::pNW4 showed that this strain exhibited generally lower activities and hydrolyzed amides in the order propionamide/acrylamide > formamide/acetamide > butyramide/urea. This profile also strongly suggested that *E. coli* JM109::pNW4 did not express acetamidase, although it was possible that the observed profile was spuriously-modified due to inauthentic folding of the heterologously-expressed protein in *E. coli* host cells. Alternatively, it was postulated that *E. coli* JM109::pNW4 expressed another recombinant protein with amidase activity, for which potential candidate enzymes included urease, which was known to be expressed by *M. methylotrophus* (M.A. Carver, personal communication), and a hypothetical hitherto uncharacterized amidase. However, it was concluded that expression of urease was unlikely, as these enzymes are generally high-MW proteins (of approximately several hundred kDa), and it was known from the small size (2.4kbp), and hence limited coding-potential of the pNW4 insert, that such a protein could not have been encoded by pNW4, unless the observed activity resulted from a novel low-MW enzyme. It was also possible that the amidase activity observed previously in *E. coli* JM109::pNW4 cultures may have resulted from contamination, i.e. from a mixed culture of *E. coli* JM109::pNW4 and a contaminating genotypically amí†/amp− organism, which was either co-isolated from the original selective medium, or which contaminated homogeneous cultures of *E. coli* JM109::pNW4 growing on solid media at a later time. If this was indeed the case then liquid cultures of *E. coli* JM109::pNW4 would have been inoculated with streaks from contaminated sub-cultures of *E. coli* JM109::pNW4 leading to the possibility that cultures exhibiting both types of organism would have been generated, and the spurious correlation of amidase activity with plasmid pNW4. However, this must be deemed unlikely, as aseptic techniques were strictly observed at all times, and because plasmid pNW4 was isolated from *E. coli* JM109::pNW4 cultures expressing amidase activity. If pNW4 did not encode amidase activity then presumably the putative contaminating organism would have outgrown the *E. coli* (ami†) inoculum in liquid batch culture, and therefore no pNW4 would have been isolated from the culture.

These anomalous results were investigated further by re-transforming cells of *E. coli* JM109 (containing no plasmid) with plasmid pNW4 and selecting putative transformants on solid glucose-M9 mineral salts-acetamide medium supplemented with amp and IPTG. Growth of transformants again suggested that cells harbouring pNW4 expressed amidase activity as the observed growth of pNW4-transformants appeared to be more rapid than control cells transformed with intact pUC19 (containing no insert DNA) in the same experiment. A single pNW4-transformant colony was re-inoculated on to identical fresh medium and incubated at 37°C and light growth was exhibited by the sub-culture after 48 h.
A heavy inoculum of cells was subsequently taken from this plate and used to inoculate a batch culture containing identical liquid medium, but this culture exhibited no growth after 72 h at 37°C with rotary shaking. It was therefore concluded that despite the correlation of amidase activity with *E. coli JM109::pNW4*, and the apparent ability to isolate plasmid pNW4 from cultures expressing amidase activity etc., the simplest explanation for the origin of the phenotype of this organism was either (i) an *ami^+*/*amp^+* contaminating organism, or (ii) a mutational event occurring after the original growth trials which putatively regenerated the intracistronic β-galactosidase α-complementation (which caused the streaks to turn blue on solid media) and which concurrently either significantly diminished, or abolished the amidase activity observed after re-transformation. Observations from restriction analysis of clone group 6 (section 5.2.3) in which putative deletion events occurred lends credence to the latter hypothesis.

Further investigation of *E. coli JM109::pNW1* and ::pNW2 using washed cells harvested either from solid (glucose-M9 mineral salts-acetamide [amp] ± IPTG) or liquid (glucose-M9 mineral salts-ammonia + acetamide [amp] ± IPTG) media showed that sonicated cell-extracts exhibited no amidase activity, indicating that the absence of activity in these clones did not result from poor induction of amidase, or impermeability of the host cell wall to acetamide. Additionally, attempted heat-reactivation of sonicated *E. coli JM109::pNW1* and ::pNW2 cell-extracts in the presence of activator fraction partially-purified from *M. methylotrophus* (Materials & Methods; see also Chapter 4) demonstrated that the absence of amidase activity was not due to the expression of a highly switched-off amidase. Furthermore, *E. coli JM109::pNW1* and ::pNW2 whole cells and cell-extracts were tested for acetyl transferase activity, with acetamide as substrate, as it had been previously demonstrated (Silman, 1990) that the acyl transferase activity of the *M. methylotrophus* amidase was much greater than its hydrolase activity under standardized conditions. This was deemed to constitute a more sensitive assay method than the hydrolase assay, and it was hoped that it might detect low-level amidase activity; however, neither clone exhibited detectable acetyl transferase activity.

In conjunction with the activity profiling described above, the possibility that *E. coli JM109::pNW1*, ::pNW2 and ::pNW4 strains expressed heterologous proteins that were insoluble within the cell was investigated by SDS-PAGE (not shown) to eliminate the possibility that putative genotypically *ami^+* clones were phenotypically *ami^-* due to inauthentic protein-processing by host cells (possibly resulting from incorrect folding of the protein as was postulated to explain the formation of insoluble aggregates of heterologously-expressed *Rhodococcus* sp. amidase in *E. coli* [Mayaux *et al.*, 1991]). However, analysis of the gels showed no significant differences in banding pattern between host cells transformed with pNW1, pNW2 or pNW4 and those transformed with intact pUC19 only indicating that inactive amidase(s) were not present in *E. coli*.

In summary, preliminary mapping data showed that each of the inserts harbouring by plasmids pNW1-4 was unique and exhibited no overlapping sequence with the other inserts. *E. coli JM109::pNW3* and ::pNW4 exhibited detectable amidase activity, but their plasmid
inserts showed essentially no homology with a probe complementary to the acetamidase N-terminus; conversely, *E. coli* JM109::pNW1 and ::pNW2 showed no detectable amidase activity even after growth in the presence of IPTG (a gratuitous inducer of the *lac* promoter) and acetamide (the physiological inducer of acetamidase), yet harboured plasmid inserts that exhibited strong homology with the probe.

*E. coli* JM109::pNW3 was shown to express formamidase, but the unexpected substrate profile and loss of activity from *E. coli* JM109::pNW4 precluded further study of this clone. However, in view of the results from Southern hybridization it could not yet be discounted that either pNW1 or pNW2 encoded the *M. methylotrophus* acetamidase. From the observed phenotypes of these clones it was concluded that (i) one of the inserts carried by pNW1 or pNW2 encoded acetamidase, or at least the N-terminal portion of it, which was expressed in an inactive form (or only at a very low-level sufficient to promote growth on solid media containing acetamide as the N-source) which possibly resulted from C-terminal truncation of the protein, or to the lack of a molecule essential for its activity, *i.e.* the absence of a putative activator molecule, or (ii) inserts were not transcribed/translated at all, or (iii) pNW1 and/or pNW2 did not encode acetamidase, but rather encoded proteins exhibiting significant N-terminal homology to acetamidase and which exhibited growth on selective media by scavenging nitrogen from sources accessory to acetamide. Therefore, the growth of recombinant clones that exhibited no detectable amidase activity on solid glucose-M9 mineral-salts acetamide medium supplemented with amp was disturbing, even if this growth did appear to be genuinely better than that exhibited by control cells (*E. coli* JM109::pUC19).

However, despite the concerns over putative growth anomalies, it was decided to proceed with this work based on the supposition that (i) either pNW1 or pNW2 encoded at least the S'-end of *amiE*, and that there was either a leaky transcriptional or translational block to amidase activity which allowed the synthesis of sufficient amidase to allow growth on acetamide as N-source, and (ii) in view of the fact that the probe was designed to be complementary to the acetamidase N-terminus, and Southern hybridization only highlighted two strong signals, it was illogical to postulate that the probe had not hybridized with the true *amiE* S'-sequence, and consequently there was no good reason to suggest that these signals were spurious. It was therefore deemed likely that the acetamidase N-terminal-encoding sequence would be isolated from one of these clones and that DNA sequencing of the plasmid insert would allow further DNA probes to be designed to it, which could be utilized to isolate the remaining C-terminal portion of the gene (if indeed the *amiE* gene was truncated). Since it was known from Southern hybridization data that the insert of pNW2 hybridized slightly more strongly with the probe than did the pNW1 insert it was decided that pNW2 was the best candidate clone for detailed investigation with a view to its eventual sub-cloning and sequencing.
5.2.5 Sub-cloning and partial DNA sequencing of the insert of plasmid pNW2

Plasmid samples (pNW1-4) were restricted with PstI, blotted to a nylon hybridization transfer membrane and subjected to Southern hybridization to confirm that the insert carried by pNW2 exhibited the strongest hybridization signal with the probe, i.e. carried at least the 5'-end of amiE. The most informative autoradiograph (Fig. 5.3) showed that except for pNW2 the probe exhibited a similar affinity for insert and parent vector (pUC19) DNA; indeed, a more intense signal was shown by vector, rather than insert DNA from pNW4. Control DNA samples exhibited signals generally as expected; the \( \lambda \)ami314 Smal digest showed a strong signal (in contrast to that shown in Fig. 5.1) which was identified as the amiE-containing fragment (at the bottom of track 5); the \( M. \) methylotrophus digest showed two faint discrete bands at the same positions as pNW1 and pNW2 inserts (thereby confirming that the inserts of the latter corresponded to the two strong hybridization signals shown in Fig. 5.1), and \( E. \) coli control DNA showed no discrete banding pattern. It was disconcerting that pUC19 appeared to hybridize as strongly with the probe as the majority of insert fragments, but taking this into account, and the fact that observed signal intensities were probably skewed due to a copy number effect, it was estimated that the insert of pNW2 hybridized approximately twice as strongly with the probe than either pUC19 or the pNW1 insert. It was therefore concluded that pNW2 was a better candidate for initial sequencing studies than pNW1.

In order to identify pNW2 insert fragments exhibiting high homology with the probe, and which were small enough to be sequenced rapidly, insert DNA was subjected to detailed restriction analysis. Restriction mapping data generated from digestion of pNW2 with endonucleases able to cleave the M13mp19 polylinker multiple cloning site of pUC19 showed that only five of these restriction enzymes were able to cleave plasmid DNA at sites within the insert (Table 5.2). Unfortunately, the fragments generated after digestion by the majority of these enzymes (AccI, BamHI & Smal) were generally relatively large, which indicated that if each restriction enzyme site of the M13mp19 sequence was considered to be fixed then the second restriction site, in relation to the first, must have been located quite close to one end of the insert DNA in each case. Thus, these enzymes did not significantly fragment the pNW2 insert and were therefore deemed unsuitable for use in sub-cloning experiments. Conversely, SphI-digestion of pNW2 generated insert restriction fragments of a favourable size for rapid sequencing (approximately 1.3kbp & 1.5kbp, and also a 3.4kbp fragment which presumably contained pUC19 DNA contiguous with the remaining 0.7kbp of insert DNA) indicating that the insert DNA contained two potentially-useful internal SphI-restriction sites, although it was not known at this stage which of the three fragments putatively contained the amiE 5'-sequence.

Similarly, digestion of pNW2 with SacI showed that a cleavage site for this enzyme might be present at a position 0.8-1.1 kbp from one end of the insert, but the exact position of this site could not be unequivocally assigned from these data alone (the sizes of restriction
Figure 5.3 Southern hybridization of plasmids pNW1-4

Plasmid samples (pNW1-4) were restricted with PstI and co-electrophoresed with samples of control DNA. DNA fragments were blotted and hybridized essentially as described above (see Legend to Fig. 5.1), except that the membrane was sequentially washed in pre-warmed (30°C) buffers with different salt concentrations (3 x & 1 x SSC buffer, pH 7.4 + 0.1% [w/v] SDS), prior to exposure to X-ray film (-70°C) for 23 h. Track 1) E. coli C600 chromosomal DNA + PstI; 2) pUC19 + PstI; 3) M. methylotrophus chromosomal DNA + PstI; 4) λami314 genomic DNA + SmaI*; 5) pNW4 + PstI; 6) pNW3 + PstI; 7) pNW2 + PstI; 8) pNW1 + PstI. (* see Drew et al., 1980).
Plasmid pNW2 was digested with each of the restriction enzymes indicated and subjected to agarose gel electrophoresis to separate DNA fragments. The sizes of fragments were determined automatically by extrapolating from a standard curve generated from BstEII-digested λ genomic DNA after image analysis of the gel (Materials & Methods). The sizes of restriction fragments are normalized to values at which linearized pUC19 = 2.7kbp. +/- indicates presence/absence of a restriction site respectively. The observed heterogeneity in the summed sizes of restriction fragments was either due to a slight over- or under-estimation of fragment sizes, or to the generation of very small restriction fragments (≤ 200bp) which went unnoticed during image analysis.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Presence of target site within insert DNA</th>
<th>Restriction fragment size(s) (kbp)</th>
<th>Sum of fragment sizes (kbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accl</td>
<td>+</td>
<td>3.10, 2.90, 0.49</td>
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</tr>
<tr>
<td>BamHI</td>
<td>+</td>
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<td>PstI</td>
<td>-</td>
<td>2.70, 3.68</td>
<td>6.38</td>
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<tr>
<td>SacI</td>
<td>+</td>
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<td>-</td>
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<td>SphiI</td>
<td>+</td>
<td>3.41, 1.53, 1.32</td>
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<tr>
<td>XbaI</td>
<td>-</td>
<td>6.12</td>
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fragments from this digest were over-estimated, which probably resulted from anomalous sample migration during agarose gel electrophoresis). However, in conjunction with PstI/SacI double-digestion of pNW2 (data not shown) the SacI site was mapped to a position approximately 0.8kbp from one end of the insert (Fig. 5.4). This suggested that SacI might be useful for sub-cloning work; furthermore the asymmetrical position of this site within the insert relative to those of the internal Spel sites allowed it to be used in a diagnostic sense to identify unequivocally the position of the second insert Spel site. Plasmid pNW2 was subjected to Spel-digestion and also SacI/Spel double-digestion. After electrophoresis of the restriction fragments and image analysis it could be seen (Fig. 5.5.a) that the approximately 1.5kbp Spel fragment had disappeared in the SacI/Spel double-digest sample, i.e. had been further digested to two smaller fragments, which indicated that since the SacI site was close to one end of the insert then the 1.5kbp fragment must also have been located terminally. Thus, by a process of elimination the approximately 1.3kbp Spel restriction fragment was identified as the central fragment of the pNW2 insert.

To determine which of the Spel-restriction fragments was the best candidate for DNA sequencing the gel shown in Fig 5.5.a was blotted and subjected to Southern hybridization (as above). The most informative autoradiograph produced from this blot (Fig. 5.5.b) clearly showed that the smallest Spel-pNW2 fragment (approximately 1.3kbp) exhibited the strongest hybridization signal. Consequently, it was decided that this band would be sub-cloned.

A sample of pNW2 DNA was digested with Spel and electrophoresed to separate the restriction fragments. The resultant three fragments were excised separately from the gel and the DNA was purified from the gel matrix. The largest fragment (approximately 3.4kbp), exhibiting cohesive ends was self-ligated, and the two smaller fragments were ligated (in separate reaction mixtures) with the Spel site of pUC19. Ligation mixes were added to competent cells of E. coli JM109 (containing no plasmid) and the putative transformants were plated on to solid glucose-M9 mineral salts-ammonia plates supplemented with amp and IPTG + X-gal. Plates were incubated at 37°C for 36 h before approximately 45 white (recombinant) colonies from each of the three groups of transformants were streaked simultaneously on to solid nutrient broth, and glucose-M9 mineral salts-acetamide plates supplemented with amp. After 48 h incubation at 37°C each of the three different groups of transformants exhibited only light growth on the latter solid medium, as had been observed for pNW2, indicating that the acetamidase activity of the putative Spel-derived sub-clones was at best very low (sub-clones also exhibited no detectable acetyl transferase activity, and showed no amidohydrolase activity after heat-reactivation at 60°C with partially-pure activator fraction). Subsequent mini-prep and Spel restriction analysis of plasmid samples (designated pNW213, pNW215 & pNW208) containing sub-cloned pNW2 insert fragments isolated from each of the three putative sub-clones (designated E. coli JM109::pNW213, ::pNW215 & ::pNW208) confirmed that the 1.3kbp and 1.5kbp Spel fragments, and the self-ligated 3.4kbp fragment isolated from pNW2 had been recovered. This was additionally confirmed by subjecting Spel digests of pNW213, pNW215 and pNW208 to Southern hybridization.
Plasmid DNA was isolated from a nutrient broth batch culture supplemented with amp and inoculated with a single colony of *E. coli* JM109::pNW2. The culture was grown overnight with rotary shaking at 37°C. Plasmid DNA was digested with various restriction enzymes (Table 5.2) and subjected to electrophoresis to separate DNA fragments. Image analysis of gels (Materials & Methods) allowed automatic-sizing of DNA fragments by comparison with a standard curve generated from *Bst*EII-digested λ genomic DNA. Only those restriction sites whose positions were unequivocally assigned are included; other restriction sites are omitted for the sake of clarity. Sub-cloned pNW2 insert DNA fragments and the pNW213 sequencing strategy are as indicated.
Plasmid pNW2 was digested with restriction enzymes singly and in pairs, as indicated below. Restriction fragments were subjected to agarose gel electrophoresis with DNA size-markers and control samples of DNA (Fig. 5.5.a) (Materials & Methods). DNA samples were then blotted from the gel and Southern hybridized essentially as described above (see Legend to Fig. 5.1), except that the membrane was sequentially washed in pre-warmed (30°C) buffer (3 x & 1 x SSC buffer, pH 7.4 + 0.1% [w/v] SDS), dried and then exposed (-70°C) to X-ray film for 16 h (Fig. 5.5.b). Track 1) λ-BstEII standards; 2) pNW2 + Sacl/SphI; 3) pNW2 + PstI/SphI; 4) pNW2 + SphI; 5) pUC19 + PstI; 6) M. methylotrophus chromosomal DNA + PstI; 7) E. coli C600 chromosomal DNA + PstI; 8) λ-BstEII standards.
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Figure 5.5.b

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which showed an identical result to that of Fig. 5.5.b, i.e. the insert of plasmid pNW213 clearly exhibited stronger hybridization with the probe than either pNW215 or pNW208. It was therefore concluded that the 1.3kbp pNW2-SphI fragment had been successfully sub-cloned from pNW2 and it was decided to sequence the insert DNA of this construct.

5.2.6 DNA sequence analysis of the insert of plasmid pNW213

The insert DNA of pNW213 (approximately 1.3kbp) was sequenced manually from denatured plasmid DNA (Materials & Methods), using the sequencing strategy shown (Fig. 5.4). Initial sequence data was produced using forward and reverse Universal primers homologous with M13mp19 sequences located just peripheral to the multiple cloning site, and then with custom-designed oligonucleotide primers complementary to internal sequences (sequence data was also produced from Universal primers using an automated DNA sequencer by Dr. K. Lilley, University of Leicester; this sequence was cross-referenced with that obtained from manual sequencing and the two sequences were found to be essentially identical). However, the sequence data indicated that the 5'-end of amilE was not present in the insert DNA of pNW213, i.e. no sequence was evident that exhibited strict identity with any of the possible versions of the mixed probe (even after taking into account the possible alterations to the sequence arising from the unequivocally assigned nucleotides), so full sequencing of both strands was not completed. Since the results from database homology searches with DNA and derived amino acid sequences (see below) indicated that the most significant sequence similarities occurred on the insert coding strand (as designated in relation to the direction of transcription from the lacZ promoter), contrary to convention, the presented DNA sequence (Fig. 5.6) is a coding strand composite derived from the fusion of the anti-parallel version of sequence data generated with reverse primers with that generated from the forward primers (N.B. the insert coding strand does not necessarily constitute the same in the M. methylotrophus genome). It was concluded that despite successful restriction mapping and sub-cloning to produce a construct that could be rapidly sequenced, the pNW213 insert fragment had been obviously mis-identified as an amilE-containing fragment.

Analysis of the DNA sequence in all reading frames initially indicated that a number of putative open reading frames (ORFs) existed on both the non-coding and coding strands (not shown), which were terminated at two or three different putative stop codons (either TGA or TAA), and which varied in length depending on the arbitrary upstream position of various putative start codons (ATG). Investigation of the homology of the complete insert DNA sequence (non-coding & coding strands), and that of the derived amino acid sequences of all putative ORFs was undertaken by searching relevant databases (Materials & Methods). DNA sequence database searching identified a multitude of apparently unrelated sequences exhibiting some similarities (presumably chance) with the query sequence under relatively low-stringency search conditions. The greatest sequence homology identified between query and database sequences involved the *Klebsiella aerogenes ppk* (polyphosphate kinase) gene.
Figure 5.6 DNA sequence of the pNW213 insert

The nucleotide sequence of plasmid pNW213 insert DNA (1297 nt) is shown on the recombinant plasmid coding strand in the 5' to 3' direction. The SphI restriction sites are singly-underlined; nucleotides shown in lower case type represent possible erroneous insertions; the order of nucleotides shown in bold type has not been unequivocally determined; the doubly-underlined nucleotide sequence represents an example of a putative hybridization probe binding site; -/I indicate non-identity/identity respectively; and putative termination codons are overlined with asterisks (putative translation initiation codons have been omitted for the sake of clarity).

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GCATGGTAACACCATATTGATGGGAGGCGATGCCGCGAGCGGTGAGACTGGA
ACTACGATCAGTAAAGCGCAATCTGTTGGGCAACAGGACCACAGGATTTTGTGCGGC
CGCTGTTCTTTAGCCCGGATGCAGTGACACAAGAAGTGATGGCATTGGTGCAGGAGCGGT
TTGCACAGCATCCCGCGCGCTGGAATMTGGTGCTCCCATATCAAGACG
-ACT-GT-T-A-T-GTA
CCATGTGGCAAGATCAGCCATATTTATGGCATTCATTGCTGGCCTCTGCCATGAACCTCA
AATTGCTTAACCAAGGAGATGTTAGCAGGCGAAGAGGTTATCTTGGACGGCAGACAGG
TGCCGCTGGGGTGTCAGGAAATGGTTATCCGCCAGATATTGGGGTGCGAATTGGTGCGC
GCCGCGTGATATTGGCTGGGAATGCCAGCACTTAAGTCGGCAAATTATTTCCAGCATCAGC
AGGATTTGCTGGCTGGTTCTGGACGGGAGACACGCATATGCAGTGTTTGTCGCAGTGTA
TCGCCAGACCTTGCGTACCGGCTATGCCCATCATATCCAGCGCCTGATGGTCATCGGCC
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TGTTCTGCACCTGCGCAGCCGCTACCCGCGCAACTTTCTGACTTTGGCTCTGGCCTACCTGGGATTTTTGCTGAC
690

ATGTCGATGCcGTAGAGTGGGTGGAACTGCCAAATGTCATGGCATGGCCTTGTATGCCAACTCA
750

CGGTGGCCGCTTTACCACGAACACCTTACGTGGCAAGCGGGGCTTACATTCAGCGCATGAGGGG
810

CAATTATTGCAAAAGCTGCCGTTATGACCCCAAGCAAAAGACAGGTGAGAATGCGTGCCC
870

ATTTACCACGCTCTATTGGGCATTCCTCATCAAGCACAGCCAGTCTTGGGGAAGTACCC
930

GCCATGGGCTCATGGTCAAGCATGTGGCTAATCTTTCAGTAGCCGAGCGTTCTGCCAT
990

CAATGACATGGCCACGCTAAGATTGCAGCACCTTGATCAATTGTAATGTGCAATCTGCTA
1050

AATTCACAAAACGTGACATAGTCCTGCACAGCCGATACAAATACGCCATGAAAAAT
1110

AATACGCGCTTCGTCATAGACAGACTACCTCAGTCTCAGAGTTAATGCTGACTGGG
1170

TCCCTGGGCGAAGCTGAAAGACTATCCGCTACTGGAACGCCTGCGTTTTTTATGTATTG
1230

TCGAAATAATCTGGAAGTTCTTTGGAGATGCGCCATGC
1290
sequence (nt 1163-, Fig. 5.6), after which no further comparisons could be made as the pNW213 insert sequence terminated at this point. In addition, protein database searches (BLAST & FASTA) also identified the corresponding amino acid sequences, i.e. polyphosphate kinase etc., as constituting part of one of the putative ORFs identified on the coding strand. These amino acid sequences each exhibited 58% strict identity (rising to 78% similarity if the single nucleotide differences between other codons which gave rise to amino acid differences were taken into account) over a 44 residue sequence, which again was terminated by truncation of the pNW213 insert sequence. Furthermore, the database polyphosphate kinase sequences were N-terminal sequences which aligned well with a putative *M. methylo trophus* start codon, indicating that the N-terminus of an *M. methylo trophus* protein exhibiting significant homology with *K. aerogenes* and *E. coli* ppk gene products was potentially encoded by the DNA sequence nt 1132-. This suggested that the 3'-end of the insert coding strand putatively encoded the 5'-region of an *M. methylo trophus* ppk gene, although no *E. coli* consensus promoter sequence could be found upstream from the putative start codon. All other searches (DNA & protein) yielded no significant sequence matches, which indicated that either the putative ORFs were genuinely unique gene sequences or were artefactual. Thus, database search results showed that the pNW213 insert did not encode a gene product that could have promoted growth of *E. coli* JM109::pNW2 on acetamide as N-source.

Further analysis of the pNW213 insert sequence also confirmed the part of the restriction map corresponding to pNW213 (Fig. 5.4) and showed that multiple putative probe binding sites, exhibiting ≥ 40% identity with the mixed 17-mer probe were apparent within the pNW213 insert sequence, and presumably the increased hybridization signal exhibited by this sequence, in comparison to that of pUC19, resulted from an approximately doubled concentration of these sequences within the pNW213 insert DNA sequence.

### 5.2.7 Further attempted direct selection of acetamidase-expressing clones

In view of the fact that the *M. methylo trophus* formamidase (*fmd*) gene had been serendipitously cloned and expressed in *E. coli* (and for which there was evidence to suggest that formamidase activity was at least partially constitutive, suggesting that *M. methylo trophus* promoter sequences were probably functional in *E. coli*; see Chapter 6), it was decided to attempt the same for acetamidase by generating 'shot-gun' libraries of *M. methylo trophus* chromosomal DNA in *E. coli* with a number of different restriction enzymes (EcoRI, *Kpn*I, *Hind*III), and a partial-digest library using *Sau*3A1. Restriction digests were ligated with pUC19 and transformed into competent cells of *E. coli* JM109 and putative transformants were plated on various types of solid media of different nutrient constitution (see Discussion) in an attempt to define empirically the conditions of stringency under which ami+ colonies could be rapidly and unambiguously discriminated from background ami- recombinants. This work was very labour-intensive as all putative ami+ colonies had to be screened individually,
rather than being screened *en masse* by colony hybridization in the absence of a reliable probe. A number of false-positive colonies were briefly investigated and identified after plasmid restriction mapping and liquid batch culture growth trials, but this work was finally abandoned due to the constraints of time.

5.3 Discussion

All attempts to clone *amiE* were eventually unsuccessful, and in one such case this led to the mis-identification of a putative *amiE*-containing DNA fragment which, despite being successfully manipulated (by detailed restriction analysis and construction of sub-clones) for DNA sequencing, was eventually shown to be a false-positive. The mis-identification of the insert fragment of pNW213 as the *amiE*-containing fragment resulted from spurious hybridization results coupled to the mis-interpretation of the observed growth phenotype of *E. coli* JM109::pNW2 (which may have additionally applied to that of other clones expressing no detectable amidase activity), although the underlying reasoning for the original interpretation remains valid. Analysis of results from pre-DNA sequencing work was deliberately interpreted cautiously in view of the anomalies concerning the growth of clones which exhibited no detectable amidase activity on solid selective media, and concerning hybridization results which showed two bands (rather than the expected one band) that hybridized strongly with the probe, in addition to the unexpected spurious hybridization signal exhibited by pUC19 vector DNA. At each stage of this work, therefore, the validity of results and conclusions were considered with respect to those from control samples, and in each case it was decided that there was a valid reason to proceed with this work. Thus, false-positive growth phenotypes and spurious hybridization results contributed conjointly to the mis-identification of *E. coli* JM109::pNW2 (::pNW213) as a genotypically *ami*+ strain. The possible reasons for false-positive results are considered further below.

Every attempt was made to minimize spurious hybridization signals by empirically altering the stringency of membrane-washes to remove as much background non-specific hybridization as possible, whilst retaining the signal exhibited by the putative *amiE*-containing band(s). In each case it was found that alteration of temperature or buffer salt concentration independently of the other parameter (which was held constant), generally improved the clarity of autoradiographs and this was deemed sufficient to give an accurate depiction of probe and target sequence homology. Due to the constraints of time it was not attempted to coincidentally alter both of the parameters as results appeared to be adequate from washes in which either single parameter was altered.

Control samples of DNA were carefully chosen for hybridization studies. *Ps. aeruginosa* EF2 chromosomal DNA (positive control) was isolated from a culture showing amidase activity which confirmed that this organism expressed *amiE* (the 1.5kbp *SmaI*-λami314 fragment that was originally isolated for use as a probe against acetamidase, in addition to the complete *SmaI*-λami314 digest, were also used as positive control samples). *E. coli* C600 DNA (negative control) was used as this organism does not express an acylamide
amidohydrolase, and linearized pUC19 vector DNA was also used. In general it was observed that E. coli C600 DNA control samples exhibited unexpectedly strong, although non-localized hybridization signals; similarly, pUC19 DNA showed a relatively high affinity for the probe which was rationalized by analysis of its DNA sequence which showed at least one sequence, 17 nucleotides in length, of which 12 were identical (spaced into a block of 7 consecutive nucleotides separated from a block of 5 consecutive nucleotides by a further 3 nucleotides) to one particular version of the degenerate 17-mer probe. This sequence, and others identified within the pUC19 sequence that exhibited a lower degree of homology with different versions of the probe, presumably constituted reasonable binding-sites for the probe and gave rise to the spurious pUC19 hybridization signal. Consideration of Ps. aeruginosa DNA hybridization with the 17-mer probe showed that it was not as strong as expected, although discrete bands were observed within the chromosomal digest, indicating that target and probe sequences exhibited significantly greater homology than that shown by negative control samples, as expected from the strong homology between the N-terminal amino acid sequences of Ps. aeruginosa and M. methylotrophus amidases (similarly, the 1.5kbp Smal-Lam1314 fragment showed a strong hybridization signal). Plasmid inserts isolated from pNW3 and pNW4 showed only a weak signal with the probe indicating that they contained very few putative probe-binding sequences and this was confirmed for pNW3 by DNA sequence analysis (Chapter 7). DNA fragments which were eventually cloned into pUC19 to form pNW1 and pNW2 exhibited higher homology with the probe, which suggested that pNW2 was a good candidate for encoding the amiE sequence. Since eventual DNA sequencing results from pNW2 showed that it contained no amiE sequence then this also presumably indicated that these fragments happened substantially contained a greater concentration of sequences complementary to the various different versions of the probe. In view of the fact that the original PstI-digested M. methylotrophus genomic sample must have contained the amiE sequence, it cannot currently be explained why it was not identified by the 17-mer probe (in addition to the two apparently spuriously hybridizing bands), unless the insert of pNW1 encodes amiE, but as a result of one of the possibilities discussed previously (section 5.2.4) this clone expresses no amidase activity. However, results from analysis of pNW2 suggest that this is unlikely.

That E. coli JM109::pUC19, the control organism, grew poorly on glucose-M9 mineral salts-acetamide (amp) was unquestionable. The potential underlying reasons for this growth are manifold, but as E. coli does not possess an acylamide amidohydrolase growth must have occurred either independently of amidase activity or as a result of the activity of another enzyme(s) with amidase activity. The former may have been true if the acetamide that was added to solid media as the N-source had either hydrolysed spontaneously prior to incorporation into media or was subsequently thermally-degraded after prolonged incubation of cultures at 37°C. The corresponding appearance of free amonia and acetate in culture media, which E. coli can utilize respectively as N- and C-sources, may then have been sufficient to support the observed growth. The fact that control cells, and those of various clones did not grow in liquid media in which acetamide was supplied as N-source
(supplemented with glucose as carbon source), but did grow on identical solid medium, was presumably a function of different medium preparatory methods, since solid and liquid cultures of identical nutrient constitution were both incubated at 37°C for similar periods of time. This may suggest that addition of acetamide to cooled molten agar, or flaming of the surface of molten agar media immediately after pouring to remove bubbles was sufficient to liberate enough free ammonia and acetate to support the growth of amr clones. Alternatively, this disparity could have arisen if control E. coli JM109::pUC19 streaks were geographically located in close proximity to streaks of E. coli JM109::pNW3 on solid media, such that excess ammonia and acetate released into the agar matrix, resulting from the (form)amidase activity of the latter organism, was sequestered by control cells and was sufficient to promote growth (see below). Since this possibility was eliminated from single-inocula liquid cultures, and control cells did not grow in this medium, this could also explain the observed growth phenotypes on solid and liquid media.

Furthermore, it is also possible that E. coli JM109::pUC19 was able to hydrolyse acetamide, albeit at a very slow rate, by virtue of its putative penicillin amidase (or the β-lactamase encoded by pUC19). Various strains of E. coli have been reported to contain at least one penicillin amidase (Cole, 1969a,b; Szewczuk et al., 1987), although their substrate specificities towards aliphatic amides have generally not been tested, so it therefore remains a possibility that E. coli JM109::pUC19 is able to hydrolyse acetamide in this way. Cole (1969b) detected no activity towards acetamide in whole cells of E. coli NCIB 8743 after 4 h incubation at 35°C (conversely, very low activity was expressed by whole cells towards hexamide and valeramide); however, this does not necessarily imply that E. coli JM109::pUC19 is unable to degrade acetamide as the duration of incubation used in the former study may have been insufficient to detect very low acetamidase activity, and in this study E. coli JM109::pUC19 was generally incubated for a much greater length of time (> 24 h) on acetamide-containing medium at 37°C. It is also of significance that a partially-purified cell extract of E. coli JM109::pUC19 exhibited very low activity (approximately 1000-fold less than a similar extract of E. coli JM109::pNW3) towards formamide after prolonged incubation with this substrate at 37°C (see Chapter 6), although the acetamidase activity of this fraction was not tested. The origin of this latter amidase activity is therefore obscure, although it may reside with the biosynthesis of an enzyme resulting from the induction of a silent catabolic gene(s). This possibility is exemplified by a number of reports (e.g. Hall & Hartl, 1974) in which E. coli has been shown to encode and express several different 'exotic' catabolic genes under conditions in which a strong directional selection pressure was applied. It is therefore feasible that incubation of E. coli JM109::pUC19 on acetamide-containing medium induced an enzyme with very low amidase activity towards acetamide which allowed growth on media containing this amide as an N-source. In combination with this, or as an alternative, the limited growth of E. coli JM109::pUC19 on glucose-M9 mineral salts-acetamide (amp) medium may have been supported by the presence of ammonia and acetate in culture media which was hydrolysed either spontaneously or thermally.
All of the clones studied which expressed no detectable amidase activity (E. coli JM109::pNW1, ::pNW2 & ::pNW4) can be considered to be metabolically/genetically identical to E. coli JM109::pUC19 except that the former organisms all harboured recombinant plasmid inserts. Similarly, if it is assumed that these inserts encoded no amidase activity, then the reproducibly-observed enhanced growth of these clones in comparison to that of E. coli JM109::pUC19 on selective media must have been due to the sequestration of ammonia and acetate present in the growth medium, as a result of spillage from the amidase activity of E. coli JM109::pNW3. Analysis of the geographical positions of inocula of each of the clones and the control organism on the same solid medium plate tended to support this hypothesis as control streaks were always happenstantially inoculated on the opposite side of the plate furthest away from E. coli JM109::pNW3. Therefore, control streaks would have encountered very little acetate and ammonia, due to their limited diffusion in the agar matrix, and consequently would have received only marginal 'assistance' with growth; conversely, other amni streaks inoculated closer to the source of acetate and ammonia would have enjoyed higher concentrations of these compounds and exhibited enhanced growth assistance.

Alternatively, it is conceivable that cloned inserts (of pNW1, pNW2 & pNW4) encoded a protein, or part of a protein, that exhibited very low cryptic acetamidase activity. This appeared unlikely from database searches concerning the pNW2 nucleotide sequence, and the sequences of amino acids in all possible reading frames, which showed no homology to any published proteins that exhibited amidase activity, even though the whole of pNW2 was not sequenced and there remained a considerable uninvestigated coding-potential associated with this insert. This possibility is also probably equally unlikely for E. coli JM109::pNW1 and ::pNW4.

The medium chosen on which to select putative transformants (glucose-M9 mineral salts-acetamide [amp]) was based on that used previously (Drew et al., 1980) during cloning of amiE from Ps. aeruginosa using bacteriophage λ. In shot-gun cloning experiments the composition and preparation of this medium was altered in order to minimize growth of false-positive colonies whilst retaining a strong selection pressure for amiE+ colonies. This was attempted by reducing the glucose concentration from 2g l⁻¹ to 0.5-1.0 g l⁻¹ and the acetamide concentration from 18mM to 5-10 mM on separate occasions. Fresh stocks of acetamide were also made just prior to addition of the amide to molten agar in order to minimize the level of ammonia and acetate present in the amide sample resulting from spontaneous hydrolysis. Furthermore, the surfaces of plates were not flamed to eliminate the possibility that bubble-removal using this method degraded the surface layer of acetamide liberating sufficient ammonia and acetate to support amidase-independent growth.

Throughout the cloning work great care was taken to maintain sterility, but on certain occasions plates became contaminated with organisms that expressed amidase activity. These contaminants posed a problem whilst employing blue/white screening of putative transformants, as these organisms appeared identical to transformant colonies at an early growth stage, before the blue/white phenotype had had chance to develop. Several false-positive contaminants were therefore pointlessly investigated. Thus, the screening of
individual putative ami+ colonies was both laborious and inefficient, and the efficacy of screening could have been greatly improved if a reliable probe had been available.

Further to the reasons postulated above to explain the absence of amidase activity in a clone that putatively encoded amiE, the likelihood of heterologously-expressing the acetamidase from *M. methylotrophus* in *E. coli* might also rely heavily on the presence/absence of flanking regulatory sequences. The *Ps. aeruginosa* amiE gene is part of an operon involving regulatory proteins exhibiting both positive (AmiR) and negative (AmiC) influences on amidase expression (see Introduction), and amiE was cloned from *Ps. aeruginosa* using DNA derived from a constitutive mutant strain which was also resistant to catabolite repression by virtue of a second (up-promoter) mutation. Presumably, if the DNA had been isolated from the wild-type strain, or if amir had been separated from amiE by restriction digestion, then the subsequent activity of ami+ clones would have been considerably lower and therefore more difficult to identify. Indeed, when the amiER genes were sub-cloned into pBR322 (Clarke et al., 1981) the level of amidase activity after transformation into *E. coli* was low, even though the positive regulator (AmiR) was present. In view of the observed N-terminal amino acid (Silman, 1990) and immunological (J. Mills & C.W. Jones, unpublished) similarities between the amidase of *Ps. aeruginosa* and the acetamidase of *M. methylotrophus* it is not unreasonable to suggest that the latter amidase might also be part of an operon containing regulatory sequences that are essential for the expression of acetamidase activity. Chance separation of these putative flanking regulatory sequences from amiE by restriction digestion prior to library-construction might totally abolish acetamidase activity (or significantly reduce it) thereby rendering selection of clones on the basis of expression of amidase activity virtually impossible. It is therefore likely that future attempts to clone acetamidase would benefit greatly from the identification of a better probe exhibiting a less-ambiguous sequence which could presumably be achieved by amino acid sequence analysis of internal acetamidase peptide sequences produced by proteolytic cleavage. Renewed efforts could also be directed at cloning attempts using libraries of DNA fragments significantly larger than those cloned in this study (2-10 kbp) in order to maximize the probability that amiE and its putatively-associated regulatory sequences would be co-cloned. Cloning DNA isolated from hyperactive mutant strains (MM6, MM8 or MM15) of *M. methylotrophus*, in conjunction with a re-appraisal of nutrient selective conditions to take advantage of the novel characteristics of these mutant acetamidases, might also prove fruitful.
CHAPTER 6

MOLECULAR CLONING AND HETEROLOGOUS EXPRESSION OF FORMAMIDASE
CHAPTER 6

MOLECULAR CLONING AND HETEROLOGOUS EXPRESSION
OF FORMAMIDASE

6.1 Introduction

6.2 Preliminary identification of a recombinant E. coli clone expressing formamidase activity

6.3 Characterization of the formamidase-expressing clone (E. coli JM109::pNW3)

   6.3.1 Purification of heterologously-expressed formamidase
   6.3.2 Properties of heterologously-expressed formamidase
   6.3.3 Restriction mapping and sub-cloning of plasmid pNW3 insert DNA
   6.3.4 The effect of growth under various conditions on heterologously-expressed formamidase activity

6.4 Discussion
6.1 Introduction

The physiological and/or genetic regulation and biochemical properties of aliphatic amidases from various bacterial species (other than *M. methylotrophus*) including *Ps. aeruginosa* (Brammar & Clarke, 1964; Kelly & Kornberg, 1962a,b; Clarke, 1970, 1984; Brown et al., 1973; Ambler et al., 1987; Brammar et al., 1987), *Alcaligenes eutrophus* (Friedrich & Mitrenga, 1981) and *Brevibacterium* sp. R312 (Thiéry et al., 1986a,b; Maestracci et al., 1988) have been extensively investigated. Detailed work has also been reported concerning ongoing molecular biology studies of the regulatory sequences controlling the expression of amidase activity in *Ps. aeruginosa*, and the molecular cloning of the wide-spectrum amidase from *Brevibacterium* sp. R312 (see Introduction, Chapter 5). Most of these enzymes are at best only poorly induced by formamide, and exhibit only low activities with formamide as substrate despite being able to hydrolyse rapidly other short-chain aliphatic amides.

Preliminary studies of the *M. methylotrophus* formamidase (D.J. Scherr, N.J. Silman, J. Mills & C.W. Jones, unpublished) showed that the enzyme was strongly induced by formamide and acetamide (formamide > acetamide), strongly repressed by ammonia and was specific for formamide as a substrate (only negligible activity apparent towards acetamide). The results from continuous culture studies indicated that the primary physiological function of formamidase concerned its role in nitrogen metabolism, involving the hydrolysis of formamide to liberate ammonia which is readily utilized as an N-source by this organism. In addition, the organic acid end-product of formamide hydrolysis, formate, can be oxidized to CO$_2$ by *M. methylotrophus* by virtue of its NAD$^+$-linked formate dehydrogenase, and the NADH resulting from the latter enzymic reaction can also be oxidized by the respiratory chain with the concomitant formation of ATP (Patchett et al., 1985; Jones et al., 1987). Thus, in contrast to the organic acids generated by the hydrolysis of acetamide and acrylamide (acetate and acrylate) catalysed by acetamidase, and for which *M. methylotrophus* possesses only a limited metabolic potential (Carver & Jones, 1993), the subsequent oxidation of formate can contribute significantly to the energy economy of the cell.

In contrast to the acylamidases active towards short-chain aliphatic amides, relatively few bacterial species have been shown to hydrolyse formamide at high rates. There is some evidence, mainly from physiological studies, that *Mycobacterium smegmatis*, *A. eutrophus* and *Brevibacterium* sp. R312 contain discrete formamidases (Draper, 1967; Friedrich & Mitrenga, 1981; Maestracci et al., 1988), but a detailed analysis of their properties has not been described. *Streptomyces parvulus* contains two types of kynurenine formamidase (arylformylamine amidohydrolase EC 3.5.1.9) (Brown et al., 1986), and a eukaryotic formamidase from *Aspergillus nidulans* (Hynes & Pateman, 1970) has also been described.
In a similar fashion to the *M. methylotrophus* formamidase, the formamidase from *A. eutrophus* was induced by both acetamide and formamide, which served as N-sources, and was completely repressed by ammonia (see Friedrich & Mitrenga, 1981). In contrast, the formamidase activity of *A. eutrophus* cells grown on acetamide as N-source was approximately ten-fold higher than cells grown on formamide and it was concluded that acetamide was a more potent inducer of formamidase than formamide; formamidase was also subject to catabolite repression by succinate. No data are available concerning the regulation of formamidase activity in *Brevibacterium* sp. R312, and acetamide was shown to be a significantly better inducer of formamidase activity than formamide in *M. smegmatis* (Draper, 1967).

In relative terms, little is known currently about the fundamental molecular biological properties of methylotrophs (see Lidstrom & Stirling, 1990) although the situation is improving at a significant rate. A paucity of data exists concerning codon-usage, for example, in many methylotrophs and study of the functioning of methylotrophic promoters in heterologous systems has so far received little attention. Preliminary investigations in this area of research identified four potential promoter sequences from *Methylomonas clara* which exhibited similarities with the *E. coli* consensus sequence, only one of which, promoter pL, was transcriptionally active in *E. coli*; however, there was no evidence for coupled translation from this promoter. Similarly, a putative promoter sequence located just upstream from the *mauF* genes of *Methylobacterium extorquens* AM1 appeared to drive expression of a reporter gene in *E. coli* after insertion into a promoter probe vector (Lidstrom & Chistoserdov, 1993). Presumably the occurrence and availability of such data will increase rapidly as more research is conducted in this field, and it is hoped that ongoing work in this area will effect a full characterization of methylotrophic molecular biology.

This Chapter describes the molecular cloning and heterologous expression of the formamidase structural gene (*fmd*) from *M. methylotrophus* in *E. coli*. A comparison of the properties of the cloned formamidase with those of the formamidase purified from *M. methylotrophus* is presented, as well as evidence to indicate that the methylotrophic *fmd* promoter is transcriptionally active in the heterologous host.
6.2 Preliminary identification of a recombinant *E. coli* clone expressing formamidase activity

Insertion of size-selected *PstI*-digested *M. methylotrophus* chromosomal DNA fragments into pUC19, and the subsequent transformation of *E. coli* JM109 with the resultant population of recombinant plasmid vectors led to the selection of an *amn* transformant which harboured a 5.9kbp plasmid and which was capable of utilizing acetamide as an N-source. This clone was designated *E. coli* JM109::pNW3 (Chapter 5).

Initial studies with *E. coli* JM109::pNW3 showed that it harboured a 3.2kbp plasmid insert, and that whole cells exhibited significantly greater amidase activity with formamide, than with propionamide, acrylamide or acetamide as substrate. *E. coli* JM109::pNW3 also synthesized a protein that co-migrated with the formamidase purified from *M. methylotrophus*, and exhibited rapid growth with formamide as N-source in both solid and liquid cultures. Retransformation of *E. coli* with plasmid pNW3 produced only white recombinant colonies on solid medium containing acetamide as N-source supplemented with IPTG + X-gal; furthermore, plasmid pNW3 was re-isolated from a batch culture which expressed formamidase activity that had been inoculated with a single transformant colony. From these results it was concluded that the observed formamidase activity was encoded by plasmid pNW3 and that the formamidase structural gene (*fmd*) had been isolated from *M. methylotrophus* and expressed in *E. coli* JM10::pNW3. The confirmation of preliminary conclusions is described below.

6.3 Characterization of the formamidase-expressing clone (*E. coli* JM109::pNW3)

6.3.1 Purification of heterologously-expressed formamidase

*E. coli* JM109::pNW3 (and a sub-clone *E. coli* JM109::pNW323; see below) were routinely maintained on glucose-M9 mineral salts (amp) medium containing acetamide as N-source, despite the fact that formamidase rather than acetamidase activity was expressed by these strains. This was due to the high levels of residual ammonia contained by lab stocks of formamide (presumably as a result of spontaneous hydrolysis), which in the presence of glucose could have supported the growth of contaminant colonies, and also because formamidase-expressing strains grew adequately overnight on acetamide-containing medium. Consequently, single colonies could be picked easily from these plates, whereas this was more difficult from formamide-containing plates as a result of the more rapid growth of the latter. Growth of these strains on acetamide as the N-source presumably also constituted a stronger selective pressure for the maintenance of formamidase-encoding plasmids than growth on formamide, as a result of the low-activity of formamidase with acetamide as substrate.
Unequivocal confirmation that the *M. methylotrophus* formamidase had been expressed in *E. coli* was sought by purifying the enzyme from *E. coli* JM109::pNW3 using anion-exchange and gel-filtration FPLC. The purification procedure was essentially identical to that of acetamidase (Chapter 3), except that the second anion-exchange gradient (0-350 mM KCl) used in acetamidase purifications was often replaced by the third multi-step gradient method (Materials & Methods). Control FPLC-purifications were also carried out from cultures of *M. methylotrophus* and *E. coli* JM109::pUC19 to confirm that the chromatographic behaviour of native and heterologously-expressed formamidases were identical, and to show that *E. coli* JM109::pUC19 (which was identical to *E. coli* JM109::pNW3, except for the absence of a plasmid insert and any polypeptide[s] encoded by the insert DNA) expressed no formamidase activity.

The uniform chromatographic behaviour of heterologously-expressed and native formamidases was confirmed after each anion-exchange and gel-filtration chromatographic step by identification of peak-activity fractions using the amidase assay with formamide as substrate (Materials & Methods). Interestingly, but unexpectedly, application of the same procedure to *E. coli* JM109::pUC19 FPLC fractions showed that certain crude fractions exhibited very low formamidase activity (approximately 1000-fold less than that shown by *E. coli* JM109::pNW3). This activity eluted at the same $V_e$ value as the native/heterologously-expressed formamidase activity and represented either (i) an artefact generated by the contamination of the control starting material with a miniscule quantity of *E. coli* JM109::pNW3 cells (which was deemed unlikely), or (ii) the genuine amidase activity of an unidentified enzyme, possible candidates for which included the pUC19-encoded $\beta$-lactamase or the putative *E. coli* JM109 penicillin amidase (see also Discussion, Chapter 5). A control 'dummy' FPLC anion-exchange step showed that the observed *E. coli* JM109::pUC19 formamidase activity could not be attributed to the retention of formamidase by the anion-exchange column and the subsequent co-elution with, and contamination of, FPLC fractions that originally contained no formamidase.

The peak-activity gel-filtration formamidase samples from heterologously-expressed and native sources were pooled separately and their purity with respect to formamidase was estimated to be $\geq 90\%$ by SDS-PAGE, which also showed a single major protein in each track, indicating that each enzyme was composed of a single type of monomer (Fig. 6.1). Both enzymes exhibited identical electrophoretic mobility and the monomer MW was estimated to be $51kDa$, which was in good agreement with that estimated previously (D.J. Scherr, unpublished). Multiple attempts to determine a more accurate monomer MW using ESMS and MALDI-TOF were unsuccessful for reasons that were obscure.

The *M. methylotrophus* formamidase purified as described above was isolated from cells in which acetamidase was in the high-activity form (switched-on). High-activity acetamidase underwent switch-off during FPLC purification (see Chapter 3), but there was no evidence that formamidase also underwent switch-off during purification, and in further contrast to high-activity acetamidase, all pure formamidase samples appeared to be stable to the effects of multiple (-20°C) freeze/thaw events. Formamidase was also purified from *M. methylotrophus*
Figure 6.1 SDS-PAGE analysis of pure heterologously-expressed and native formamidases

Heterologously-expressed formamidase was purified from a 200ml batch culture (glucose-M9 mineral salts-acetamide [amp] medium) inoculated with a single colony of \textit{E. coli} JM109::pNW3 and grown overnight at 37°C with rotary shaking, using anion-exchange and gel-filtration FPLC. Native \textit{M. methylotrophus} formamidase was similarly purified from an approximately equivalent volume of cells (kindly supplied by J. Mills) grown in continuous culture (D = 0.1 h\textsuperscript{-1}) at low cell density under dual methanol-acetamide limitation. After gel-filtration, peak-activity protein samples were subjected to SDS-PAGE, and specific activity was measured with formamide as substrate at 37°C (Materials & Methods). The numbers under the tracks indicate the corrected specific activities of the pure enzymes (μmol min\textsuperscript{-1} [mg protein]\textsuperscript{-1}) calculated from the assumption (SDS-PAGE) that enzyme samples were only 90% pure. Track 1) MW standards; 2) \textit{E. coli} JM109::pNW3 formamidase; 3) \textit{M. methylotrophus} formamidase; 4) MW standards.
grown in continuous culture at high cell density under pseudo-dual methanol-acetamide limitation (under which conditions acetamidase is in the low-activity [switched-off] form) and the kinetic characteristics of the pure formamidase were found to be essentially identical to those determined previously. Furthermore, whole cell formamidase activity was not stimulated by heating at 60°C; rather the formamidase activity decreased by 0-20% under these conditions, presumably due to thermal denaturation. These results indicated that the *M. methylotrophus* formamidase is probably not subject to switch-on/switch-off activity cycling in vivo, unless this process occurs by an alternative mechanism to that regulating acetamidase activity, which cannot be mimicked in vitro, by heat-reactivation.

6.3.2 Properties of heterologously-expressed formamidase

The specific activities of pure heterologously-expressed and native formamidase samples were essentially identical (34.4 cf. 37.4 μmol min⁻¹ [mg protein]⁻¹; Fig. 6.1), as were the N-terminal amino acid sequences, MKTIV- (Materials & Methods). Similarly, both enzymes showed essentially identical substrate profiles (Table 6.1), which were in agreement with that exhibited by whole cells of *E. coli* JM109::pNW3 after overnight growth in glucose-M9 mineral salts-acetamide (amp) batch culture (section 6.3.4). Control cultures (*E. coli* JM109::pUC19) grown with ammonia as N-source in the presence of acetamide showed no activity with any of the amides tested under identical experimental conditions.

The native MW of pure heterologously-expressed formamidase was determined by gel-filtration chromatography (Materials & Methods) and the observed MW (123kDa) was in good agreement with that reported previously (130kDa) for the *M. methylotrophus* formamidase (D.J. Scherr, unpublished), even though the former determination was extrapolated from a standard curve constructed without Dextran blue (MW 2000kDa) data pertaining to the column void volume. In view of the estimated monomer MW from SDS-PAGE (51kDa) and the native MW determined above, it was concluded that formamidase was probably a homodimer (α₂), or much less-likely a homotrimer (α₃), as very few trimeric enzymes have been reported in the literature.

The kinetic properties of pure heterologously-expressed and native formamidases were investigated (Table 6.2). Hanes plot data apparently showed that the *Vₘₐₓ* of the heterologously-expressed enzyme was significantly lower than that of the native enzyme. However, this was probably an artefact generated by differences in the positions of lines of best fit of s/v versus s (fitted by eye) between the two data sets which would have had a direct effect on the relative gradients of these lines (equivalent to the reciprocal of *Vₘₐₓ*), and therefore the calculated *Vₘₐₓ* values also. Similarly, *Kₐₗₕ* values and specificity constants (*Kₐₗₕ/Kₐₗ₆*) calculated from Hanes plot *Vₘₐₓ* values as shown would also have been skewed by the differences in *Vₘₐₓ*. However, the heterologously-expressed and native specific formamidase activities, which had been determined previously by amidase assay with 50mM formamide as substrate at 37°C, produced *Kₐₗₕ* values (58 s⁻¹ cf. 64 s⁻¹) and specificity constants (36 s⁻¹ mM⁻¹ cf. 30 s⁻¹ mM⁻¹) which were in much closer agreement with each
Table 6.1 Substrate profiles of heterologously-expressed and native formamidases

The relative amidase activities of pure native and heterologously-expressed formamidases were determined at 37°C (Materials & Methods) with a range of amide substrates (all 50mM final concentration). Data pertaining to the native enzyme is taken from (i) this study, and (ii) D.J. Scherr (unpublished). The activities with different amides are expressed relative to the activity of each enzyme with formamide as substrate (100%).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Native activity (%)</th>
<th>Heterologously-expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(i)</td>
<td>(ii)</td>
</tr>
<tr>
<td>Formamide</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Propionamide</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Butyramide</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Acetamide</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Urea</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 6.2 Kinetic properties of heterologously-expressed and native formamidases

The $K_m$, $K_{cat}$ and $V_{max}$ values were determined for formamidase purified from *E. coli* JM109::pNW3 and *M. methylotrophus* with formamide as substrate at 37°C (Materials & Methods). $K_{cat}$ values were calculated from (a) $V_{max}$ values (Hanes plot) and (b) from the measured specific activity (50mM formamide, 37°C) using a native MW of 102kDa. $V_{max} = \mu$mol min$^{-1}$ (mg protein)$^{-1}$; $K_m = \text{mM}$; $K_{cat} = \text{s}^{-1}$; $K_{cat}/K_m = \text{mM}^{-1}$. Measured *E. coli* JM109::pNW3 and *M. methylotrophus* specific activities were 34.4 and 37.4$\mu$mol min$^{-1}$ (mg protein)$^{-1}$ respectively.

<table>
<thead>
<tr>
<th>Formamidase</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$K_{cat}$</th>
<th>$K_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. methylotrophus</em></td>
<td>(a) 44</td>
<td>2.1</td>
<td>75</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>(b) —</td>
<td>—</td>
<td>64</td>
<td>30</td>
</tr>
<tr>
<td><em>E. coli</em> JM109::pNW3</td>
<td>(a) 23</td>
<td>1.6</td>
<td>39</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>(b) —</td>
<td>—</td>
<td>58</td>
<td>36</td>
</tr>
</tbody>
</table>
other, and with the equivalent values calculated from Hanes plot data for the native \textit{M. methylotrophus} enzyme. Thus, the kinetic properties of heterologously-expressed and native formamidases were very similar, and the catalytic efficiency of formamidase was demonstrated to be approximately equal to that of acetamidase hydrolysing either acetamide or acrylamide (Chapter 3). In addition, the kinetic characteristics of \textit{M. methylotrophus} formamidase (data not shown) purified from cells which contained low-activity acetamidase were essentially identical to those of the formamidase purified from cells containing high-activity acetamidase. This re-affirmed the kinetic properties of the latter formamidase sample and suggested that in contrast to acetamidase, formamidase was probably not subject to switch-on/switch-off \textit{in vivo}. It was concluded from these results that the \textit{M. methylotrophus} formamidase had been successfully expressed in \textit{E. coli}, and that this enzyme was distinct from the short-chain aliphatic acetamidase described previously for this organism (Silman, 1990; Silman et al., 1989, 1991).

6.3.3 Restriction mapping and sub-cloning of plasmid pNW3 insert DNA

Plasmid pNW3 was digested with each of the common restriction enzymes (excluding XmaI & HincII) which were able to cleave the M13mp19 polylinker sequence of pUC19 in an attempt to identify potentially-useful restriction sites for use in the generation of sub-clones. The rationale behind this work was to identify a sub-clone expressing formamidase activity which contained the smallest insert DNA fragment possible for rapid DNA sequencing of the formamidase structural (\textit{fmd}) gene.

Image analysis of restriction digests of pNW3 indicated that only two endonucleases (AccI & HindIII) of the ten that were tested cut insert DNA. HindIII cleaved pNW3 insert DNA once to liberate restriction fragments of 3.6kbp (2.7kbp of pUC19 DNA contiguous with 0.9kbp of insert DNA) and 2.3kbp (insert DNA only), and AccI cleaved the insert at two (or possibly three) sites yielding fragments of 3.7kbp (2.7kbp of pUC19 DNA contiguous with 1.0kbp of insert DNA), 1.4kbp and 0.7kbp, with the possibility that a fragment < 0.3kbp was also generated (see Chapter 7). The exact positions of the majority of the AccI sites could not be assigned from these data alone, although one of the AccI sites was mapped to a position approximately 1kb from one end of the insert (Fig. 6.2; see also below). Since the \textit{fmd} gene was predicted to be encoded by approximately 1.4kbp of DNA, and in view of the fact that the probability of isolating a sub-clone expressing formamidase activity would be enhanced if the putative regulatory sequences flanking \textit{fmd} were also present, it was concluded that the 2.3kbp HindIII fragment represented the best candidate DNA fragment for sub-cloning.

Plasmid pNW3 was therefore digested with HindIII (this led to the artificial incorporation of the polylinker SphI restriction site into the sequence of the smaller [2.3kbp] restriction fragment) and the two restriction fragments were purified separately from the agarose gel matrix after electrophoresis. The larger fragment (3.6kbp), exhibiting cohesive ends, was ligated upon itself and the smaller fragment (2.3kbp) was ligated with HindIII-digested
pUC19. The resultant covalently-closed recombinant vector molecules were subsequently transformed separately into competent cells of *E. coli* JM109 and putative transformants (designated group 1 and group 2 transformants) were plated on to solid nutrient broth medium supplemented with acetamide and amp. After overnight incubation at 37°C all plates exhibited good growth, as expected, and three single transformant colonies were arbitrarily chosen from each of the two groups of transformants for plasmid restriction analysis (Fig. 6.3). The results clearly showed that the sub-cloning procedure was successful as both the larger (group 1) and the smaller (group 2) *Hind*III-generated pNW3 fragments were recovered in transformants, and group 1 and group 2 clones harbouring these fragments were respectively re-designated *E. coli* JM109::pNW310 and *E. coli* JM109::pNW323. It was subsequently found that *E. coli* JM109::pNW310 exhibited no growth in liquid glucose-M9 mineral salts-acetamide (amp) medium ± IPTG, whereas *E. coli* JM109::pNW323 exhibited good overnight growth in both types of media (see below). Thus, the size of the *fmd*-containing insert was successfully reduced.

A more detailed restriction analysis of pNW323 (5.0kbp) was undertaken in an attempt to reduce still-further the size of the *fmd*-containing insert DNA fragment to be sequenced. Plasmid pNW323 was incubated with various restriction enzymes (*Accl*, *Aval*, *BglII*, *ClaI*, *Hind*II, and *PvuI*), for some of which the corresponding restriction site was known to be absent from pUC19. Analysis of putative digests showed that after incubation with *BglII*, *ClaI* and *PvuI* the plasmid remained undigested, indicating that the pNW323 insert contained no restriction sites for these enzymes (Fig. 6.2). Furthermore, digestion of pNW323 with *Hind*II (which cleaves a unique site within pUC19) apparently linearized the plasmid which indicated that there was no restriction site for this enzyme within the insert DNA of pNW323. In contrast, *Accl* apparently liberated three restriction fragments (3.7kbp & 2 x 0.6kbp), and mapping of the insert *Accl* site that remained contiguous with pUC19 DNA after digestion showed that the pNW323 insert had been co-orientated within pUC19, as in plasmid pNW3, with respect to flanking polylinker restriction sites. Therefore, identification of the position of this *Accl* site within the pNW323 insert sequence, in conjunction with restriction data from pNW3 *Hind*III and *Accl* digestions, also allowed the position of the second and third *Accl* sites (see above) within the pNW3 sequence to be mapped unequivocally. *Aval*-digestion of pNW323 liberated two restriction fragments (3.6kbp & 1.5kbp) indicating that an *Aval* site was present approximately 0.9kbp from one end of the insert.

After re-appraisal of the unfavourable positions of restriction sites that were available for sub-cloning, and because the position of *fmd* within the pNW323 sequence was unknown, it was decided not to attempt further sub-cloning. It was deemed probable that work of this nature would be time-consuming and unlikely to reduce significantly the amount of DNA sequencing required to elucidate the complete *fmd* sequence, without perturbing expression from the insert and adversely affecting whole cell formamidase activity, which constituted the only means of identifying the *fmd*-containing fragment. In addition, it was not known whether pNW310 insert DNA represented upstream or downstream sequences in relation to
Figure 6.2 Restriction map of plasmids pNW3, pNW310 and pNW323

Plasmid DNA was digested with various restriction enzymes and subjected to electrophoresis to separate DNA fragments. Image analysis of gels (Materials & Methods) allowed automatic-sizing of DNA fragments by comparison with a standard curve generated from BstEII-digested λ genomic DNA. The positions of further sites that were unequivocally mapped (BstEII) and the artificially-incorporated SphI site are omitted for the sake of clarity. Several other sites (BamHI, HindII & AccI) have also been omitted as they were not originally unequivocally identified by restriction mapping (see Chapter 7).
Figure 6.3 Restriction analysis of sub-cloned DNA from pNW3

Samples of plasmid DNA were isolated from overnight (30ml) nutrient broth batch cultures (supplemented with acetamide and amp) inoculated with single colonies of the putative *E. coli* group 1 and group 2 transformants. Plasmid DNA (including control samples) was digested with *HindIII*, electrophoresed and the sizes of restriction fragments were determined by image analysis of restriction patterns versus a standard curve constructed from *Bst*EII-digested λ DNA standards (Materials & Methods). The numbers below the tracks indicate the average whole cell specific formamidase activities (μmol min⁻¹ [mg dry wt]⁻¹) from at least 3 independent determinations from group1 and group 2 transformants after growth in glucose-M9 mineral salts-acetamide (amp) medium + IPTG. Track 1) *Bst*EII-digested λ DNA standards; 2) pNW3 + *Hind*III; 3) Group 1 transformant plasmid + *Hind*III; 4) Group 2 transformant plasmid + *Hind*III; 5) pUC19 + *Hind*III; 6) *Bst*EII-digested λ DNA standards.

![Image of gel electrophoresis](image-url)
fmd. It was therefore decided to sequence the insert DNA carried by plasmids pNW310 and pNW323 to identify putative regulatory and ORF(s) sequences upstream/downstream from the fmd translation initiation site, and to elucidate the structural gene sequence of fmd (Chapter 7).

6.3.4 The effect of growth under various conditions on heterologously-expressed formamidase activity

E. coli JM109::pNW3 grew well in batch culture in glucose-M9 mineral salts (amp) medium containing either ammonia, formamide or acetamide as the N-source independently of the presence of IPTG. Washed cells from all three cultures exhibited substantial formamidase activity (Table 6.3), even though the concentration of ammonia in culture supernatants was generally > 0.5mM at the time of harvesting (see also Fig. 6.5) (formamidase is repressed in M. methylotrophus in vivo at ammonia concentrations ≥ 0.1mM), either via inclusion in the original medium or resulting from catalysis during growth.

Original determinations of the specific formamidase activity of E. coli JM109::pNW3 grown in batch culture with formamide as the N-source reproducibly showed inexplicably twenty-fold lower activities (0.023 ± 0.016 [±] 4 μmol min⁻¹ [mg dry wt]⁻¹) in comparison to those measured reproducibly on later occasions (Table 6.3). Since little is known about the detailed nature of the regulation in vivo of fmd in M. methylotrophus it was impossible to predict what effect the heterologous system exerted on fmd regulation, particularly during ill-defined batch growth, even if it was assumed that all of the putative elements necessary for the correct regulatory pattern were present and functioning. A speculative interpretation of these observations postulates that E. coli JM109::pNW3 was originally subject to regulation of formamidase activity by an ammonia- or formate-responsive element (possibly encoded by either ORF2 or ORF3 [which was truncated]; see Chapter 7) which monitored the intracellular concentration of ammonia/formate. The down-regulation of formamidase activity resulting from the high concentrations of ammonia/formate generated by the rapid hydrolysis of formamide in formamide-grown cultures could then explain the exceptionally low formamidase activity of such cultures. This putative ammonia/formate-responsive element may have been subsequently subject to a mutation that abolished its regulatory function, thereby resulting in the elevation of the specific activities of identical cultures observed at a later time. This specific activity increase, to a level commensurate with that of E. coli JM109::pNW3 grown on ammonia (in the absence of either amide inducers or their organic acid hydrolytic end-products), would therefore be consistent with the uncoupling of the regulation of formamidase activity from the putative ammonia/formate-responsive element.

No formamidase activity was detected in washed cells of E. coli JM109 or E. coli JM109::pUC19, i.e. in untransformed host cells or in host cells transformed with non-recombinant pUC19. However, washed cells of E. coli JM109::pNW3 exhibited the substrate profile expected of the M. methylotrophus formamidase, i.e. activities with acetamide and
Table 6.3 The effect of growth under various conditions on heterologously expressed formamidase activity

_E. coli_ JM109::pUC19 and ::pNW3 were grown in (20ml) batch culture in glucose-M9 mineral salts (amp) medium supplemented with either ammonia, formamide or acetamide as N-source. Cultures were inoculated with single bacterial colonies and incubated overnight with rotary shaking at 37°C. Washed cells were assayed for amidase activity with formamide as substrate at 37°C (Materials & Methods).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nitrogen source</th>
<th>Formamidase activity (µmol min⁻¹ [mg dry wt]⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109::pUC19</td>
<td>ammonia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ammonia + formamide</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ammonia + acetamide</td>
<td>0</td>
</tr>
<tr>
<td>JM109::pNW3</td>
<td>ammonia</td>
<td>0.500 ± 0.049 (8)</td>
</tr>
<tr>
<td></td>
<td>formamide</td>
<td>0.551 ± 0.049 (8)</td>
</tr>
<tr>
<td></td>
<td>acetamide</td>
<td>1.250 ± 0.049 (17)</td>
</tr>
</tbody>
</table>
Figure 6.4 Heterologous-expression of formamidase by *E. coli* JM109::pNW3

Untransformed *E. coli* JM109, and *E. coli* JM109::pUC19 and ::pNW3 were grown in batch culture in glucose-M9 mineral salts medium with ammonia and/or acetamide as N-source. The medium was supplemented with amp for the growth of strains harbouring plasmids, and cultures were grown overnight with rotary shaking at 37°C. Washed cell samples were assayed for formamidase activity at 37°C and subjected to SDS-PAGE (Materials & Methods). The numbers under the tracks indicate whole cell specific formamidase activities (µmol min⁻¹ [mg dry wt]⁻¹) and the arrow shows the position of formamidase. Tracks 1) MW standards; 2) Untransformed *E. coli* JM109; 3) *E. coli* JM109::pUC19; 4) *E. coli* JM109::pNW3; 5) *M. methylotrophus* formamidase; 6) MW standards.
other aliphatic amides were typically < 8% of the activity with formamide (no activity was observed with urea), and SDS-PAGE of cellular proteins from E. coli JM109::pNW3 showed the presence of a polypeptide of 51kDa which co-migrated with M. methylotrophus formamidase, but was absent from E. coli JM109 and E. coli JM109::pUC19 (Fig. 6.4). Since expression of formamidase in E. coli JM109::pNW3 occurred following growth in the absence of both physiological inducers of formamidase (acetamide and formamide) and also of IPTG (a gratuitous inducer of the lacZ promoter), it was concluded that expression of fmd was at least partially constitutive and was probably initiated from its own promoter (see below).

An attempt to correlate the observed whole cell specific activities of E. coli JM109::pNW3 grown in batch culture (glucose-M9 mineral salts [amp] medium) supplied with ammonia, formamide or acetamide as N-source and the formamidase concentration of each culture as determined by SDS-PAGE (not shown) was complicated by the presence of a protein in E. coli JM109::pUC19 which happenstantially co-migrated with formamidase. However, the amount of formamidase in E. coli JM109::pNW3 grown on each of the different N-sources was higher than that of the control, and the amount of formamidase in the culture grown on acetamide was approximately twice as high as that in cultures grown on either ammonia or formamide (i.e. approximately as expected from the specific activities). This enhanced formamidase expression may have resulted from acetamide acting as a stronger inducer of fmd than formamide (as has been observed for the formamidase of A. eutrophus; Friedrich & Mitrenga, 1981); however, if this was indeed the case, since whole cell activities exhibited by E. coli JM109::pNW3 after growth on ammonia and formamide were equal, this suggested that the inductive strength of formamide must have been virtually nil, which conflicted with observations concerning the M. methylotrophus formamidase which is known to be more highly induced by formamide than acetamide (D.J. Scherr, J. Mills & C.W. Jones, unpublished). Thus, it was deemed possible that the putative fmd induction system was not necessarily functioning in the heterologous system exactly as it would in M. methylotrophus.

An investigation of the relationship between the whole cell formamidase activities of E. coli JM109::pNW3 cultures grown on various different N-sources (acetamide, formamide & ammonia) and the ammonia concentration of culture supernatants (Fig. 6.5) suggested that the specific activity was inversely related to the ammonia concentration, although the relationship was not linear. A preliminary study of the effects on whole cell formamidase activity of the addition of variable amounts of exogenous ammonia, formate and formamide to glucose-M9 mineral salts-acetamide (amp) batch cultures inoculated with E. coli JM109::pNW3 was also undertaken. The composition of the media was rigged to give a range of initial starting concentrations of each compound as follows: (i) ammonia (ammonium chloride) (0-50 mM), (ii) formate (potassium salt) (0-20 mM), (iii) equimolar ammonia + formate (0-20 mM), and (iv) formamide (0-20 mM). The results (not shown) were difficult to interpret due to the multitude of variables acting potentially in concert and in opposing directions, viz. putatively variable strengths of induction of fmd by acetamide and formamide versus putative repression by ammonia and/or formate, in addition to the observed
Figure 6.5 The effect of ammonia concentration on whole cell formamidase activity

*E. coli* JM109::pNW3 was grown in (20ml) batch culture in glucose-M9 mineral salts (amp) medium supplemented with either ammonia (○), formamide (●) or acetamide (△) as N-source. Cultures were inoculated with single bacterial colonies and incubated overnight with rotary shaking at 37°C. Washed cells were assayed for amidase activity with formamide as substrate at 37°C. The concentration of ammonia in culture supernatants was also determined (Materials & Methods).
constitutivity of the system and the ill-defined nature of batch growth. However, several
trends were tentatively identified: (a) it was generally true that the average whole cell
formamidase activity was inversely related to the ammonia concentration of the culture
supernatant, although this relationship was not linear (as above); (b) addition of exogenous
formate exerted no significant effect on formamidase activity, but equimolar amounts of
formate + ammonia depressed formamidase activity; (c) addition of formamide exerted a
curious effect on formamidase activity, such that the specific activity was maximal in cultures
originally containing approximately 10mM formamide, but at initial formamide
concentrations greater or lesser than 10mM the final observed activity was lower. These
results suggested that *fnd* was subject to repression by ammonia (but that the heterologous
system was not as sensitive to the ammonia concentration as the native system), and its
activity may also have been modified by formate, possibly acting in concert with ammonia,
but this latter effect apparently necessitated that the formate be generated intracellularly.

The batch growth of *E. coli* JM109::pNW323 and ::pNW310 was also investigated briefly
to determine the effects of sub-cloning on formamidase activity (Table 6.4). *E. coli*
JM109::pNW323 grew well in glucose-M9 mineral salts (amp) medium ± IPTG containing
ammonia or acetamide as N-source in 24 h, but grew more slowly when formamide replaced
acetamide or ammonia as the N-source. In contrast, *E. coli* JM109::pNW310 exhibited no
growth on either formamide or acetamide as N-source, but grew well when ammonia replaced
these amides. As expected from these growth phenotypes, it was found that *E. coli*
JM109::pNW310 showed no formamidase activity, whereas the whole cell specific
formamidase activities of each of the *E. coli* JM109::pNW323 cultures grown in the presence
of IPTG were very similar (0.28-0.38 μmol min⁻¹ [mg dry wt]⁻¹), and as for *E. coli*
JM109::pNW3 the highest activity correlated with growth on acetamide. These results
indicated that *fnd* expression remained at least partially constitutive, as *E. coli*
JM109::pNW323 exhibited good growth on acetamide, formamide and ammonia
independently of the presence of IPTG, but whole cell activities of cultures grown on
acetamide + IPTG were significantly lower than those determined previously for identical
cultures of *E. coli* JM109::pNW3 (≥ 0.5μmol min⁻¹ [mg dry wt]⁻¹; see also Fig. 6.6). This
indicated that either sub-cloning, or the presence of IPTG, or a combination of both factors
exerted a deleterious effect on culture activity, and since the specific activities of cultures
grown without IPTG were not determined the effect of the latter on *E. coli* JM109::pNW323
formamidase activity could not be ascertained. However, the diminution in activity was not
due solely to an insert-orientation effect, as it was known that *E. coli* JM109::pNW3 and
::pNW323 inserts were orientated co-linearly, and it therefore also remained a possibility that
the removal of the 0.9kbp fragment that was contiguous with the *fnd*-containing fragment
during construction of pNW310 had separated a regulatory element from *fnd*. Batch
culture results from *E. coli* JM109::pNW323 cultures also indicated that formamidase
activity decreased as the culture supernatant ammonia concentration increased in a similar
fashion to that elucidated for *E. coli* JM109::pNW3.
Table 6.4 The effect of growth under various conditions on heterologously expressed formamidase activity of sub-clones

_E. coli_ JM109::pNW323 and ::pNW310 were grown in (20ml) batch culture in glucose-M9 mineral salts (amp) medium supplemented with ammonia, formamide, acetamide or a combination of ammonia + acetamide as the N-source. IPTG was added to cultures as indicated (+/- = presence/absence). Washed cells were assayed for amidase activity with formamide as substrate at 37°C, and the ammonia concentration of culture supernatants was also determined (Materials & Methods). Very good growth (+++); good growth (++); no growth (-); nd = not done. All data items represent single experimental determinations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nitrogen source</th>
<th>IPTG</th>
<th>Growth</th>
<th>Formamidase activity [ammonia] (μmol min⁻¹ [mg dry wt]⁻¹)</th>
<th>Supernatant ammonia (mM)</th>
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<tr>
<td>JM109:: ammonia</td>
<td>+/-</td>
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<td>pNW310 formamide</td>
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SDS-PAGE analysis of the intracellular formamidase concentration of *E. coli* JM109::pNW323 and ::pNW310 cultures indicated that the former contained a protein that co-migrated with *M. methylotrophus* formamidase, which was absent from *E. coli* JM109::pNW310. The banding patterns from different cultures otherwise appeared essentially identical (not shown) from which it was concluded that any polypeptide(s) expressed by pNW310 must have been present only at a relatively low level. SDS-PAGE also indicated that the elevated formamidase activity of *E. coli* JM109::pNW323 grown on acetamide (cf. ammonia or formamide) was probably due to a greater concentration of formamidase, which re-affirmed the suggestion that acetamide was probably a stronger inducer of *fmd* than formamide. Furthermore, the fact that growth of *E. coli* JM109::pNW323 on formamide as N-source was slower than on either acetamide or ammonia may suggest that the rapid generation of excess ammonia and/or formate in this culture strongly repressed biosynthesis of formamidase.

The apparently deleterious effect of IPTG on the whole cell formamidase activity of *E. coli* JM109::pNW3 was investigated. The inclusion of IPTG in liquid glucose-M9 mineral salts-acetamide (amp) medium reproducibly diminished the specific formamidase activity of *E. coli* JM109::pNW3 cells by suppressing expression from *fmd*, as evinced by SDS-PAGE analysis which clearly showed the greater intracellular formamidase concentration of cultures grown in the absence of IPTG (Fig. 6.6). These results suggested that the IPTG-induced diminution in activity was either a result of the titration of transcription factors by the *lacZ* promoter from the *fmd* promoter, or steric hindrance of transcription from the *fmd* promoter, for example, by RNA polymerase read-through from the *lacZ* promoter.

In conclusion, the simplest mechanistic explanation of the observed pattern of *E. coli* JM109::pNW3 whole cell formamidase activities (Table 6.3) is that the heterologous expression of *fmd* is fully constitutive (and is not subject to induction by amides), and that expression is subject only to repression by increasing ammonia concentration, but in a non-linear fashion. Thus, the specific activity of acetamide-grown cells, which exhibited the lowest levels of supernatant ammonia, would represent the maximal derepressed activity under the imposed experimental conditions. Conversely, the reduced specific activities of ammonia- and formamide-grown cells could be explained by the significantly increased supernatant ammonia concentrations of these cultures which would repress *fmd* expression. Alternatively, a more complex system may be operational in which the system is considered to be only partially constitutive, yet is subject to the further putative effects of variable strengths of induction by acetamide and formamide, and repression by ammonia and/or formate.

### 6.4 Discussion

The expression of formamidase in *E. coli* JM109::pNW3 and ::pNW323 in a manner that was independent of amide-inducers and IPTG strongly suggested that expression of *fmd* occurred from its own promoter. However, the precise position of *fmd* within the pNW3(23) insert DNA sequence and its orientation with respect to the direction of transcription from the
The specific formamidase activities of batch cultures of *E. coli* JM109::pNW3 grown in the presence or absence of IPTG were determined, and whole cells from each culture were subjected to SDS-PAGE (section 2.21, Materials & Methods). The numbers below each track indicate whole cell specific formamidase activities (μmol min⁻¹ [mg dry wt]⁻¹) and the arrow indicates the position of formamidase. Track 1) MW standards; 2) Nutrient broth + glucose (amp) starter culture; 3) & 4) glucose-M9 mineral salts-acetamide (amp) -IPTG; 5) & 6) glucose-M9 mineral salts-acetamide (amp) + IPTG; 7) *M. methylotrophus* formamidase; 8) MW standards.

**Figure 6.6 The effect of IPTG on heterologous-expression of formamidase**

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MW (kDa)   1    2    3    4    5    6    7    8

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lacZ promoter was unknown at this time. This fact rendered *fmd* expression from the *lacZ* promoter (which remained a possibility), rather than the putative *fmd* promoter, conditional upon the identical orientation of regulatory (*lacZ*) and structural (*fmd*) sequences. If this was indeed the basis for formamidase expression then this suggested that there was only a 50% chance that transcription would actually have occurred (that *fmd* and the *lacZ* promoter exhibited alternative orientations was later shown by DNA sequencing; see Chapter 7). Since in relative terms very little is known about the structure of methylotrophic promoters (cf *E. coli* promoters) and their ability to direct the heterologous expression of methylotrophic genes in foreign hosts (see Chapter 1) the putative expression of the *M. methylotrophus* formamidase structural gene in *E. coli* probably constitutes one of the first demonstrations of the efficient functioning of a methylotrophic promoter in *E. coli*. In addition, further study of the putative *fmd* promoter region has potential applications with respect to the development of shuttle vectors and heterologous expression systems involving methylotrophic control elements.

The strong similarities in the properties of the purified heterologously-expressed and native formamidases (kinetic properties, substrate profile, monomer and native MW) suggested that the heterologously-expressed formamidase was translated and processed authentically in *E. coli*. It would have been interesting to compare ESMS and/or MALDI-TOF MW determinations for the two enzymes to confirm that these proteins exhibited identical masses, and that one or other of these enzymes had not been subject to post-transcriptional processing that was absent from the other. Either of these techniques would presumably have also confirmed, or otherwise, the measured monomer MW and the MW predicted from the *fmd* DNA sequence (see Chapter 7). Unfortunately, ESMS and MALDI-TOF work was unsuccessful; however, it is likely that improved diafiltration methods and different solvent systems might enhance the probability of future success with this work.

Interpretation of results from *E. coli* JM109::pNW3 and ::pNW323 batch growths was difficult and largely speculative, and it is possible that the observed whole cell specific activities were subject to the effects of the putatively-variable inducer strengths of formamide versus acetamide, the potentially variable effects of different formate and acetate concentrations, and the effect of repression by ammonia. In addition, it must also be taken into account that the rates of formation and disappearance of ammonia, amide and organic acids from culture media would also exhibit significant variation, and that the influence of the growth stage of each organism at the time of harvesting was of importance, as cultures were not necessarily harvested after growth to equivalent cell densities.

A more controlled investigation of the regulation of heterologous formamidase expression using cultures grown under better-defined conditions would be necessary to probe further the effects of ammonia, acetate and formate on *fmd* expression. The constantly fluctuating levels of medium constituents in batch culture renders this a highly variable system, in which the magnitude of the change in experimental variables is virtually impossible to predict at a given point in time. Therefore, it is suggested that future work concerning formamidase regulation centres on fed-batch, or ideally, continuous culture (i.e. techniques which allow culture
parameters to be accurately defined, and in which the effects of perturbation of the culture by alteration of a single experimental parameter can be readily identified and studied).

As far as the author is aware, the only acylamidase other than the formamidase reported in this study to have been cloned and identified solely on the basis of the direct selection of a recombinant clone actively expressing an ami\textsuperscript{+} phenotype was the \textit{Ps. aeruginosa} amidase (Drew \textit{et al.}, 1980). The cloning and direct selection of formamidase from \textit{M. methylotrophus} therefore constitutes an essentially novel, albeit fortuitous, amidase cloning method, and the chance element underlying this work highlights the potential problems which were associated with the attempted use of this method to clone the acetamidase gene, especially in view of the apparently more complex acetamidase regulatory mechanism (Chapter 5). Thus, the ability to heterologously express formamidase from a relatively small DNA fragment (2.3kbp) suggests that regulation of \textit{fmd} expression is probably not as complicated as that of \textit{amiE}. 


CHAPTER 7

NUCLEOTIDE SEQUENCE OF THE FORMAMIDASE STRUCTURAL GENE
CHAPTER 7

NUCLEOTIDE SEQUENCE OF THE FORMAMIDASE STRUCTURAL GENE

7.1 Introduction

7.2 Analysis of the nucleotide sequence of plasmid pNW3 insert DNA

7.2.1 Introduction
7.2.2 Nucleotide sequence of the formamidase structural gene (fmd)
7.2.3 Analysis of the DNA sequence of ORF1
7.2.4 Analysis of the DNA sequence of ORF2
7.2.5 Analysis of the DNA sequence of ORF3
7.2.6 Analysis of restriction sites contained within the pNW3 insert sequence

7.3 Discussion
NUCLEOTIDE SEQUENCE OF THE FORMAMIDASE
STRUCTURAL GENE

7.1 Introduction

Very few organisms possess amidases that are able to hydrolyse formamide at high rates. Organisms that do exhibit this ability include \textit{M. methylotrophus}, \textit{Mycobacterium smegmatis}, \textit{Acaligenes eutrophus}, \textit{Brevibacterium} sp. R312 and \textit{Aspergillus nidulans} (see Chapter 1; Introduction, Chapter 6). The mycobacterial formamidase activity was first characterized by Draper (1967) and it resides with an amidase which has been referred to as an 'acetamidase' in a recent report detailing the cloning and DNA sequencing of its structural gene (Mahenthiralingam \textit{et al.}, 1993). Previous cloning and sequencing studies of bacterial amidases have characterized the acylamidases of \textit{Ps. aeruginosa} and \textit{Brevibacterium} sp. R312 (Chapter 1; Chapter 5), and more recently the sequence of a very wide-spectrum acylamidase from \textit{R. rhodochrous} J1, which is maximally active towards longer-chain aliphatic amides (\textit{e.g.} isobutyramide > valeramide > butyramide > propionamide) in addition to certain aryl amides (\textit{e.g.} benzamide & \textit{p}-toluamide) was reported (Kobayashi \textit{et al.}, 1993). Moreover, the sequences of the enantiomer-selective amidases of \textit{Rhodococcus} sp. (Mayaux \textit{et al.}, 1991) and \textit{Brevibacterium} sp. R312 (Mayaux \textit{et al.}, 1990), and the indole-3-acetamide hydrolase of \textit{Ps. savastanoi} (Yamada \textit{et al.}, 1985) have also been elucidated. These studies have shown that the amidase structural genes sequenced thus far can be divided into two groups (families) on the basis of DNA sequence (dis)similarities (Mayaux \textit{et al.}, 1990; Soubrier \textit{et al.}, 1992). Amidases which occur in each group exhibit high homology with the other amidases within that group, but negligible homology with the amidases of the alternative group. The two groups respectively encompass (i) the enantiomer-selective amidases plus the indole-3-acetamide hydrolases and the acetamidase of \textit{Aspergillus nidulans}, and (ii) the acylamidases of \textit{Brevibacterium} sp. R312 and \textit{Ps. aeruginosa}. The former group is apparently characterized by the consensus amino acid motif 'GGSSGG' (Mayaux \textit{et al.}, 1991) which is conspicuously absent from the latter.

Genes whose products function coordinately in a given catabolic pathway are often clustered at a particular chromosomal locus. This genetic arrangement is true for the short-chain aliphatic amidase operon of \textit{Ps. aeruginosa} (see Drew & Wilson, 1992; Chapter 5), and is likely to be true also for the 'wide-spectrum' amidase from \textit{Brevibacterium} sp. R312, although no such formal statement has been made in view of the limited information concerning its flanking sequences (Soubrier \textit{et al.}, 1992).

The \textit{Ps. aeruginosa} operon contains foremost the structural gene \textit{amiE}, downstream from which are located sequentially \textit{amiB} (which encodes a protein possessing a consensus nucleotide-binding site), \textit{amiC} (a protein that regulates the activity of the transcriptional antiterminator, AmiR, in an amide-dependent fashion), \textit{amiR} (which encodes the antiterminator protein) and \textit{amiS} (which encodes a putative transmembrane protein). In addition to the regulatory activities of AmiCR, it has been suggested that the AmiBS proteins...
associate to form an energy-dependent amide-uptake system, although the necessity for such a system, and evidence supporting its existence appears to be lacking in this organism. Further investigations into the biological roles of AmiBS are currently underway (Drew & Wilson, 1992) to clarify this paradoxical situation. Thus, the close proximity of these genes on the chromosome, in addition to the presence of various intrinsic non-coding regulatory DNA sequences that are specifically recognized by AmiR and regulatory proteins of the ntr system, allows transcription of the genes contained therein to be tightly and coordinately regulated in response to the nutrient environment. This sensor-regulator coupling of gene expression to environmental stimuli prevents the wasteful expenditure of ATP that would otherwise result from the inappropriate transcription and translation of unnecessary gene products.

This Chapter describes the molecular cloning and DNA sequencing of insert DNA fragments of plasmids pNW323 and pNW310 containing the formamidase structural gene (fmd) and its flanking regions from M. methylotrophus. A striking homology between the fmd gene sequence and the mycobacterial 'acetamidase' structural gene sequence is demonstrated and this is discussed in terms of a wider evolutionary context with respect to the different families of amidases mentioned above. Gene sequences putatively encoding a zinc finger DNA-binding protein and a regulatory protein analogous to the Ps. aeruginosa AmiC protein were also identified, and their possible roles in the regulation of fmd are discussed.
7.2 Analysis of the nucleotide sequence of plasmid pNW3 insert DNA

7.2.1 Introduction

The mapping of restriction sites within the insert DNA of pNW3 identified a HindIII site which was used to generate two restriction fragments (0.9kbp & 2.3kbp). These fragments were separated and re-inserted into pUC19 to form the recombinant plasmids pNW310 and pNW323 (see Fig. 6.2) and the latter was shown to encode formamidase activity. The insert DNA fragments of pNW310 and pNW323 were subjected to automated DNA sequencing in both directions using highly-purified double-stranded plasmid templates in conjunction with Universal primers complementary to M13mp19 vector DNA and custom-designed primers complementary to internal insert DNA sequences (Materials & Methods). The sequencing strategy and a fine-detail restriction map of pNW3 are shown below (Fig. 7.1), as is also the complete nucleotide sequence of insert DNA (3194 base pairs [bp] containing four ORFs) from plasmid pNW3 which was produced by combining the sequence data from pNW310 and pNW323 insert fragments (Fig. 7.2).

7.2.2 Nucleotide sequence of the formamidase structural gene (fmd)

A nucleotide sequence complementary to the N-terminal amino acid sequence (MKTIV-) which was derived from both heterologously-expressed and native formamidases by Edman degradation chemistry was identified within the pNW3 sequence starting at nucleotide (nt) position 1184. This sequence extended in the 5' to 3' direction to form an ORF (fmd) 1224 nt in length which terminated at an ochre stop codon (TAA). The fmd ORF encoded a protein 407 amino acids in length with a predicted MW of 44,438Da cf. 51,000Da estimated previously for the formamidase monomer by SDS-PAGE (Chapter 6). Although the predicted MW appeared to be significantly lower than that expected from SDS-PAGE, this was unlikely to have arisen from premature truncation of the reading frame resulting from sequence data error (which could have introduced a spurious termination codon into the ORF), since another putative ORF (ORF2, see below) started immediately downstream from the fmd termination codon. It must therefore be assumed that the difference in the predicted and measured MW values is a function of an electrophoretic anomaly, although its basis cannot currently be explained.

A putative ribosome binding site (AAGGAGA) which exhibited high homology to the E. coli consensus sequence (AAGGAGG) was identified at a position centred approximately 12 nt upstream from the initiation codon (ATG), in addition to putative upstream '-10' and '-35' promoter sequences showing good homology to the E. coli consensus sequences (i.e. TATAACC cf. TATAATG at '-10' & TTGAGG cf. TTGACA at '-35'; Rosenberg & Court, 1979). Unfortunately, since very little is currently known about methylotroph promoter structure, and in the absence of primer transcript extension sequence data, the significance of these sequences is doubtful, as it is currently unknown whether methylotroph promoters
Figure 7.1 Restriction map, sequencing strategy and genetic organization of plasmid pNW3 insert DNA

The fine-detail restriction map of plasmid pNW3 insert DNA derived from DNA sequence analysis is shown below, including those restriction sites (BamHI, HincII and AccI) whose presence and/or position could not be unequivocally assigned from original restriction mapping data alone (see Chapter 6; see also section 7.6). The pNW310 and pNW323 insert DNA sequencing strategies and the position of fmd and ORFs 1, 2 and 3 are also indicated.

[Diagram showing restriction map and sequencing strategies]
The complete nucleotide sequence (3194 nt) of plasmid pNW3 insert DNA, which was derived from sequencing studies of plasmid pNW310 and pNW323 insert DNA fragments, is shown below on the *M. methylotrophus* non-coding DNA strand in the 5' to 3' direction. The orientation and position of sub-cloned DNA insert sequences was determined by sequencing through the internal *HindIII* site (which was used to generate sub-clones) of the pNW3 insert DNA. The derived amino acid sequences from each ORF are indicated underneath the corresponding DNA sequence. Potential Shine-Dalgarno ribosome binding sites are singly-underlined and the *HindIII* restriction site is doubly-underlined. The positions of putative ORFs (1, 2 & 3) are identified in bold-type and the dot (•) marks the position of the first nt of each ORF. Bold-type and overlining (^^^) also denotes the position of putative '-10' and '-35' *E. coli* promoter consensus sequences. Chevrons (>>>) indicate the original pNW3 *PstI* cloning sites delimiting *M. methylotrophus* insert DNA, and dashed underlining (---) indicates the positions of the (3') artificially-incorporated *SphI* site and the *HindIII* cloning site of pNW323. Nonsense (termination) codons are overlined with asterisks (**). Underlined amino acid residues indicate the N-terminal sequences of native formamidase and of peptide fragments generated by V8 protease digestion. The 'P' at nt 3139 represents a purine (A or G) and lower case nucleotides (g) in the terminal 3' sequence region represent possible errors. No ORFs of a significant size preceded by a good ribosome binding site were identified on the coding strand.

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**ORF1**

CTGCAAGAGCAATGCTCTGCTGCTGGCTCCTGACCAATTGGCAAAATGCAGGGGCCACGATCA

AVAMACGLGAQLANAGATI

GCTTGGCGAGATAAAGTATATCATGTGGGCCGCCCTGCTGTTTATCGAGATTTATACTCTG

SFGEKYSAGFGPFTSYNS

TTGAAAGATGCTGCTGCCAATGAGCGTACCCATACAGGATTTTACTGAGCTGACGACAC

VEDAAANGSDRSNFDTLD

GGTGATTTGCTGACGCTATTTTAACAAATACACAGGATGGATGGGACCGAAATAT

RLYSFSGSNKYIKGMLNTEK

CTGGGCCTGCTGGCAACAGTAATTTAGAAGATGATGATGCAAATGTCGCGTTTCTGCGCC

SGGGSANSNSNFEDVIDANVQFQ

TGACCCTTGAAGTGCAATTGGCAAGTTTCTCTTCCACAGTACCCTGCGCCAAATA

LTPENVAIWAGRFLESPSDRAN

TGCCAGCGCTTTACTCACTCCATGCGGCTGTTAAGATTAGCCAAATCCCTCCCTCCTAC

HAGPYTSMGGGTYWANJASRY

GCTGAAACGGCGCGCTTGGCTGCTGGTAGATAGGAGTACGGGAAATTATG

GWNGGVIGRDGDGVAVVTSMF
AGGACCCACGCTGCGGTTTCTTCTTTGAGGCGAATATTTTTCCGCCCTTCGAGG
EDHVAVSPFGAEGDNIIFRFSP

GCGTAGTGCCCCATGTCAGTCAAGGCTACTAAGAAGCTGGAATGCTGATCGGC
GVGAGQSEANGTSDKLMYAGR

TACAAATGATTCGGGATGCCAGGGCTACATGGCAGTAATTTCGGCG
VQIDFDWAEPGYGYGTGNYPG

CAAAGATAATTCTGCCGCTGGAATCCGCGGTGAAGAGATGAGTTGAGTAAGA
AKDILIAIGARMKKGDGVVS

CTATTCTCTGGATTGGACTACAATACAGTGTGGATTCCCTTTTGGAGAAAA
TIPGTVGVDKSYVSDFELLEK

AGACGCGCCCAGTCCTCTGAGGCTTGTGAAGTTCTGAAGCCACGCTTAGG
KDVGPQFSAEAAAYYVDTD

ATGCATTCTGAGAGGACACAGAGATAAAAGCTTCTCGGTGAGTTCTTACGATG
DVFLGEQGKAYSAALGYLFN

ATCCCCTGGTGGGCAAGACATCTACGCTACTCGGAAATTTGAGCCACG
DPGWGGKIMFIVRYQKFDAD

GCTGGAGCACCACAGTGGGCTGGAACACTCTGTTTATCCGAGTTTCTGCG
GTLTTAAVSNRTSADTKRFEE

TAGGATCCCACTACGCTGCGCCTCCGCTCATAACCTCTGTGATACAGCCACCTAAGC
IGANYVIAPYNHSVITAAYGK

' -35' """

CAGATACGCGGTGCTCTCAGGCTAAACCTTTAGATGCTGGTTGCGAGATGCAATTT
TDTTAPAASSDFRPLALQMF

' -10' """

ATTCACACCTTTATATAACCTCTCCTCACAAGGAGATGAGC
KMKTVK

GGTGATGATATAAAAACCATGGGGAGGAGCGGGATATGCTAGGAGCTGACC
LDDLKKFPEEQDGQIHNHRWHIP

CGACCTGGCCGACTGTCAGTGAGCTCACAAGGAGGACAGATTCCGCTGCTGAGTGA
DLPMIAMVVKPGDDEFRVECMD

CTGGATCGAGTTGCTCAGTAGTGAACACATGAGCCAAATGACGCGGGCTGAAGCTAGG
WGGQIGNNDSANDVRDVDL

GACCCAGGTGACTCTCCTGCTGCTGGCTATTGGCGATGGCAATGCTGAGGCC
TQVHYLSGPIGVEGAGAEPGDL

GATGGTCTGAGACATTCTGAGTGGGACATTGATGGCTACAATGGGGGATGCA
MVVDILDVGGTVFDDSQWGFNG

CTTGGATGCAAGGAAAATGGGGCGGCTTCGTCAACCATTCTGGAGCAGCAAA
LPKENGGGFLLTDHFPEASK
TGGCTGTACCGGATCACATACTTGTAAAACCAAGCCGCCGGTGAACCAGGATACAGGGAA
GCTGSHTCRKFKPVNOQDTGK
***
ACCAGGTGCAATGCAGACCAAGAAACGGCAGCGGCCATGATGCGGG
PLQMKTARPMVGHPGCC
AGCGACATCAATCCCTTAATTAAITATTGATTTTATCCATAAAGGGAGAAGTAA
ORF3
TGAGTACCTCAAACAGCCCGTATTTTATGAAAGGGCAGCTGCTGCTGCTGCA
MSTSNRGRGFMKGAALVGMMA
AGLISGHAFADYPTAKVNT
CTGGCTTGCACTGTATTCTCCAGAATGAAAGGGATATTCTGCACCTGACCACATGCC
TGLAVTDSTVKGILHSAHTG
CCATGCAGATGAAAAGTTCCAATCCAGGCTGAAGAAGGTGCGA
TMAISETGSIQAEKLAILIQI
ATGCCATGCGCCATTTTTGGCCCAAGATGAAATCTCCAGGGAAGCTGCTGCTG
NAMGIILGRKIEIEIQEDGAS
<<<<
ACTGGCGACTTTCCAGFAAAAGGGGAAGGAAATTGCGGAGAAAGCAAAAGTGCTG
DWPTFAEKKAKEIAWRKTKWL
<<<<<<
AGGCCATGCAAGCTT
Q
2700
2760
2820
2780
2940
3000
3060
3120
3180
220
should be expected to conform to the *E. coli* consensus sequences or whether methylotroph promoters are more likely to exhibit slightly different consensus sequences which are characteristic of this particular type of organism.

A computer search for potential hairpin/stemloop structures upstream from *fmd*, and in the vicinity of the 3' end of the *fmd* sequence and the 5' region of ORF2 showed a number of reasonable candidates, although these were only identified when the relative search stringency was reduced by increasing the maximum allowed number of bp mismatches and the loop size, and by decreasing the minimum stem length. In view of the fact that very few of these possible secondary structure elements contained poly T tracts, which are known to aid transcription termination (Rosenberg & Court, 1979), and bearing in mind the method used to identify these sequences, it was concluded that these sequences were probably not involved with transcription termination and were therefore chance functions of this particular region of DNA sequence. A similar situation was evident during a search for hairpin/stemloop structures between the 3' end of ORF2 and the 5' end of ORF3. However, as for the DNA sequence immediately upstream from the ATG initiation codon of the *Ps. aeruginosa* *amlE* gene (Brammar *et al.*, 1987), a number of nonsense codons (TAA & TGA) were found closely preceding the *fmd* initiation codon. Moreover, the *fmd* initiation codon (ATG; see below) overlapped with two consecutive termination codons (TAA, TGA) in an alternative reading frame, viz. 5'-TAATGA-3', which suggested that the latter served to prevent transcriptional read-through from upstream DNA sequences. In view of this possibility, and the location of the putative *E. coli* -10' and -35' consensus promoter sequences just upstream from *fmd*, it may therefore follow that ORF1 is not an element of the putative *fmd* operon.

DNA sequences corresponding to the N-terminal amino acid sequences of peptide fragments generated by digestion of native formamidase with V8 protease (K. Hall, J. Mills & C.W. Jones, unpublished; see Fig. 7.2) were identified and mapped within the *fmd* ORF. These amino acid sequences confirmed that the *fmd* DNA sequence was in the correct reading frame at least up until the point at which the last residue of these sequences occurred.

The overall % G + C content of *fmd* was 53.5% (cf. 52.3% G + C overall for the complete pHw3 sequence) which was in close agreement with the estimated global % G + C content of the *M. methylotrophus* genome (50-53 % G + C; Jenkins *et al.*, 1987). Furthermore, the third position of all codons was generally biased towards G or C, and the complete codon repertoire was not utilized, TTA and CT(T/A) (Leu), CG(A/G) and AG(A/G) (Arg), and GGA (Gly) codons being absent. However, the significance of this latter finding was unclear due to the paucity of published methylotroph codon usage data.

A hydropathy profile (Kyte-Doolittle assignment) of the translated amino acid sequence from *fmd* (Fig. 7.3.a) showed the general absence of highly hydrophobic regions and the overall negative hydropathic index intimated that this protein was essentially a globular hydrophilic molecule, a conclusion which was in keeping with its soluble nature and its presumed cytoplasmic location (due to the absence of a hydrophobic N-terminal peptide leader sequence characteristic of membrane-associated and periplasmic proteins).
A database search was conducted to compare the \textit{fmd} DNA and derived amino acid sequence with other published sequences. DNA database searches (BLAST & FASTA; Materials & Methods) on both coding and non-coding strands returned a strong match with the 'acetamidase' structural gene of \textit{Mycobacterium smegmatis} to which \textit{fmd} exhibited an overall 63.1\% strict identity at the DNA level (determined using the GAP sequence alignment algorithm; Irix). Less-striking homologies were also identified between \textit{fmd} and DNA sequences encoding a number of diverse proteins, although the significance of these matches was difficult to determine without conducting a massive search of databases, and carrying out multiple sequence alignment comparisons. However, quite a high \% of such cases involved proteases (e.g. porcine endopeptidase & \textit{E. coli} aminopeptidase) which typically exhibited approximately 55-85 \% identity with \textit{fmd} over localized DNA sequences which were approximately 35-80 nt in length; this was interesting in view of the low aliphatic amidase activity exhibited by some of these enzymes. Other noteworthy sequence matches included: (i) 52.5\% identity with a mouse mRNA sequence encoding a GTP-binding protein over a 204bp region, and (ii) 55.6\% identity over a 117bp region with a \textit{Salmonella typhimurium} gene \textit{(cysE)}, which encodes a serine acetyl transferase. The latter was also interesting in view of the widespread acyl transferase activity of many amidases (see Chapter 1).

Protein database searches (BLAST & FASTA) for homology between the \textit{fmd}-derived formamidase amino acid sequence and published sequences returned similar results to those from DNA databases, and again the \textit{M. smegmatis} 'acetamidase' showed the highest homology with formamidase by a considerable margin. It was also of note that porcine endopeptidase exhibited 31.3\% identity to formamidase over a short sixteen amino acid residue sequence, and the \textit{E. coli} aminopeptidase showed 71.4\% identity in an even shorter amino acid sequence (seven residues). Both of these sequences contained residues (not shown) which were homologous with the formamidase amino acid sequence (-CMDWT-) starting at residue 44 (Fig. 7.2), and since this group of residues appeared in both a eukaryotic and a prokaryotic protease this suggested that they had been strongly conserved. Furthermore, the apparent conservation of these residues indicated that they were important for the biological function of the protease, and it is tempting to speculate that these residues may be involved with the formation of the protease active site, and hence also with the putative acylamidase activity of these proteases. Bearing this in mind, it is also tempting to speculate that these residues may be involved with the formation of the formamidase active site; however, this must remain an interesting possibility only, in the absence of further evidence to substantiate this notion.

A GAP sequence alignment between the \textit{M. methylotrophus} formamidase and the \textit{M. smegmatis} acetamidase amino acid sequences (Fig. 7.4) demonstrated 57.3\% overall homology (which was equivalent to 71.2\% similarity) between these two enzymes at the primary sequence level, indicating that they were very similar. This alignment highlighted discrete regions of relatively strong and weak homology between the sequences; the weakest regions of homology spanned approximately the first twenty and the last forty five amino acid residues of the proteins, but the intervening amino acid sequences between the protein
termini were strongly-conserved and the sequences exhibited high homology in this region. This pattern of conserved residues may indicate that the N- and C-terminal amino acid residues are not essential for the physical formation of the amidase active site, and have therefore been subjected to a weaker selective pressure during evolutionary time, such that these oligopeptide sequences have undergone more marked divergent evolution.

Consideration of the physico-chemical properties of the mycobacterial 'acetamidase' highlighted some interesting points. The original substrate profile that was reported for this enzyme (Draper, 1967; see Chapter 1) showed that it was at least forty-fold more active towards formamide than to the next best substrate (n-butyramide). This substrate profile was confirmed in this laboratory using a sample of *M. smegmatis* acetamidase (kindly gifted by P. Draper). Furthermore, immunological studies confirmed that the mycobacterial 'acetamidase' and *M. methylotrophus* formamidase were closely related (J. Mills & C.W. Jones, unpublished). Thus, in view of the striking similarities between these two enzymes it is suggested that the mycobacterial enzyme was originally mis-classified as an 'acetamidase' and is more accurately described as a formamidase. This notion was further substantiated by a recent report of the cloning and DNA sequencing of the mycobacterial 'acetamidase' structural gene (Mahenthiralingam et al., 1993) which showed that it encoded a protein of 406 amino acids with a predicted MW of 43,965Da, i.e. very similar to the *M. methylotrophus* formamidase system described above. Furthermore, a small discrepancy between the predicted and measured mycobacterial 'acetamidase' monomer MW values (43,965Da cf. 47,000Da by SDS-PAGE) was evident, as for the *M. methylotrophus* formamidase; however, the difference was significantly less than for formamidase, although both 'acetamidase' and formamidase SDS-PAGE MW values were overestimated with respect to that predicted from their respective gene sequences. These observations gave further credence to the idea that the mycobacterial 'acetamidase' is really a formamidase. The wider implications of these observations are considered in the Discussion.

The derived *M. methylotrophus* formamidase primary sequence was also searched at relatively high and low stringency for known amino acid motifs (MOTIF algorithm; Irix) which are often indicative of the biological function of a given protein. A high stringency search identified a sequence ([R] YAGIMH [P] GL [I] [G] [C]) which was similar to, but not identical with, a prokaryotic membrane lipoprotein lipid consensus attachment site ([D,E,R,K] xxxxxx [L,I,V,M,F,S,T,A,G] xx [I,V,S,T,A,G,Q] [A,G,S] [C]; 'x' represents any amino acid and cleavage occurs before the C residue prior to lipid attachment); however, this sequence was situated centrally within the formamidase primary sequence starting at amino acid residue 145, which in view of the typical N-terminal location of the prokaryotic lipid attachment sites strongly suggested that this was a spurious 'motif' generated by chance. It was therefore concluded that this was probably not an authentic lipid attachment motif and that on the strength of this observation alone there was no reason to suggest that formamidase was a lipoprotein. In addition, a low stringency search identified a multitude of different amino acid motifs which were all involved in covalent protein modification (e.g. N-glycosylation,
Figure 7.3 Hydropathy analysis of pNW3-encoded ORFs

The hydropathy profiles for \textit{fmd} and the putative ORFs 1, 2 and 3 encoded by plasmid pNW3 insert DNA are shown below based on Kyte-Doolittle assignments. The ordinate and abscissa respectively represent the hydropathic index of the protein and the amino acid residue number (proceeding from the N-terminus to the C-terminus) in each case: (a), \textit{fmd}; (b), ORF1; (c), ORF2; (d), ORF3. The vertical axis represents the hydropathic index of the protein and the horizontal axis shows the amino acid residue number.
Figure 7.4 Formamidase and mycobacterial 'acetamidase' amino acid sequence alignment

The *M. methylotrophus* formamidase amino acid sequence was readily aligned (GAP sequence alignment algorithm; Irix operating system) with the *M. smegmatis* 'acetamidase' amino acid sequence (taken from Mahenthiralingam et al., 1993), a process which necessitated the insertion of only a very few sequence gaps to maintain the close alignment between the two sequences. This indicated that these proteins exhibited significant homology (57.3% strict identity) at the primary sequence level. A 'I' represents a strictly conserved amino acid residue, and the numbers represent the position of amino acid residues within the protein sequence relative to the N-terminal Met residue (designated residue 1) of each protein. The *M. methylotrophus* formamidase sequence is the uppermost strand.

```
1   MKTIVKLDDLKKSFEGQDGQIHNRWHPDLPMIAMVKPGDEPRVECMMDTG 49
1   MFEPYVPSVSDKSMDPAAVPGRNRMHFQIDFAAVPXPGPFEKEMTD 50
50  GQIQGNDSDANVDVNLTOVHTLSGPICVGEASREVPSDLKMDILDVGLGDYFDD 99
51  GQIQGNDSDANVDVNLACPMHLSGPICVGEASEFQDGPLLVIDDLIGFVPQ 100
100 SQ ........WFPGQFEXKGGGFLTDMPEASSTYDTPHGYTVTSRQHP 142
101 TNGPWCSEGQWGPSGIFARKVNGGGFQVPPDVPAQKAIWDFGQHQTSGHP 150
143  KVRYAG1MPGLIGCPKELDVNRREGLNAPDPDVPVPLACPTSQ 192
144  GVYTG1THPGFTAPSFLADBKN6KKEKQATIDPQDPVPPPLPPLPLD 200
149  SAVMPHLSGIIAAGEGTVPQPDHGQNCDDKNLGGSSRRVYVYV 242
151  TLOQSTAGQDQAIAANDQARTQVTNFPQRGIFVQVVE 250
243  DGLSMGDLFSQGQG8ITFCOAEMAGYLIDKQGKVGYI4NP 291
245  GAMLSGDLFQGQG8EINFOCAEMGGFIDMVDLIGGKMGYVTVTPN 300
292  VFQPSPHTFYKSYMFELGYSVGRAGQKH.YLDPHIANYPQLNLAPNL 340
301  IFPGVRPVEEMLTLP5GSDHARHNNAYMDATMAYNRALCLA يا 350
341  KFGYGEQAVSILGTAVPKHCISGIDPACATWIEFIFDPFPNA 390
351  EWGYTQDVYQALLGTSFEG ...........ASAASNTSRTYVPRCSRFPRE 391
391  DGPK1MVFPGVDVSPTS 407
392  STSTSFPRQQPEGR... 406
```
N-myristoylation and protein kinase C phosphorylation sites etc.), rather than motifs which might have been more informative with respect to the biological function of formamidase, or which might have identified conserved residues possibly implicated in amide-binding. Thus, the significance of the motifs that were identified in this search was questionable in view of the high frequency at which they occurred within both the formamidase primary sequence and other unrelated amino acid sequences (not shown). It was therefore concluded that, in the absence of further protein chemistry investigations, these motifs were at best interesting, and therefore worthy of further investigation at a later date, and at worst artefactual. A specific search of the formamidase sequence for the 'amidase consensus' sequence (GGSSGG; Mayaux et al., 1991) was negative. Similar motif searches conducted for the *M. smegmatis* 'acetamidase' amino acid sequence (Mahenthiralingam et al., 1993) yielded essentially identical results to those described above, indicating that this enzyme contained no motifs that were absent from the *M. methylotrophus* formamidase sequence.

Finally, in view of the great similarity between the *M. methylotrophus* formamidase and the *M. smegmatis* 'acetamidase', plus the observed lack of homology between the latter enzyme and the *P. aeruginosa* acylamidase (see Mahenthiralingam et al., 1993), the *M. methylotrophus* formamidase sequence was compared by eye with that of the *P. aeruginosa* 'acetamidase' amino acid sequence (Mahenthiralingam et al., 1993) yielded essentially identical results to those described above, indicating that this enzyme contained no motifs that were absent from the *M. methylotrophus* formamidase sequence.

7.2.3 Analysis of the DNA sequence of ORF1

A large ORF (ORF1) was identified which was terminated by a stop codon (TAA) located just upstream from *fmd* at nt 1140. This ORF started either with a GCA codon (nt 3) (indicating that it was truncated at its 5' end and therefore its ribosome binding site and initiation codon were presumed to be missing) or with one of the many downstream putative ATG start codons (or less-likely at one of the many putative downstream GTG or TTG start codons which have also been shown to function as initiation codons in *E. coli* [see Kozak, 1983]). If the latter was the case, then it was likely that such an initiation codon would be closely preceded by a good *E. coli* consensus ribosome binding site, as was the case for the other ORFs (*fmd*, ORF2 & ORF3). A systematic search of the ORF1 DNA sequence indicated that of the possible downstream start codons only a few were preceded by potential ribosome binding sites, none of which exhibited very high homology with the *E. coli* consensus sequence. Moreover, in each case it was found that the putative start codon was in
frame with the largest possible version of ORF1, and that each possible ORF generated in this way always terminated at the same ochre stop codon (TAA) located at nt 1140. It was therefore concluded that the simplest explanation was that ORF1 had been truncated at its 5' end and that the putative ribosome binding sites downstream from this point were chance occurrences and did not represent true regulatory elements.

Characterization of ORF1 showed that it was 1137 nt in length and that it encoded part of a protein of > 379 amino acids (MW > 40,475Da). Its % G + C content was 51.2% which was slightly lower than that of \textit{fmd}, but which was still in keeping with the overall % G + C content of the \textit{M. methylotrophus} genome. Once again, the third codon position was biased towards G or C and the full codon repertoire was not utilized (CTA [Leu], TCG [Ser], CCG [Pro], CAT [His], CG[A/G] and AGG [Arg] and TGC [Cys] being notably absent).

A hydropathy profile (Kyte-Doolittle assignment) of the translated amino acid sequence from ORF1 (Fig. 7.3.b) showed the general absence of highly hydrophobic protein domains and the overall negative hydropathic index intimated that this protein was essentially a globular hydrophilic molecule, as concluded for formamidase. However, it appeared feasible from the hydropathy plot that the N-terminal region of this protein was more hydrophobic than the rest of the molecule, even though the initial N-terminal amino acid residues were missing.

DNA and protein database searches were undertaken (exactly as for \textit{fmd}) to identify any published sequences exhibiting homology with ORF1. A number of apparent homologies were reported with a diverse range of proteins, most of which were probably of dubious significance. However, several DNA matches were potentially of interest and these included (i) 74% identity over a 43 nt sequence with a \textit{Mycobacterium leprae} cosmid library sequence, and (ii) homologies of approximately the same magnitude between ORF1 and several Fe-S proteins (human) and proteins exhibiting ATPase activity (yeast, human, bacterial). Similarly, interesting amino acid homologies included (i) 15.4% identity with an outer membrane porin protein (bacterial) over a 175 amino acid overlap, and (ii) 15.6% identity with an alcohol dehydrogenase precursor (bacterial). An amino acid motif search of ORF1 identified no significant consensus motifs that might have indicated the biological function of the ORF1 gene product.

The \textit{Ps. aeruginosa} amidase regulatory sequences (\textit{amiRC}) and putative structural sequences (\textit{amiBS}) also presumed to be involved with amide assimilation by AmiE in this organism are all clustered downstream from the \textit{amiE} structural gene to form an operon (Introduction; Chapter 5). Similarly, two ORFs are again present downstream from the \textit{Brevibacterium} sp. R312 \textit{amiE} sequence, one of which (designated as ORF1) exhibits high homology to the regulatory sub-unit of an ATP-dependent protease and the other encodes an apparently unique gene product (Soubrier \textit{et al.}, 1992); however, these workers did not speculate on the inter-relationship between the downstream flanking regions and \textit{amiE}. In view of the flanking nature of ORF1 in relation to the position of \textit{fmd} in the pNW3 DNA sequence it is possible that ORF1 encodes a gene product involved with formamide catabolism, despite possible indications to the contrary (see section 7.2.2). Since database and
motif searches suggested that the ORF1 protein was essentially unrelated to the database sequences it was decided to align the ORF1 protein sequence (using the GAP algorithm) with that of AmiCR from *Ps. aeruginosa* (see Lowe *et al.*, 1989; Wilson & Drew, 1991; Drew & Wilson, 1992), and the ORF1 sequence occurring downstream from the *Brevibacterium* sp. R312 amiE sequence (*i.e.* known and suspected amidase regulatory sequences) in order to identify any short, conserved regions of amino acid sequence which might be indicative of some motif common to each of these proteins. None were found and the overall homology between the *M. methylotrophus* ORF1 amino acid sequence and each of the other sequences was low, *i.e.* the ORF1 derived amino acid sequence exhibited respectively 14.6%, 13.3% and 15.4% identity with the sequences of AmiC, AmiR and ORF1 from *Brevibacterium* sp. R312. Furthermore, similar alignments of the *M. methylotrophus* ORF1 amino acid sequence with each of the putative ORFs (ORFPl, ORFP2 & ORFP3) located upstream from the mycobacterial 'acetamidase' sequence (Mahenthiralingam *et al.*, 1993) showed little similarity between ORF1 and either ORFPl or ORFP2; however, the mycobacterial ORFP3 sequence exhibited 21.8% identity with ORF1 (not shown). This is of possible interest as ORFP3 showed some homology to a number of membrane-associated proteins, as did the ORF1 protein. This similarity suggested that the ORF1 protein was possibly membrane-associated, as did the likely hydrophobic nature of its missing N-terminal region (as deduced from its hydropathy profile). ORFP3 and ORF1 were also similar in that neither exhibited any detectable ATP-binding consensus motifs (Walker *et al.*, 1982; Higgins *et al.*, 1986, 1988). Thus, despite these observations and inferences, the biological function of the ORF1-encoded protein remains obscure.

7.2.4 Analysis of the DNA sequence of ORF2

A third, relatively-short ORF (ORF2) was identified immediately 3' to *fmd* at nt 2420, starting with an ATG initiation codon and extending 339 nt downstream to terminate at an ochre stop codon (TAA). A putative ribosome binding site (AAGGAGT) which exhibited high homology with the *E. coli* consensus ribosome binding site (AAGGAGG) was centred approximately 12 nt upstream from the ATG start codon and overlapped with the TAA termination codon of *fmd*. The presence of a good ribosome binding site, and the close proximity of ORF2 to *fmd* was taken as good evidence that the *fmd* sequence was correct and that its reading frame had not been prematurely terminated by a sequencing error.

Characterization of ORF2 showed that it encoded a protein of 112 amino acids with a predicted MW of 12,306Da. The third codon position generally favoured G or C and its overall % G + C content was 55.2%, which was higher than for ORF1 or *fmd*, although this may have resulted from statistical bias due to the relatively short sequence of ORF2 in comparison with the upstream ORFs. In addition, as described for the upstream sequences, this ORF did not utilize all possible codon variations, but again this may have been exacerbated by the short length of the ORF2 DNA sequence. A hydropathy profile (Kyte-
Doolittle assignment) of the translated amino acid sequence from ORF2 (Fig. 7.3.c) showed that this protein was probably a globular hydrophilic molecule.

Protein database searches showed that the ORF2 gene product showed strong homology with a number of different zinc finger DNA-binding proteins (mouse, human) (see Freemont et al., 1991), which was generally localized at amino acid residues 6-12 (-YECDSCEG-) of ORF2. These sequences typically exhibited approximately 71% identity over these seven amino acids, indicating that this sequence was strongly conserved among these proteins. The -CDSC- amino acid sequence also represents an example of a putative metal-binding motif, CxxC (where x represents any amino acid), which has been implicated in the regulation of gene expression in eukaryotes (Latchman, 1990), and which occurs twice within the complete ORF2 sequence. Significant homology was also identified between longer amino acid sequences in ORF2 (including the region already mentioned) and zinc finger proteins, e.g. 27.5% identity over a forty amino acid overlap. Further examples of proteins that are known to associate with DNA and which exhibited significant homology with the ORF2 protein included a viral DNA replication protein, trypanosomal DNA polymerase 1 and a Caenorhabditis elegans transcription factor. These findings were particularly interesting as the CxxC motif also occurred twice within the amino acid sequence of a short ORF (ORFP2; encoding a protein of MW 10,000Da) which was identified upstream of the mycobacterial 'acetamidase' structural gene (see Mahenthiralingam et al., 1993). A GAP local homology alignment of the mycobacterial ORFP2 sequence with that of the M. methylotrophus ORF2 sequence (Fig. 7.5) showed that these two proteins exhibited 24.2% identity. It is possible therefore, that the M. methylotrophus ORF2 and M. smegmatis ORFP2 both encode a metalloenzyme (e.g. a zinc finger-type protein) that is involved with the regulation of fmd expression. Alternatively, it is also possible that ORF2 encodes a thioredoxin, a protein disulphide isomerase, or a cytochrome as these proteins also contain CxxC motifs (Loferer & Hennecke, 1994; Nunn & Anthony, 1988; Anthony, 1991). However, in view of the results from the database searches it would appear that the DNA-binding function is more likely to represent the true biological role of the ORF2 protein. Moreover, the apparent hydrophilic nature of this protein supports the notion that it is located in the cytoplasm rather than being in association with the membrane, a location which is at least consistent with its putative gene-regulatory function. Finally, the ORF2 amino acid sequence did not exhibit strong homology with AmiCR from Ps. aeruginosa (≤ 17.4% identity after GAP alignment).

7.2.5 Analysis of the DNA sequence of ORF3

A fourth putative ORF (ORF3) was located downstream from ORF2. ORF3 initiated at an ATG codon (nt 2820) and was preceded by a ribosome binding site (AAGGAGA) which again exhibited high homology with the E. coli consensus sequence (AAGGAGG). ORF3 extended downstream until the PstI cloning site of pNW3 was reached at which point the M. methylotrophus insert DNA sequence ends (the SphI & HindIII sites located 3' to this restriction site are vector DNA sequences). Thus, ORF3 was truncated at the 3' end and must
Figure 7.5 Alignment of the ORF2 gene product with that of ORFP2 from *M. smegmatis*

The *M. methylotrophus* ORF2 amino acid sequence was aligned (GAP sequence alignment algorithm; Irix operating system) with the *M. smegmatis* ORFP2 amino acid sequence (taken from Mahenthiralingam et al., 1993). This indicated that these proteins exhibited significant homology (24.2% strict identity) at the primary sequence level. A 'I' represents a strictly conserved amino acid residue, and the numbers represent the position of amino acid residues within the protein sequence relative to the N-terminal Met residue (designated residue 1) of each protein. The *M. methylotrophus* ORF2 sequence is the uppermost strand. The positions of the C residues of the CxxC motifs putatively involved with metal-binding are marked with asterisks (*).
therefore presumably encode a protein of at least 121 amino acids (MW > 12,788 Da).

The G/C bias in the third codon position was slightly less than that described for the other ORFs and the same general pattern of codon usage was evident. Hydropathy analysis of ORF3 (Fig. 7.3.d) showed that the protein contained an essentially hydrophobic region close to its N-terminus after which point the sequence tended to become more hydrophilic towards the presumed middle/C-terminal end of the protein. This hydrophobic region is potentially indicative of either (i) a membrane-association patch which may suggest that this protein is anchored to the cytoplasmic membrane, or (ii) a hydrophobic leader-sequence involved with protein translocation across the cell membrane.

DNA and protein database searches were conducted and a FASTA protein search showed relatively strong homology, 32.9% identity in a 73 amino acid overlap, between the ORF3 derived amino acid sequence and the primary sequence of AmiC from *P. aeruginosa* (Wilson & Drew, 1991). This similarity translated to an overall 28.9% identity between the truncated ORF3 gene product and AmiC after GAP alignment (Fig. 7.6). The ORF3 protein also exhibited weaker, although possibly still significant matches with, for example, (i) the N-terminus of a sub-unit of the nitrate-inducible formate dehydrogenase of *E. coli* K-12 (22.6% overall identity), which is encoded by the *fdnG* structural gene (Berg et al., 1991); (ii) the α-sub-unit precursor of the Rubisco sub-unit binding protein from wheat (*Triticum aestivum*; 25.0% identity in a 52 amino acid overlap); and, (iii) a 60kDa chaperonin from *Synechococcus* sp. (24.1% identity in an 87 amino acid overlap). However, the significance of these matches is difficult to determine and they must presumably be happenstential, although it is tempting to speculate on the relevance and importance of the formate dehydrogenase match, in view of its potential role in formamide catabolism. It therefore seems highly likely that ORF3 encodes a regulatory protein that may perform a functionally-analogous sensory role to AmiC with respect to amide-binding. Moreover, the size of the ORF3 protein (MW > 12,788 Da) is at least consistent with that of AmiC (42,834 Da), as is its putative hydrophobic N-terminal domain, which is characteristic of the family of periplasmic binding proteins to which AmiC is related (see Wilson et al., 1993; Tam & Saier, 1993). However, it was unsafe to conclude that the ORF3 protein functions exactly as for AmiC, i.e. as an amide-binding protein involved with the regulation of transcription termination, due to the apparent absence of an ORF encoding a protein similar to AmiR, the apparent lack of good candidate stemloop terminator structures upstream from the start codon of *fmd*, and the general absence of other information concerning the regulation of *fmd* expression. It was, of course, quite possible that the putative missing amiR sequence, which can be reasonably expected to be located downstream from ORF3 by analogy with the *P. aeruginosa* amidase operon, was separated from ORF3 during the generation of the pNW3 insert. Alternatively, amiR may genuinely be absent from the *M. methylotrophus* chromosome, and the ORF3 protein functions in an alternative regulatory mode, perhaps by modulating the activity of the putative DNA-binding protein encoded by ORF2. The possible implications of the genetic constitution of the pNW3 insert fragment is considered further in the Discussion.

It is also important to note that the DNA sequence of ORF3 has not been determined
Figure 7.6 Alignment of the ORF3 primary sequence with that of AmiC

The *M. methylotrophus* ORF3 amino acid sequence was aligned (GAP sequence alignment algorithm; Irix operating system) with the *Ps. aeruginosa* AmiC amino acid sequence (taken from Wilson & Drew, 1991). This indicated that these proteins exhibited significant homology (28.9% strict identity) at the primary sequence level. A T represents a strictly conserved amino acid residue, and the numbers represent the position of amino acid residues within the protein sequence relative to the N-terminal Met residue (designated residue 1) of each protein. The *M. methylotrophus* ORF3 sequence is the uppermost strand.

1 MTSNRRGFMKALVGAMMAAGLISCHAPADYPTAKVTGAVTDSTV 50
1 ..........................................................MGSHQERP 8

51 KGILHSATGTAIEGQAIEQINAMGIGKBRRIIIGQEDGAS 100
9 LIGLLFSETGTVADSHAYGALLAVEQNLREGVORPIETLSQDPGG 58

101 DWPTFAEKEZIAN.RTKWLQACKL.................................................. 125

59 DFDYRLCAEDFIRNVRVFLVCYSHTRKAVMPVERADALLCYTPY 108
unequivocally after nt 3138, which has a subtle bearing on the observed relationship between the ORF3 protein sequence and that of AmiC. If the purine (P) at nt 3139 is changed from an 'A' residue (which is inferred from the sequence, as shown) to the alternative 'G' residue then this causes the substitution of a single G for an E residue in the amino acid sequence, but does not alter the reading frame. However, by removing one or other, or both, of the 'g' residues which constitute possible sequence errors, then a 'frameshift mutation' can be introduced which either generates a stop codon or alters the number and identities of the terminal amino acid sequence encoded by ORF3. It will therefore be of prime future importance to re-sequence this region of DNA and confirm its true sequence, in addition to the isolation and DNA sequencing of the missing ORF3 downstream DNA sequence.

7.2.6 Analysis of restriction sites contained within the pNW3 insert sequence

The accuracy of the restriction map of the insert DNA of pNW3 was checked (Materials & Methods) to confirm the interpretation of restriction patterns which were generated previously (Chapter 6). In general, the sequence data was in good agreement with mapping data, but a few minor omissions from the original map (Fig. 6.2) were identified and subsequently accounted for, viz. (i) a single BamHI site within the sequence of pNW3(23) which was not originally identified (subsequent digestion of plasmids pNW3 and pNW323 with BamHI confirmed the presence of this site and yielded restriction fragments of the correct size as predicted from the sequence data); (ii) a pNW323 insert HincII site very close to the vector HincI site, thus the apparent linearization of plasmid pNW323 which was originally reported with this enzyme resulted from the cleavage of the recombinant plasmid at both of these sites (rather than at the vector site only as was initially concluded), which resulted in the formation of a small restriction fragment (approximately 100bp), the presence of which was not determined during gel electrophoresis; and (iii) a third putative AccI site within the pNW3 sequence was confirmed.

7.3 Discussion

The DNA sequence of fmd and its upstream (ORF1) and downstream (ORF2, ORF3) flanking regions was determined. Since the orientation of fmd within the pNW3 insert sequence was shown to be opposite to the direction of transcription from the vector lacZ promoter, this observation strongly suggested that fmd was expressed from its own promoter rather than from lacZ. It therefore followed from this that an M. methylotrophus promoter was likely to exist in the pNW3(23) sequence upstream from the fmd initiation codon and that this promoter must have been functional in the heterologous host. This notion was further substantiated by the finding that E. coli JM109::pNW323 expressed good formamidase activity (Chapter 6), even though the majority of ORF1 (which is encoded by the insert DNA of pNW310 and which is situated 5' to fmd) was absent from this clone. If fmd expression
was being directed from a vector sequence upstream from the pNW310 sequence (and fmd) then it would have been expected that the removal of this sequence during the construction of pNW323 would have brought the 5' end of fmd closer to the putative vector promoter which would probably have increased the level of fmd expression, but this was not observed. The maintenance of good formamidase activity by the sub-cloning procedure therefore intimated that a functional \textit{M. methylotrophus} promoter existed somewhere between the \textit{HindIII} site located approximately at nt 865 and the \textit{fmd} initiation codon at nt 1184. This region has been postulated to contain putative '-10' and '-35' \textit{E. coli} consensus promoter sequences (see above) which may be responsible for directing \textit{fmd} expression from pNW3(23). This hypothesis could be further investigated by primer extension transcript mapping to locate the transcription start point upstream from \textit{fmd}.

The third position codon bias towards G or C which was identified in the \textit{M. methylotrophus} fmd, ORF1, ORF2 and ORF3 sequences may represent an analogous situation to that described for the \textit{amiE} sequence of \textit{Ps. aeruginosa} (Brammar \textit{et al.}, 1987) in which the biased codon usage was taken to be indicative of genes that were expressible at high rates. Formamidase is expressed at high rates and may also constitute up to approximately 4\% of the total cell protein when fully derepressed in the wild-type organism. However, since the \textit{M. methylotrophus} codon bias was not as pronounced as that of \textit{Ps. aeruginosa}, the codon usage pattern of further \textit{M. methylotrophus} genes would need to be elucidated to clarify this point.

The striking homology between the \textit{M. smegmatis} 'acetamidase' and \textit{M. methylotrophus} formamidase structural gene sequences and derived amino acid sequences intimated that the mycobacterial enzyme is really a formamidase. In view of the negligible similarity between these two amidases and members of the two different amidase genotypic groups (see Introduction) which contain respectively the acylamidases of \textit{Ps. aeruginosa} and \textit{Brevibacterium} sp. R312 and the enantiomer-selective amidases of \textit{Rhodococcus} sp. and \textit{Brevibacterium} etc., it seems highly likely that the formamidases constitute a third discrete amidase group. In addition, the expression of both a formamidase and an acetamidase (exhibiting high N-terminal homology to the acylamidases of \textit{Ps. aeruginosa} and \textit{Brevibacterium} sp. R312) by \textit{M. methylotrophus} would therefore seem to provide an answer to the query of Mayaux \textit{et al.} (1991) who pondered whether a single organism might express amidases from different groups. Moreover the strong homology demonstrated between the \textit{M. methylotrophus} formamidase and the mycobacterial 'acetamidase' is a directly-analogous situation to that of the \textit{Ps. aeruginosa} and \textit{Brevibacterium} sp. R312 \textit{amiE} relationship which was considered by Soubrier \textit{et al.} (1992). These workers pointed out that the latter two bacterial species are respectively Gram-negative and Gram-positive organisms, and speculated that the exceptional homology between the \textit{amiE} sequences of these organisms was probably the result of a horizontal gene transfer event. Furthermore, the enantiomer-selective amidases of \textit{Brevibacterium} sp. R312 and \textit{Rhodococcus} sp. (both Gram-positive) and the indole-3-acetamide hydrolases of \textit{Ps. savastanoi} and \textit{Agrobacterium tumefaciens} (both Gram-negative) also exhibit the same relationship. By the same reasoning, it therefore seems possible that the
observed similarity between the formamidase of *M. methylotrophus* (Gram-negative) and the 'acetamidase' of *M. smegmatis* (Gram-positive) may also have a similar genetic basis. Thus, the significant differences in the DNA sequences of the three putative amidase groups appears to suggest that either (i) a common progenitor amidase structural gene existed before the evolutionary divergence of Gram-positive and Gram-negative bacteria and that the observed contemporary DNA sequence differences have resulted from divergent evolution, or (ii) a horizontal gene transfer event occurred well after the evolutionary divergence of Gram-positive and Gram-negative bacteria. The latter hypothesis was favoured by Soubrier et al. (1992) in view of the fact that gene sequences known to be strongly conserved throughout evolution (e.g. glyceraldehyde-3-phosphate dehydrogenase) only exhibited approximately 60% homology between Gram-positive and Gram-negative organisms. At least 81% strict identity demonstrated between *P. aeruginosa* and *Brevibacterium sp.* R312 amiE sequences. The experimental confirmation that horizontal gene transfer could occur between Gram-positive and Gram-negative organisms also lent further support to the idea that this mechanism was responsible for the observed homology.

The comparison of the *M. methylotrophus* ORFl, ORF2 and ORF3 DNA and derived amino acid sequences with database sequences yielded some interesting results. The ORFl amino acid sequence showed reasonable homology with the mycobacterial ORFP3 gene product, but since the MW of the ORFl gene product (>40,475Da) is probably at least twice that of the ORFP3 gene product (approximately 22,000Da) then this may be a chance similarity. ORFl did not show any other striking similarities to sequences published previously and therefore appears to constitute a unique sequence.

The protein encoded by ORF2 appears to be a DNA-binding protein and it shows quite a strong similarity to the mycobacterial ORFP2 gene product. Since both of these ORFs are located in close proximity to the amidase structural gene it is likely that these proteins are somehow involved with the regulation of its expression in each case. These proteins may either exhibit an analogous regulatory function to AmiR, and act as positive regulators of *fm* expression, or alternatively they may function as negative regulators of gene expression in a manner akin to the *lacI* repressor. The presence of ORF3 just downstream from ORF2, and the relatively high homology of the ORF3 protein with AmiC may suggest that transcription antitermination is also involved in the regulation of *fm* activity. This process could be mediated by the interaction of the ORF3 protein with the ORF2 protein (essentially as for the AmiCR system) or perhaps the *M. methylotrophus* system in vivo involves the interaction of the ORF3 protein with a protein more closely related to AmiR, but whose structural gene sequence is not contained by pNW3. Alternatively, in view of its two CxxC motifs and the presence of this motif in a number of proteins not necessarily involved with DNA-binding (see section 7.2.4), it is tempting to speculate that the ORF2 protein may function as a protein disulphide oxidoreductase (see Loferer & Hennecke, 1994). These proteins are able to effect the reversible oxidation and reduction of Cys residues in various protein substrates. In general, amidases appear to be sensitive to sulphhydryl reagents which demonstrates the importance of thiol groups to the maintenance of amidase activity (although no data are
currently available concerning the effect of thiol reagents on the *M. methylotrophus* formamidase). It is not entirely implausible to suggest, therefore, that the activity of formamidase might be regulated at the protein level by conformational changes induced by the reversible oxido-reduction of Cys mediated by the ORF2 gene product. However, it must be stated that on the strength of evidence from database sequence comparisons the DNA-binding function of this protein would appear to define the more probable biological role of this protein.

The *M. methylotrophus* ORF3 gene product exhibited significant homology with AmiC, as stated above, and the presence of *amiC* within the putative *fmd* operon suggested that transcription antitermination may be a feature of *fmd* regulation by analogy with the *amiE* operon in *Ps. aeruginosa*. However, as yet there is little experimental data available concerning the regulation of formamidase activity in either *M. methylotrophus* or *E. coli* JM109::pNW3. It is known from physiological studies using continuous culture that the *M. methylotrophus* formamidase is induced by acetamide and formamide, and repressed by ammonia, that the heterologously-expressed formamidase activity of *E. coli* JM109::pNW3 grown in batch culture appears to be subject to down-regulation in response to high ammonia concentrations. However, the sensitivity of the latter system to ammonia appears to have been diminished in comparison to that of the intact native system as recombinant formamidase activity is not fully repressed by very high supernatant ammonia concentrations, *i.e.* > 15mM (cf. native wild-type formamidase repressed by ammonia concentrations approximately > 0.1mM; J. Mills & C.W. Jones, unpublished). Essentially nothing is known about the regulatory elements and modes involved with the regulation of formamidase activity, or whether formamidase is subject to wider control, particularly in response to the global nitrogen control system (*ntr*) which has been implicated in the control of *amiC* (and hence *amiE*) expression in *Ps. aeruginosa* (see Drew & Wilson, 1992). Thus, despite gaining some knowledge of the possible control elements involved with the regulation of formamidase activity from superficial studies of native and recombinant formamidase systems, no firm conclusions concerning the regulation of formamidase activity can be drawn. The fact that the putative *fmd* operon DNA sequence which is presented in this Chapter is incomplete also essentially precludes meaningful conclusions from being drawn from the batch studies of formamidase activity in *E. coli* JM109::pNW3 in the absence of further work (see Chapter 8). However, it is possible that the apparently altered response of formamidase to repression by ammonia may have resulted from a functionally-perturbed or inactive sensor-regulator system resulting from the truncation of putative regulatory sequences (possibly including ORF1, but more likely to result from the truncation of ORF3).

Comparison of the genetic organization and constitution of the putative *fmd* operon encoded by pNW3 suggested that it may be similar, but not identical to either the *amiE* operon of *Ps. aeruginosa*, or the putative 'acetamidase' operon of *M. smegmatis*. The pseudomonal *amiBS* genes appeared to be absent from the *fmd* operon, unless either of these genes equates with the *M. methylotrophus* ORF1 sequence, or ORF2 represents an evolutionary divergent (and significantly compacted) version of *amiB*. In addition, *amiR*
appeared to be missing from the pNW3 fragment although it may exist downstream from ORF3 and therefore have been removed during chromosomal restriction (as might also be true for amiBS). The arrangement of the mycobacterial operon also appeared to exhibit significant differences to either the M. methylotrophus or the Ps. aeruginosa systems, as it contained three relatively short ORFs all of which were located upstream in relation to the position of the 'acetamidase' structural gene. However, as for the \textit{fmd} operon, further sequences which are likely to be involved with amide catabolism and which are probably located downstream from the amidase structural gene are likely to be missing from this sequence. The isolation and DNA sequencing of flanking regions to both the mycobacterial and \textit{M. methylotrophus} amidase-containing sequences will presumably clarify the relationship between the genetic constitution and organization of these tentatively ascribed operons with respect to that of \textit{Ps. aeruginosa}.

The absence of protein bands on SDS-PAGE gels (not shown), at positions commensurate with the predicted MWs of the ORF1, ORF2 and ORF3 gene products either suggests that these products were expressed at a very low level (or not at all) or simply that these proteins were unstable in \textit{E. coli}. Low-level expression of the ORF2 and ORF3 gene products would be consistent with their putative regulatory functions, particularly so in the case of ORF3 as there exists a relatively long intergenic region between the 3' end of ORF2 and the ORF3 initiation codon. The apparent absence of good promoter sequences in this region coupled with its length may provide considerable scope for transcription to be terminated in this region, thereby ensuring only low-level expression of the ORF3 gene product. However, the putative low-level of expression of the ORF2 gene product is more difficult to explain, as there is only a short intergenic region between the stop codon of the \textit{fmd} sequence and the ORF2 initiation codon. Thus, without postulating the presence of a stemloop terminator structure in this region and/or degradation of the ORF2 protein, a mechanism accounting for the apparent down-regulation of ORF2 expression is lacking. Further work is therefore required to clarify this point. Finally, the ORF1 gene product was deemed unlikely to have been transcribed in any case, due to its lack of an upstream ribosome binding site/promoter sequences and its location upstream from the putative \textit{fmd} promoter, which was presumably responsible for driving the expression of ORF2 and ORF3, albeit at a low level only.
CHAPTER 8

SUMMARY AND DISCUSSION
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SUMMARY AND DISCUSSION

8.1 Summary of conclusions from biochemical work

The data from studies of the physico-chemical attributes of high- and low-activity acetamidases in vitro and the heat-reactivation phenomenon strongly suggest that the regulation of acetamidase activity is unlikely to exhibit an exclusively covalent basis. All of the available evidence suggests that acetamidase does not exist in discrete switched-on and switched-off states, but rather its activity is likely to be modulated over a wide and continuous range. Since pure high-activity acetamidase can apparently spontaneously re-convert to the low-activity form (which is the most robust and physically stable activity state of the enzyme) it is suggested that this re-conversion probably results from the slow dissociation of an allosteric ligand from acetamidase whose function is to maintain the high-activity state. It therefore follows that the switch-off process is presumably characterized by a conformational change. Acetamidase activity can be transiently heat-reactivated, i.e. switched-on, in the presence of an activator component, which suggests that this process reverses the conformational change which occurs during switch-off. In the absence of kinetic studies defining and confirming a mathematical model for the regulation of acetamidase activity the key observation which militates against the covalent regulatory model is the lability of the high-activity state. It is therefore concluded that the simplest interpretation of experimental results is that acetamidase activity is primarily subject to allosteric regulation.

8.2 Physico-chemical analysis of acetamidase

The ability to reproducibly isolate pure enzyme samples from either extreme of the acetamidase activity spectrum is of prime importance to further investigations of the nature of the putative acetamidase 'modification' and its role in the reversible regulation of acetamidase activity. The magnitude of any physico-chemical differences between high- and low-activity acetamidases would presumably be maximized in such samples, thereby making these differences easier to detect. Thus, an ideal starting point from which to elucidate the intrinsic nature of the particular physico-chemical states that define the in vitro properties of switched-on/high-activity acetamidase and switched-off/low-activity acetamidase, would require the purification of acetamidase samples exhibiting essentially wholly switched-on (i.e. very high) and switched-off (i.e. very low) specific activities, that were also demonstrably stable to the effects of freeze-thaw and long-term storage at -20°C. The subsequent characterization of potentially important variables for each pure sample, viz. the degree of switch-off and heat-reactivation exhibited by a given high-activity sample in particular (as low-activity samples generally exhibited constant and more stable physico-chemical properties), might then allow the standardization of experiments conducted on different occasions. This could be achieved using the same acetamidase sample (exhibiting defined kinetic properties) in a series of
different experiments which would minimize experimental variations arising from the use of different acetamidase samples exhibiting potentially variable kinetic properties. The 'luxury' of being able to use such well-defined and stable samples was generally unavailable throughout the course of the physico-chemical work described previously (Chapter 3) due to the inexplicable inability to isolate reproducibly a pure high-activity enzyme with stable kinetic properties. All attempts to achieve this were thwarted by the highly-variable switch-off of acetamidase samples purified from cells of *M. methylotrophus* which originally exhibited genuinely switched-on whole cell specific acetamidase activities. Switch-off was observed in both partially-pure and pure acetamidase preparations. Thus, the physico-chemical comparisons undertaken in this study used high-activity preparations which exhibited unavoidably and potentially diverse levels of switch-off in conjunction with (good) low-activity samples which exhibited essentially constant properties. The interpretation of results from experiments conducted at different times throughout this research was therefore complicated by the non-uniform properties of different high-activity acetamidase samples.

Improvements to this type of work, (i.e. being able to conduct more-controlled and better-defined experiments) would necessitate a complete characterization of the switch-off event, both in vivo and in vitro. It is therefore suggested that a systematic and detailed study of the conditions that promote switch-off should be undertaken to define this process prior to further investigation of the physico-chemical attributes of high- and low-activity enzymic forms, so that this problem can be prevented or avoided. Whether or not the latter is theoretically possible depends largely on the nature of the switch-off event, as it is envisaged that a covalent enzyme alteration would probably be an easier event to define and perhaps prevent, whereas the involvement of allostery (which is deemed likely) with its potential for multiple control elements would complicate matters considerably.

The study of the physiological regulation of switch-off in vivo is ongoing in this laboratory and will not be discussed here, but possible areas of investigation that might clarify the conditions 'triggering' switch-off in vitro include: i) an investigation of the relationship between ionic strength and buffer composition and their relative influences on the degree of switch-off, as removal of salt and buffer exchange (diafiltration) appeared to alter the level of switch-off; (ii) characterization of multiple purifications of acetamidase from high-activity whole cells cultured under different nutrient limitations in order to highlight any trends concerning the effects of the latter on the stability of the high-activity state; (iii) a study of the long term effects of storage at -20°C on the kinetic properties of the pure high-activity enzyme, as pure high-activity enzymes appeared to switch-off spontaneously during switch-off or possibly as a result of multiple freeze-thaw cycles; (iv) an investigation of the ability of compounds (such as DTT and β-mercaptoethanol) which are used to stabilize pure enzyme samples to prevent switch-off of high-activity acetamidases once purified; and, (v) experiments designed with appropriate controls to determine whether switch-off occurs solely in a spontaneous fashion or whether it can be accelerated by various physico-chemical treatments.
Other work that might contribute to the elucidation of the switch-off mechanism could involve: (i) a full characterization of the kinetic and heat-reactivation properties of multiple pure intermediate-activity forms of acetamidase (i.e. 'high' activity acetamidases exhibiting various degrees of switch-off, which would presumably be generated during the optimization of the purification protocol to prevent switch-off) to confirm, or otherwise, the switch-off hypothesis (see Discussion, Chapter 3); (ii) biophysical studies of high- and low-activity acetamidases potentially involving techniques such as circular dichroism and differential scanning calorimetry to investigate putative conformational differences, in addition to crystallography and NMR studies of acetamidase in the presence/absence of amide substrates and potential allosteric ligands (e.g. ammonia, acetate and acetyl CoA); (iii) spectrofluorimetric studies of surface hydrophobicity using fluorescent probes; (iv) an investigation of the direct effect of the incubation of high-activity acetamidase with various potential allosteric ligands to determine their effect on the level of switch-off (in view of the fact that ammonia appeared to destabilize high-activity acetamidase selectively in vitro); (v) optimization of ESMS/MALDI-TOF MW determinations for high- and low-activity acetamidases (current results suggest that a MW difference of ≤ 52Da exists between different activity forms of acetamidase, but it is possible that this reflects only the maximum sensitivity that has been achieved with ESMS thus far, and this value may therefore represent an approximation only to the true [lower] mass difference); and (vi) an investigation into the nature of switch-off within a population of acetamidase molecules using ESMS potentially in coupling with capillary electrophoresis, to determine if, for example, a 90% switched-on preparation is a homogeneous system in which all molecules equally exhibit 10% switch-off, or whether the system exhibits molecular heterogeneity in which the observed 10% switch-off is an average value for the overall population derived from individual molecules exhibiting greater or lesser degrees of switch-off (unfortunately, the potential success of this work would depend on the stability of the acetamidase modification to these techniques, as is also true for ESMS MW determinations).

Finally, in view of the possible mutational basis of the production of high-activity acetamidase samples that rapidly switched-off in vitro, a thorough investigation of the genetic and molecular biological properties of mutant M. methylotrophus strains (e.g. MM6, MM8 & MM15; see Chapters 1 & 3) producing acetamidases with altered physico-chemical properties could be undertaken. The identification of acetamidase structural and regulatory mutations influencing switch-off would provide a means of relating phenotypic changes at the protein level to genotypic changes at the DNA level (as has been demonstrated for the Ps. aeruginosa amidase). The discovery of mutations affecting switch-off which may be located at loci distinct from the putative acetamidase operon may also indicate the involvement of other gene products (e.g. putative enzyme-cascade converter enzymes; Chapter 1) in the reversible regulation of acetamidase activity. These studies, in conjunction with a physico-chemical investigation of mutant acetamidases conducted exactly as for the wild-type enzymes described above, would allow the results from mutant and wild-type enzyme systems to be compared and the underlying reasons for any differences to be inferred.
8.3 Studies concerning heat-reactivation of low-activity acetamidase

Heat-reactivation of acetamidase activity was shown to be a very complex and ill-defined process. It is likely that this area of work could potentially yield greater insight into the regulation of acetamidase activity only if the multitude of variables influencing this system (see Chapter 4) could be rigorously-defined, so that heat-reactivation experiments could be standardized which would greatly clarify the interpretation of results.

Work that could contribute to the achievement of this goal might involve the generation of a quantitative purification protocol for the activator component so that it can be isolated and subjected to a battery of biochemical tests in order to identify its properties, and to elucidate its role in the heat-reactivation of acetamidase activity using better-defined mixtures of pure acetamidase and activator component only. Identification of the N-terminal amino acid sequence of the activator component (assuming that it is a protein or at least part of a protein) would allow an oligonucleotide probe to be synthesized with which the gene encoding this protein could be isolated and cloned, with the eventual aim of determining its DNA sequence and over-expressing the activator component to increase its availability for physico-chemical studies. It is hoped that the likely biological function of the activator component could be inferred from homology comparisons to proteins of known function and/or from amino acid motif searches.

Further studies involving the purified activator component might attempt to characterize putative physical interactions between acetamidase and the activator component. This work might include (i) acetamidase/activator component kinetic binding studies, and (ii) attempts to demonstrate acetamidase/activator component complexes, for example, by detecting an alteration in the rate of acetamidase sedimentation during ultracentrifugation in the presence of the activator component or by X-ray diffraction studies involving crystallization of the putative acetamidase/activator component complex. Work of this nature might also clarify the stoichiometric versus catalytic argument concerning the basis of heat-reactivation. Furthermore, confirmation of the apparently spontaneously-reversible nature of the heat-reactivation of acetamidase activity in vitro could be sought, which might lend support to the notion that the activator component and acetamidase interact both transiently and non-covalently during reactivation.

A comparison of the properties (e.g. MW determined by ESMS, hydrolase substrate profile, pI etc.) of heat-reactivated high-activity acetamidase to those of high-activity acetamidase purified from switched-on whole cells might also prove interesting, and help to determine whether these two functionally analogous enzyme activity states are physico-chemically identical or not. An extension of studies concerning the effects of potential allosteric ligands on acetamidase activity could also be achieved by conducting a kinetic study of the heat-reactivation or autoreactivation of acetamidase activity, in order to elucidate kinetic evidence which would confirm the putative allosteric and/or hysteretic properties of acetamidase. The effects on reactivation (spontaneous or heat-induced) of potential allosteric ligands could also be investigated by adding various candidate effectors (e.g. cations, acetate,
ammonia, ATP) to desalted acetamidase/partially-pure activator fractions, and/or MW size-
fractionated partially-pure activator fractions, and then incubating these with untreated low-
activity acetamidase with or without heat, prior to measuring the specific hydrolase activities
of these samples.

Since all cell-extracts prepared from cells of *M. methylotrophus* grown under diverse
nutrient limitations apparently contained the activator component it would be interesting to
prepare a crude cell extract from an unrelated ami' bacterial species and test its ability to effect
heat-reactivation of acetamidase activity. This might provide further evidence to suggest that
this process is either artefactual or that it is mediated by a common intracellular non-
proteinaceous metabolite (e.g. acetyl CoA), as it would be expected that no reactivation would
occur in this instance, due to the absence of the *M. methylotrophus* regulatory products which
have presumably specifically evolved in this organism to regulate acetamidase activity.

Finally, a genetic approach could also be employed to investigate the regulation of
acetamidase activity by attempting to isolate and characterize spontaneous or chemically-
induced *M. methylotrophus* mutant strains which are unable to switch-on and/or switch-off
acetamidase activity.

8.4 Molecular biology of acetamidase and formamidase

Further attempts to clone the acetamidase structural gene could be based on the use of
better, *i.e.* less-degenerated and longer (e.g. 40-mer) oligonucleotide probes. These could be
designed to be complementary to internal acetamidase amino acid sequences identified by the
N-terminal sequencing of peptide fragments generated after the proteolytic digestion of
acetamidase. Alternatively, a probe could be designed to certain of the strongly conserved
sequences common to the acylamidases of both *Ps. aeruginosa* and *Brevibacterium* sp. R312,
since *M. methylotrophus* exhibits very high N-terminal homology with the analogous *Ps.
aeruginosa* and *Brevibacterium* sp. R312 sequences, and this homology can be reasonably
expected to extend to the rest of the amino acid sequence. This would hopefully minimize
non-specific hybridization signals during Southern analysis of chromosomal DNA and allow
the unambiguous identification of a DNA fragment containing amiE. Moreover a highly-
specific probe could accelerate analysis of putative amiE clones by allowing the use of colony
hybridization to screen transformants, which would also increase the number of potential
clones that could be screened per unit time.

Direct selection of amiE clones could also be attempted using *Sau3AI* gene libraries
constructed using alternative cloning vectors which allow the insertion of larger DNA
fragments into the vector (e.g. λ-replacement vectors or cosmids). The cloning of relatively
large DNA fragments (as was used to clone the *Ps. aeruginosa* amidase; Chapter 5) would
increase the chance of subsequently expressing acetamidase in the heterologous host due to
the likely co-cloning of cis-acting regulatory elements involved with the regulation of
acetamidase activity in the native system. The likelihood of expressing acetamidase could also
be increased by inserting DNA fragments into an expression vector under the control of a
strong promoter and/or by using restricted \textit{M. methylotrophus} chromosomal insert DNA isolated from constitutive strains expressing high-levels of acetamidase (e.g. MM8). Furthermore, improvements could also be introduced by defining selective media on which the \textit{E. coli} host is unable to grow and by using an \textit{E. coli} penicillin amidase-mutant host for direct selection work to eliminate the possibility that false-positive colonies could grow as a result of the putative amidohydrolase activity of the latter enzyme. Alternatively, insertion of DNA fragments into a broad-host range vector and its subsequent mobilization into an \textit{ami}\textsuperscript{i} host other than \textit{E. coli} (e.g. an amidase-negative \textit{Ps. aeruginosa} strain or ideally a phenotypically-identical \textit{M. methylotrophus} strain; however, the latter would be very difficult to generate in view of its multiple amidases [see Chapter 1]) might alleviate potential expression problems which could result in the translation of an inactive acetamidase (however, this would probably be unnecessary as the \textit{M. methylotrophus} \textit{fmd} promoter appeared to function efficiently in \textit{E. coli} suggesting that the same would probably be true for the acetamidase promoter). DNA sequence data derived from the molecular cloning of \textit{amiE} from \textit{M. methylotrophus} could then be added to that determined previously for other bacterial amidases, and formamidase (see Chapter 7), to confirm and extend the knowledge of evolutionary aspects of amidase activity in prokaryotes.

The successful cloning, heterologous expression and DNA sequencing of the \textit{M. methylotrophus} formamidase structural gene also identified three ORFs whose identities require further clarification. Further molecular biological studies could be directed at the isolation and elucidation of the full ORF1 DNA sequence in order to determine its complete amino acid sequence, using an oligonucleotide probe designed to a non-degenerated ORF1 sequence. Identical work could also be undertaken to effect the same for ORF3. A repeat database search could then be undertaken to confirm, or otherwise, that ORF1 encodes a unique gene product, and that the ORF3 protein is most similar to AmiC. It would also be interesting to extend DNA sequencing from the pNW3 insert fragment in either direction, and to 'walk' along the \textit{M. methylotrophus} chromosome in an attempt to confirm the likely presence of accessory \textit{fmd} operon elements (e.g. sequences potentially equivalent to \textit{amiR} or \textit{amiS}).

It would also be of particular interest to investigate the biological function of the ORF2 gene product and its relationship to formamidase. The presence of the two CxxC motifs within its primary sequence suggests that the ORF2 protein may be related to certain eukaryotic DNA-binding proteins, and database searches showed that this protein was probably a zinc finger-type DNA-binding protein. However, this motif is also characteristic of thioredoxins (and related proteins), cytochromes and some Fe-S proteins. Thus, initial studies concerning the ORF2 protein would most-usefully be directed at the over-expression of its structural gene under the control of a strong promoter (e.g. \textit{ptac}), in order to purify significant quantities of this protein for subsequent analysis. The possibility that ORF2 encodes a DNA-binding protein could then be tested further by gel-retardation assays and its binding site elucidated by DNA footprint analysis. Crystallization and X-ray diffraction studies might also confirm the putative zinc finger structure of this protein.
As for ORF2, the over-expression and purification of the complete ORF3 gene product would be of immediate importance to future work. In view of its homology with AmiC the amide-binding capacity of the ORF3 protein could be investigated using equilibrium dialysis, and X-ray diffraction studies of the crystallized protein could be undertaken. An investigation of the putative formation of stoichiometric ORF2/ORF3 protein complexes, and their possible role in fryd regulation (as for AmiCR associations) might also prove fruitful. Further analysis of the pNW3 insert fragment could involve the isolation and insertion of the putative fryd promoter sequences into a promoter probe vector in order to characterize the attributes of the latter. Information gained from this study has potential applications in the development of a broad host range shuttle vector facilitating the cloning of DNA sequences in both E. coli-based heterologous expression systems and methylotrophic systems.

After the complete DNA sequence of the putative fryd operon has been determined, an investigation of the regulation of formamidase expression could be undertaken. Formamidase is induced by formamide and acetamide and repressed by ammonia, and it would be interesting to determine the precise molecular interactions governing these processes. Presumably, induction/repression of fryd is mediated by a proteinaceous sensor-regulator partnership, as has been essentially proven for the regulation of the Ps. aeruginosa amidase (see Drew & Wilson, 1992), however, it would also be of great interest to determine if fryd was under positive control (as for amiE in Ps. aeruginosa) or negative control (as for lacZ in E. coli). It would therefore be of prime importance to elucidate the precise molecular interactions (if any) between the ORF1, ORF2 and ORF3 gene products. Hopefully, studies of this nature will eventually lead to the complete characterization of the putative fryd operon and ultimately to an understanding of the regulation of formamidase activity at the genetic level.

Sequencing studies could be complemented by studies of the regulation of fryd expression in E. coli JM109::pNW3 and ::pNW323, grown under different nutrient limitations in continuous or fed-batch culture. Results from these studies could be cross-referenced to determine any differences in formamidase activity resulting from the absence of the majority of ORF1 from pNW323. This system could also be used to test sequence data predictions concerning the likely function of each ORF gene product, and their putative responses to the nutrient environment.

Finally, it would be advantageous to be able to confirm the predicted formamidase MW from the fryd gene sequence. This could be achieved either by ESMS to determine the MW of formamidase directly, or by identification of the C-terminal amino acid of formamidase using carboxypeptidase Y, to ensure that it is the same as that predicted from the DNA sequence (Ser).

8.5 Closing remarks

This work has provided a fuller biochemical picture of amidase activity in M. methylotrophus and contributed to the broadening of knowledge concerning amidase
properties in general. More specifically, the DNA sequence of the *M. methylotrophus* formamidase structural gene and its flanking regions have been determined which will undoubtedly provide further insight into the evolutionary relationships of amidases and their regulatory properties. Furthermore, a detailed characterization of the nature of the reversible regulation of acetamidase activity *in vitro* has been produced. However, the validity of the conclusions from work *in vitro*, with respect to the regulation of acetamidase activity *in vivo* remains to be seen. Consequently, a considerable body of work concerning this interesting and intellectually-challenging conundrum still demands attention, and it is suggested that the most fruitful experimental pathway will involve an interdisciplinary approach, utilizing a wide range of biochemical, molecular biological and biophysical techniques.
REFERENCES


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