PHYSIOLOGY AND BIOCHEMISTRY OF AMIDASES FROM
METHYLOPHILUS METHYLOTROPHUS

James Mills

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ABSTRACT

PHYSIOLOGY AND BIOCHEMISTRY OF AMIDASES FROM METHYLOPHILUS METHYLOTROPHUS

James Mills

Physiological regulation of acetamidase, formamidase and urease expression in M. methylotrophus was investigated using continuous culture under various nutrient limitations. Acetamidase was maximally induced by acetamide (acetamide > formamide > urea), formamidase by urea (urea > formamide > acetamide) and urease by urea and formamidase (urea/formamide > acetamide). All three enzymes were repressed by ammonia.

The ability of acetamidase to undergo a rapid, heat-reversible loss of activity (switch-off) was extensively investigated. No switch-off was observed during growth under any steady-state continuous culture growth regime. However, significant switch-off (a decrease in $k_{\text{cat}}$ from $147 \text{ s}^{-1}$ to $66 \text{ s}^{-1}$) was observed following the addition of ammonia pulses to dual acetamide/methanol-limited cultures. Similar decreases in $k_{\text{cat}}$ were also seen when the growth regime was transiently changed from dual acetamide/methanol limitation to dual ammonia/methanol limitation, while maintaining a constant nitrogen input. Switched-off acetamidase was purified from an ammonia-pulsed culture and found to remain in the low-activity form during purification. Increased switch-off was observed during purification and also in response to various in vitro manipulations. Experiments carried out with non-growing cultures in an attempt to further investigate the biochemistry of the switch-off event were inconclusive. Formamidase also appeared to undergo putative switch-off in response to similar physiological signals as acetamidase.

Prolonged growth of wild-type M. methylotrophus in formamide-limited continuous culture led to the selection of a mutant strain (MM25). This strain produced formamidase at the wild-type concentration but with a significantly increased specific activity. Purification of the enzyme showed that this was due to an increased $k_{\text{cat}}$. MM25 also exhibited an increased rate of formate oxidation and over-expressed two proteins with subunit $M_r$ of approximately 40,000, and another with a subunit $M_r$ of approximately 32,000. The $M_r$ 40,000 proteins were identified as the products of the fmdC and fmdD genes. FmdD was purified from MM25 and shown to be a monomeric urea/formamide-binding protein (U/F-BP) with an $M_r$ of 41,000 (41,870), which bound urea stoichiometrically with a $K_D$ of 7.15μM. U/F-BP was found to belong to a structurally related family of amino acid- and amide-binding proteins.

Microbial amidases were classified into three groups using specific antibodies raised against M. methylotrophus acetamidase and formamidase.
ACKNOWLEDGEMENTS

I would firstly like to sincerely thank my supervisor during this work Dr. Colin Jones, without whom the satisfactory completion of the research described in this thesis would not have been possible. During my work at Leicester Colin has been a constant source of sound advice, support and enthusiasm, even at times when things were going horribly wrong and it appeared that we had discovered a new discipline of "bioalchemy."

I would also like to thank the members of lab. 109 during the past three years, especially my "partner" on the amidase project Dr. Neil (DP) Wyborn for useful discussions, not only on the weird science of acetamidase switch-off, but also philosophical discussions on life, the universe and bourbon; Dr. Steve (DS) Williams for practical hints and helping this simple microbial physiologist come to terms with some molecular biology; Mrs. Jacquie (CT) Greenwood for invaluable technical advice and assistance; and finally Mr. Robin (GYHC) Henderson for being generally Terry Christian like.

Many thanks are also extended to the members of my postgraduate committee, Professor W. J. Brammar and Dr. Mark Carver, for their additional guidance and input during this project. I must also thank the past and present members of the Department of Biochemistry, including Dr. Steve (CL) Cairns, Dr. Kath Lilly and Miss Liz Cavanagh, and many others far too numerous to mention by name, but you know who you are!

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I would finally like to thank my family, especially Mum and Dad, for their continued support during yet another three/four years of me being an "eternal student," the members of Westleigh RFC for giving me a vent for my frustrations and Jo for her love and understanding over the last twelve months.

The Science and Engineering Research Council and ZENECA Bio Products are gratefully acknowledged for financial support of this research.

This thesis is dedicated to my teachers; Graham Causton, Ken Kenward, Nigel Allison and Colin Jones.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>6-APA</td>
<td>6-aminopenicillanic acid</td>
</tr>
<tr>
<td>ATase</td>
<td>Adenyl transferase</td>
</tr>
<tr>
<td>ATCase</td>
<td>Aspartate transcarbamoylase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine triphosphate</td>
</tr>
<tr>
<td>D</td>
<td>Dilution rate (h⁻¹)</td>
</tr>
<tr>
<td>DRAG</td>
<td>Dinitrogenase reductase-activating glycohydrolase</td>
</tr>
<tr>
<td>DRAT</td>
<td>Dinitrogenase reductase ADP-ribosyl transferase</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EBG</td>
<td>Evolved β-galactosidase</td>
</tr>
<tr>
<td>EcLivJ</td>
<td>E. coli leucine-isoleucine-valine-binding protein</td>
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<tr>
<td>EDTA</td>
<td>Ethylene-diaminetetra-acetic acid</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethylmethane sulphonate</td>
</tr>
<tr>
<td>F</td>
<td>Flow rate (ml h⁻¹)</td>
</tr>
<tr>
<td>FCCP</td>
<td>Carbonyl cyanide p-trifluoro methoxyphenyl hydrazone</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>GBP</td>
<td>Glucose-binding protein</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>GOGAT</td>
<td>Glutamate synthase (glutamine amide-2-oxoglutarate aminotransferase)</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>HAS</td>
<td>N-(3-oxohexanoyl) homoserine lactone</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>KDO</td>
<td>Keto-3-deoxyheptonic acid</td>
</tr>
<tr>
<td>KDPG</td>
<td>2-Keto 3-deoxy 6-phosphogluconate</td>
</tr>
<tr>
<td>Kₛ</td>
<td>Saturation constant (mM)</td>
</tr>
<tr>
<td>LAO</td>
<td>lysine-arginine-ornithine-binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LBP</td>
<td>Lactose-binding protein</td>
</tr>
<tr>
<td>LIV-BP</td>
<td>Leucine-isoleucine-valine-binding protein</td>
</tr>
<tr>
<td>LIVT-BP</td>
<td>Leucine-isoleucine-valine-threonine-binding protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>Mdh</td>
<td>Methanol dehydrogenase</td>
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<tr>
<td>MES</td>
<td>2-(N-Morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMG (NTG)</td>
<td>N-methyl-N-nitro-N-nitroso-guanidine</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-Nitrophenyl galactopyranoside</td>
</tr>
<tr>
<td>orf</td>
<td>Open-reading frame</td>
</tr>
<tr>
<td>PaAmiC</td>
<td><em>P. aeruginosa</em> AmiC</td>
</tr>
<tr>
<td>PaLiv</td>
<td><em>P. aeruginosa</em> leucine-isoleucine-valine-binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</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>PTS</td>
<td>Phosphotransferase system</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidine difluoride</td>
</tr>
<tr>
<td>Rdh</td>
<td>Ribitol dehydrogenase</td>
</tr>
<tr>
<td>RubisCO</td>
<td>Ribulose 1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>RuMP</td>
<td>Ribulose monophosphate</td>
</tr>
<tr>
<td>s</td>
<td>Growth-limiting nutrient concentration (g l⁻¹)</td>
</tr>
<tr>
<td>ŝ</td>
<td>Standing growth-limiting nutrient concentration (g l⁻¹)</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEII</td>
<td>Seed II (mineral salts)</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S/F</td>
<td>Succinate/formamide</td>
</tr>
<tr>
<td>S/L</td>
<td>Succinate/lactamide</td>
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</table>
StLivC  *Salmonella typhimurium* leucine-isoleucine-valine-binding protein

TA  Transaldolase

TBS  Tris buffered saline

TEMED  *N*, *N*, *N*, *N*-tetramethyl-ethylenediamine

μ  Specific growth rate (h⁻¹)

U/F-BP  Urea/formamide-binding protein

μmax  Maximum specific growth rate (h⁻¹)

UTase  Uridylyl transferase

V  Volume (ml)

x  Biomass concentration (g l⁻¹)

Yx/s  Biomass yield (g g⁻¹)
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Science must have originated in the feeling of something being wrong.

-Carlyle
CHAPTER 1

INTRODUCTION
CHAPTER 1

INTRODUCTION

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1.1.2 The oxidation of C1-compounds by M. methylotrophus
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INTRODUCTION

1.1 The microbiology and biochemistry of Methylophilus methylotrophus

1.1.1 The methylotrophs

Methylotrophs are microorganisms which can grow at the expense of reduced C\textsubscript{1}-compounds, the latter being defined as carbon compounds containing one or more carbon atoms but no carbon-carbon bonds (Anthony, 1982). The first methylotroph, \textit{Bacillus methanicus}, was described almost 90 years ago (Sohngen, 1906; quoted in Anthony, 1982; 1986) and since its discovery numerous C\textsubscript{1}-utilising microorganisms, both prokaryote and eukaryote, have been discovered. In general methylotrophs are ubiquitous and can be isolated from soil, sewage, and river and pond water. The eukaryotic methylotrophs consist mainly of methylotrophic yeasts and will not be considered further here. Microorganisms able to use C\textsubscript{1}-compounds can be (i) obligate methylotrophs, which can only utilise C\textsubscript{1} substrates for growth; (ii) restricted facultative methylotrophs, which can also grow at the expense of a very limited range of multi-carbon compounds in addition to C\textsubscript{1} substrates; (iii) facultative methylotrophs, which can utilise a wide range of multi-carbon compounds in addition to C\textsubscript{1}-compounds. Methylotrophs can alternatively be divided into two groups based upon their ability to utilise methane as sole source of carbon and energy; those able to utilise methane are also referred to as methanotrophs, and are usually obligate methylotrophs, growing well on methane but often poorly on methanol. Those unable to use methane as a carbon and energy source utilise methanol and/or methylated amines as carbon sources (Anthony, 1982; 1986). Other C\textsubscript{1} substrates which can be utilised by such organisms (\textit{e.g. Ps. oxalaticus}, \textit{P. denitrificans} and \textit{T. novelli}) include formate and formamide which will be discussed further below.

Methanotrophs (\textit{e.g. Methyllococcus} sp., \textit{Methylomonas} sp. and \textit{Methylosinus} sp.) share common structural and biochemical characteristics such as being strictly aerobic, possessing a complex intracellular arrangement of membranes, the ability to form a differentiated resting body (exospore or cyst) and most importantly the possession of the enzyme methane mono-oxygenase, which catalyses the first step in methane utilisation.
Methanotrophs can be sub-divided into two groups dependent upon their route of carbon assimilation and arrangement of internal membranes. Group I methanotrophs utilise the ribulose monophosphate pathway of carbon fixation and have internal membranes arranged in bundles of vesicular discs. Group II methanotrophs utilise the serine pathway and have paired, peripheral internal membranes. The nitrogen sources which can be used by methanotrophs are diverse; all use ammonia, most use nitrate and nitrite, some use urea, amino acids and yeast extract. All type II methanotrophs (and some type I organisms) can also fix molecular nitrogen. The methanotrophs will not be considered in further detail in this study.

The remaining methylotrophs are those unable to grow upon methane, and represent a relatively large group of microorganisms including members of the genera *Methylophilus, Pseudomonas, Methylophilus* and *Hyphomicrobium*. This group of methylotrophs is characterised by the absence of methane mono-oxygenase (hence their inability to grow upon methane) and the other characteristics of methanotrophs described above. However, the general biochemistry (i.e. routes of carbon assimilation and dissimilation) of methylotrophs from both groups are essentially similar. For simplicity only *Methylophilus methylotrophus*, the amidases and urease of which are investigated in this study, will be considered here. *Methylophilus methylotrophus* (literal meaning methyl radical-loving, methyl radical-consuming; Jenkins et al., 1987) is a strictly aerobic, Gram-negative rod-shaped bacterium possessing a relatively-thick cell wall. Cells occur singly or in pairs and are motile by way of a single polar flagellum. The organism is able to grow rapidly and with high biomass yield with methanol as the carbon and energy source, and it was these characteristics which led to its use in the production of single-cell protein (see Vasey & Powell, 1984; Sharp, 1989). Methylated amines, glucose and, to a lesser extent, fructose can also be utilised. Due to its carbon substrate profile *M. methylotrophus* is most accurately classified as a restricted facultative methylotroph, although it is frequently referred to in the literature as an obligate methylotroph due to its very restricted range of carbon growth substrates. A thorough taxonomic study of the genus has previously been reported by Jenkins et al. (1987). The organism was originally isolated from activated sludge, and its C1-substrates are found widely in natural habitats. Methanol is produced in nature by the oxidation of methane by methane mono-oxygenase and by the hydrolysis of...
methyl ethers and esters present in pectin and lignin which are major structural components of plants. \textit{M. methylotrophus} also grows well on methylated amines, which act as both carbon and nitrogen sources (Large & Haywood, 1981; Burton \textit{et al.}, 1983). Trimethylamine is formed in nature by the microbial degradation of choline derivatives and also from trimethylamine N-oxide during the decomposition of fish. Dimethylamine is produced during the degradation of some pesticides and is a product of trimethylamine oxidation. Methylamine is a constituent of some plant material and is produced during the oxidation of all other methylated amines (Anthony, 1982). Formate can be produced from other C$_1$-compounds (see section 1.1.2) and can be a product of microbial fermentative metabolism. \textit{M. methylotrophus} is unable to utilise formate as a sole source of carbon and energy, but as it contains an NAD$^+$-linked formate dehydrogenase, formate oxidation can add to the overall energy economy of the cell (Beardsmore \textit{et al.}, 1982; Patchett \textit{et al.}, 1985; Jones \textit{et al.}, 1987; Wyborn \textit{et al.}, 1994).

1.1.2 The oxidation of C$_1$-compounds by \textit{M. methylotrophus}

The first step in the metabolism of methanol in \textit{M. methylotrophus} is the oxidation of methanol to formaldehyde. This reaction is catalysed by an NAD$^+$-independent alcohol dehydrogenase with a broad substrate specificity for primary alcohols. However, as its role \textit{in vivo} is usually the oxidation of methanol it is referred to as methanol dehydrogenase (Mdh) (Ghosh & Quayle, 1981; Anthony, 1982; 1986; 1990). The possession of this enzyme appears to be a common feature of all methane- and methanol-oxidising bacteria. Mdh is a heterotetramer of the $\alpha_2\beta_2$ conformation. The “large” (a) subunit of Mdh has an $M_r$ of approximately 60,000, and the “small” (b) subunit has an $M_r$ of approximately 8,500 (Anthony, 1990). Mdh is a periplasmic protein which is associated with the cell membrane and constitutes approximately 5-15\% of soluble cell protein, dependent upon growth conditions (Anthony, 1982; 1986; 1990; Greenwood & Jones, 1986). The enzyme \textit{in vitro} has an absolute requirement for ammonium salts or methylamine for activity, however, it is unclear whether this activation by ammonia is important \textit{in vivo}. Each molecule of Mdh has two noncovalently-bound molecules of pyrroloquinoline quinone (PQQ), which act as prosthetic groups (Anthony, 1982; 1986; Anthony \textit{et al.}, 1994), and also a single atom of
calcium which is thought to be involved in maintaining PQQ in the correct conformation in
the active site of the enzyme and may also be involved in the catalytic mechanism
(Anthony, 1993; Anthony et al., 1994). Recent crystallographic studies of Mdh from
Methylobacterium extorquens, M. methylotrophus and Methylophilus W3A1 have shown
that the small β-subunit of Mdh is wrapped around the surface of the larger α-subunit. The
three-dimensional structure of the α-subunit is a superbarrel made up of eight four-stranded
antiparallel β-sheets arranged radially like the blades of a propeller (Xia et al., 1992; White
et al., 1993; Anthony et al., 1994; Ghosh et al., 1995). The PQQ prosthetic group is buried
in the interior of the superbarrel within a chamber, representing the active site, that
communicates with the exterior via a hydrophobic funnel-shaped depression in the surface
of the molecule. The floor of the active site is formed by the plane of a tryptophan residue
and the ceiling is formed by a novel ring structure made from a disulphide bridge between
adjacent cysteine residues. The disulphide ring does not function as a redox component
during the oxidation of methanol by Mdh. It has been suggested, however, that this novel
structure might function in the stabilisation or protection of the free radical semiquinone
form of PQQ from solvent at the entrance of the active site during the reaction cycle of the
enzyme (Avezoux et al., 1995).

The oxidation of methanol by Mdh is regulated by a modifier protein (M-protein). Mdh
not only has the ability to oxidise a wide range of primary alcohols, but it can also oxidise
formaldehyde to formate in vitro (this oxidation represents a waste of carbon since formate
cannot be assimilated by M. methylotrophus, although it can be oxidised to CO₂ with the
concomitant production of NADH). It is not known whether this second oxidation is
catalysed by Mdh in vivo, but M-protein increases the affinity of Mdh for alcohols and
decreases its affinity for formaldehyde and may therefore control this wasteful oxidation of
formaldehyde to formate (Page & Anthony, 1986; Anthony, 1990; Long & Anthony,
1991). The specific electron acceptor of Mdh is the soluble cytochrome c₅₅, to which Mdh
"docks" by electrostatic interactions (Anthony et al., 1993). Cytochrome c₅₅ then interacts
(by similar electrostatic interactions) with the typical class I c-type cytochrome,
cytochrome c₅₅ which is the substrate for a membrane-bound terminal oxidase (cytochrome
aa₃ or cytochrome co dependent upon growth conditions; see Greenwood & Jones, 1986;
M. methylotrophus also contains the enzymes trimethylamine dehydrogenase, dimethylamine mono-xygenase and methylamine dehydrogenase, which collectively allow growth upon methylated amines, by catalysing the oxidation of these substrates to formaldehyde and ammonia (Large & Haywood, 1981; Burton et al., 1983; Dawson et al., 1990).

Formaldehyde produced from methanol or methylated amine oxidation can be dissimilated by a linear pathway or the dissimilatory RuMP cycle with the concomitant formation of CO$_2$ and NAD(P)H. The linear pathway involves the oxidation of formaldehyde to formate which is catalysed by either Mdh or an NAD$^+$-linked formaldehyde dehydrogenase, and the subsequent oxidation of formate to CO$_2$ (Large & Haywood, 1981; Anthony, 1982; Beardsmore et al., 1982; Jones et al., 1987). The dissimilatory RuMP cycle (Fig. 1.1) involves some of the enzymes of the KDPG aldolase/transaldolase version of the assimilatory RuMP cycle used by M. methylotrophus for formaldehyde fixation, in addition to the dissimilatory enzyme 6-phosphogluconate dehydrogenase (Anthony, 1982; Beardsmore et al., 1982). The resultant NAD(P)H from both pathways can then be fed into the respiratory chain with the concomitant production of metabolic energy.

1.1.3 Carbon assimilation by M. methylotrophus

M. methylotrophus assimilates carbon at the oxidation level of formaldehyde by way of the KDPG aldolase/transaldolase variation of the RuMP cycle (Fig. 1.2; Anthony, 1982; Beardsmore et al., 1982). The overall reaction cycle syntheseses one molecule of pyruvate (C$_3$), which can subsequently be assimilated into biomass, from three molecules of formaldehyde (C$_1$) by way of a three stage process. The first stage of the cycle is fixation; three molecules of formaldehyde (C$_1$) are condensed with three molecules of ribulose 5-phosphate (C$_5$) by hexulose 6-phosphate synthase to produce three molecules of hexulose 6-phosphate (C$_6$); the hexulose 6-phosphate is then isomerised to fructose 6-phosphate. The second stage of the cycle is cleavage; one molecule of fructose 6-phosphate is converted to 2-keto 3-deoxy 6-phosphogluconate (KDPG; C$_9$) by the Entner/Doudoroff
The enzymes of the cycle are those of the assimilatory pathway plus the dissimilatory enzyme 6-phosphogluconate dehydrogenase (reaction 23). Other enzymes are: (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:

$\text{HCHO} + 2\text{NAD}(P)^+ \rightarrow \text{CO}_2 + 2\text{NAD}(P)\text{H} + 2\text{H}^+$
Figure 1.2 The KDPG aldolase/transaldolase variant of the RuMP cycle of C-assimilation

The enzymes of the pathway 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 dehydrogenase; (19) 2-keto, 3-deoxy, 6-phosphogluconate (KDPG) aldolase; (20) PEP synthase or equivalent enzyme(s); (21) enolase; (22) phosphoglyceromutase (taken from Anthony, 1982)

The overall reaction describing the formation of phosphoglycerate from formaldehyde can be summarised as follows:

$$3\text{HCHO} + \text{NAD(P)}^+ + 2\text{ATP} \rightarrow \text{phosphoglycerate} + \text{NAD(P)}\text{H} + \text{H}^+ + 2\text{ADP} + \text{P}_i$$
enzymes; KDPG is then cleaved by the enzyme KDPG aldolase to produce one molecule of pyruvate (C₃; the "product" of the cycle) and one molecule of glyceraldehyde 3-phosphate (C₃). The final stage of the cycle is rearrangement in order to regenerate the three molecules of ribulose 5-phosphate required for fixation; the two molecules of fructose 6-phosphate which did not enter cleavage, and the glyceraldehyde 3-phosphate, undergo a series of rearrangement reactions catalysed by transaldolase and transketolase to produce 3 molecules of ribulose 5-phosphate, thus allowing the cycle to repeat.

It is essential for biosynthesis that triose phosphate is synthesised in addition to pyruvate. To this end 3-phosphoglycerate is synthesised from pyruvate at the expense of 2 ATP via phosphoenolpyruvate by PEP synthase (or equivalent enzymes), enolase and phosphoglyceromutase. Furthermore, oxaloacetate can be formed from pyruvate by the action of pyruvate carboxylase, which can be fed into the TCA cycle. However, due to the incomplete nature of the TCA cycle in M. methylotrophus it acts purely in an anabolic role generating carbon skeletons for biosynthesis and has no role in catabolism (Anthony, 1992; Lloyd et al., 1993).

1.1.4 Nitrogen assimilation by M. methylotrophus

The nitrogen sources which can be utilised by M. methylotrophus are relatively limited when compared with other bacteria. Ammonia, nitrate, methylated amines, urea, formamide and aliphatic amides can act as sources of nitrogen for growth (Jenkins et al., 1987; Large & Haywood, 1981; Silman, 1990; Silman et al., 1989, 1991; Wyborn, 1994; Wyborn et al., 1994). However, many other nitrogenous compounds (e.g. amino acids, nitriles, pyrimidines, purines, N₂ etc.) which can act as nitrogen sources for various other bacteria cannot be utilised by this organism. Nitrogen is assimilated by M. methylotrophus at the level of ammonia by the glutamine synthetase/glutamine-oxoglutarate aminotransferase (glutamate synthase) or GS/GOGAT system (Windass et al., 1980). Hence, the nitrogen sources listed above must first be metabolised to ammonia before assimilation can occur. Ammonia is produced from aliphatic amides, formamide and urea by hydrolysis catalysed by the enzymes acetamidase, formamidase and urease respectively, which are described further in this thesis.
The GS/GOGAT system has not been studied in detail in *M. methylotrophus*, although, the system operating in enteric bacteria has been well characterised (Magasanik, 1982; Merrick, 1988). The key intermediates in nitrogen assimilation are glutamate and glutamine which play a central role in the biosynthesis of cellular nitrogen-containing compounds. Glutamate is the primary amino-group donor for practically all amino acids and is also the nitrogen donor for purines and pyrimidines. Three routes of glutamate synthesis exist in enteric bacteria; (i) from ammonia and α-ketoglutarate by the action of either glutamate dehydrogenase (GDH) or by a coupled reaction catalysed by GS and GOGAT, (ii) from the amino group of another amino acid and α-ketoglutarate by a transamination reaction and (iii) as a direct product of the degradation of another amino acid. Only the route catalysed by GS/GOGAT will be considered here as wild-type *M. methylotrophus* does not possess GDH. GS catalyses the energy-dependent amidation of glutamate to glutamine by ammonia:

\[
\text{L-glutamate} + \text{NH}_4^+ + \text{ATP} \rightarrow \text{L-glutamine} + \text{ADP} + P_i + H^+ 
\]

GS from enteric bacteria is a homomultimer comprising 12 identical subunits, with an Mr of 600,000. In *E. coli* GS activity is regulated at both the transcriptional and post-translational levels. Cellular concentrations of GS are regulated transcriptionally in such a manner that during nitrogen-limited growth the concentration of the protein can be ten-fold higher than during growth under nitrogen-excess conditions (Merrick, 1988). This level of regulation is mediated by a gene expression system known as the nitrogen regulation (*ntr*) system which has been intimated in the transcriptional control of numerous proteins involved in nitrogen assimilation/metabolism, and is described further in the introduction to Chapter 3. The activity of GS is also regulated post-translationally by the nitrogen status of the cell. This control of GS activity is brought about by covalent modification involving reversible adenylylation of a specific tyrosyl residue on each of the 12 subunits, maximum biosynthetic activity being observed when the enzyme is completely deadenylylated. The modification of GS is potentiated by an elaborate cascade of events which are ultimately determined by the intracellular glutamine:α-ketoglutarate ratio, and hence the nitrogen
status of the cell. The post-translational regulation of GS activity is further described in the introduction to Chapter 4.

The second stage of nitrogen assimilation by the GS/GOGAT system is the reductive, energy-independent amination of α-ketoglutarate using glutamine as the N-donor catalysed by GOGAT:

\[ \alpha\text{-ketoglutarate} + \text{L-glutamine} + \text{NAD(P)H} + \text{H}^+ \rightarrow 2 \text{L-glutamate} + \text{NAD(P)}^+ \]

GOGAT from enteric bacteria is a multimer made-up of heterodimeric iron-sulphur protein subunits consisting of large (Mr 135,000-175,000) and small (Mr 53,000) polypeptides; the GOGAT of \textit{E. coli} is comprised of four dimers. In contrast to the regulation of GS, there appears to be no simple correlation between the activity and/or concentration of GOGAT and the nitrogen status of the cell (Merrick, 1988; see also Silman \textit{et al.}, 1989). The overall reaction catalysed by the GS/GOGAT system is as follows:

\[ \text{NH}_4^+ + \alpha\text{-ketoglutarate} \rightarrow \text{NAD(P)H} + \text{H}^+ + \text{ATP} \rightarrow 2 \text{L-glutamate} + \text{NAD(P)}^+ + \text{ADP} + \text{P}_i \]

1.2 Enzymes hydrolysing amide bonds

1.2.1 The diversity of amidases

Amidases (amidohydrolases) are enzymes which hydrolyse the amide bond of various organic compounds. Amides have the following general structure:

\[ R_1\text{CO-NH}R_2 \]

Where the dash represents the amide bond and \( R \) can be a variety of groups ranging from a hydrogen atom upwards. If the \( R \) groups are one or more amino acids then the bond is referred to as a peptide bond, rather than an amide bond, and enzymes hydrolysing it,
although amidases, are usually referred to as proteases. The general reaction carried out by amidases is:

\[
R_1.CO.NH.R_2 + H_2O \rightarrow R_1.COOH + R_2.NH_2
\]

Numerous enzymes which demonstrate amidase activity have been reported in the literature and it is broadly possible to classify amidases according to their substrate specificity (Clarke, 1970; Maestracci et al., 1988). These groups include: (i) enzymes hydrolysing peptide and amino acid amide groups (e.g. proteases, asparaginase, glutaminase and \(\alpha\)-amidase); (ii) microbial cell wall hydrolases (with \(N\)-acyluramoyl-L-alanine amidase activity); (iii) ureases (see below); (iv) biotinidases; (v) nicotinamid deaminases; (vi) 5-aminovaleramidases; (vii) allantoinases; (viii) penicillin amidases (see below) and (ix) acylamide amidohydrolases (see below). Many amidases not only demonstrate hydrolase activity but also acyl transferase activity, i.e. they are able to transfer the acyl moiety of the amide to hydroxylamine to form hydroxamates which can be measured by a colour reaction with ferric chloride. This property of amidases has been used in conjunction with, or in place of, the hydrolase assay to measure amidase activity (Brammar & Clarke, 1964; Kelly & Kornberg, 1964; Clarke, 1970).

Amidases appear to be relatively abundant in nature, being found in both prokaryotes and eukaryotes. Within microorganisms a number of acylamide amidohydrolases (EC 3.5.1.4; hereafter referred to as amidase) have been identified in bacteria, yeast and fungi (Thiery et al., 1986a). A large number of bacterial species have been found to possess amidases of this type (Maestracci et al., 1988) including: Corynebacterium, Mycobacterium, Pseudomonas, Bacillus, Micrococcus, Brevibacterium, Nocardia, Streptomyces, Arthrobacter, Rhodococcus, Alcaligenes and Methylophilus. Several of these enzymes, particularly those which are most active with short-chain aliphatic amides, have been characterised. All of these hydrolyse amides to the corresponding carboxylic acid and ammonia by the following general reaction:

\[
R.COHN_2 + H_2O \rightarrow R.COOH + NH_3
\]
1.2.2 The amidase of *Pseudomonas aeruginosa*

1.2.2.1 Activity and physiological regulation

The amidase of *Ps. aeruginosa* has been extensively investigated by Patricia Clarke and her colleagues since the early 1960's (for reviews see Clarke, 1970; 1984; Clarke & Drew, 1988). The first suggestion that *Pseudomonas* sp. possessed an aliphatic amidase was made by Den Dooren de Jong (1926; quoted in Clarke, 1970; 1984) following his observation that some species could utilise amides as carbon and nitrogen sources for growth. However, the first extensive investigation of this characteristic of *Pseudomonads* was carried out by Kelly & Clarke (1962) with *Ps. aeruginosa* 8602/A (subsequently referred to as strain PAC1). They found that *Ps. aeruginosa* 8602/A was able to grow on mineral salts medium containing acetamide or propionamide as the sole source of carbon and nitrogen via its ability to produce a discreet inducible amidase with both amidohydrolase and acetyl transferase activity (Kelly & Kornberg, 1962; 1964). The substrate specificity of the enzyme was very limited in that it only rapidly hydrolysed aliphatic amides containing three or less carbon atoms, (propionamide, acetamide and the side-chain substituted amides glycollamide and acrylamide), the highest activity being seen with propionamide. The enzyme also hydrolysed, albeit much more slowly, formamide and butyramide (at 7.5% and 1.2% respectively of the rate seen with propionamide in whole-cells; Kelly & Clarke, 1962). Acetamide and propionamide were the only amides which could act as sole sources of carbon and nitrogen for growth; formamide could not be used as a carbon source as *Ps. aeruginosa* is unable to assimilate C1-compounds, and acrylamide progressively inhibited the enzyme and was a powerful growth inhibitor. It was also discovered that inducer and substrate specificities were distinct as several non-substrate amides acted as good inducers. These included lactamide and the N-substituted amides N-methylacetamide and N-acetylacrylamide. This allowed the use of these amides as gratuitous inducers during the investigation of the kinetics of amidase induction in the absence of substrate metabolism. Interestingly, acetate was also found to induce amidase activity for reasons which remain obscure, although W. J. Brammar has suggested that sufficient acetamide to produce induction can be formed spontaneously from acetate and ammonia (see Clarke, 1970).
few amides such as cyanoacetamide and thioacetamide are able to compete with substrate or gratuitous inducers and prevent amidase induction; this phenomenon was termed amide analogue repression. Brammar & Clarke (1964) demonstrated that not only is induction of amidase inhibited/repressed by cyanoacetamide, but that amidase is sensitive to catabolite repression by several intermediates of the TCA cycle, glucose and related compounds (e.g. propionate, citrate, pyruvate, malate, succinate and lactate). These observations were later confirmed by more detailed studies carried out using continuous cultures (Boddy et al., 1967; Clarke et al., 1968). However, the amidase of *Ps. aeruginosa* is not repressed by ammonia, the other product of amide hydrolysis. The physiological rationale behind this is probably that the enzyme provides both carbon and nitrogen for growth and therefore, even in the presence of excess ammonia, is still required to provide carbon.

1.2.2.2 Experimental evolution of *Ps. aeruginosa* amidase

The amidase of *Ps. aeruginosa* has been used as a “tool” to investigate the evolution of catabolic enzymes in bacteria (see Clarke, 1970; 1984; Clarke & Drew, 1988). This was carried out using selective media to isolate mutant strains which demonstrated changes in amidase regulation or which had evolved new functions (i.e. the ability to hydrolyse amides which were not substrates of the wild-type amidase). These studies also ultimately lead to the elucidation of the regulatory mechanism of amidase synthesis at the molecular level. In general the mutants which were isolated can be grouped into three classes viz. regulatory mutants, amidase-negative mutants and mutants containing an altered enzyme (Clarke, 1984).

Regulatory mutants were the first group of amidase mutants isolated and were of three types; *viz.* (i) constitutive, (ii) altered in inducer specificity, and (iii) resistant to catabolite repression. The process of isolating amidase mutants began with the selection of constitutive strains on succinate/formamide (S/F) plates (Brammar et al., 1967). The wild-type (*Ps. aeruginosa* 8602/A: PAC1) does not grow on this medium since formamide is a poor substrate and only a very weak inducer of amidase. Formamide was also only used as a nitrogen source, as *Ps. aeruginosa* is unable to assimilate formate; succinate was therefore used as the carbon source. Succinate also imposed an additional selective
pressure as it acts as a catabolite repressor of amidase synthesis. It had been predicted that constitutive mutants would be able to outgrow the wild-type on S/F plates since they would not be dependent on induction by formamide and would also be insensitive to repression by succinate. Several classes of mutant were isolated spontaneously or following chemical mutagenesis on S/F plates, viz. magnoconstitutive strains, which synthesise high levels of amidase in the absence of inducer; semiconstitutive mutants, which synthesise lower levels of amidase in the absence of inducer, but which could produce higher levels if an inducing amide was present; formamide-inducible mutants, which were altered in their inducer specificity in such a way that amidase was induced to similar or higher levels in the presence of formamide as compared to acetamide. All of the amidases produced by these mutants were identical to the wild-type enzyme, and it was therefore concluded that these strains carried mutations in the amidase regulatory gene \textit{amiR}. Transductional analysis showed that \textit{amiR} was closely linked to the amidase structural gene, \textit{amiE} (Brammar \textit{et al.}, 1967).

Mutants which were resistant to repression by butyramide were isolated by plating wild-type and constitutive strains on butyramide-agar plates (Brown & Clarke, 1970). Butyramide is a very poor substrate for the wild-type amidase, is not an inducer and has the ability to prevent induction by inducing amides. All of the resulting mutants produced wild-type amidase constitutively (a second group of mutants isolated on butyramide but producing an altered amidase will be considered below) and possessed mutations in the amidase regulator gene which allowed them to synthesise large amounts of wild-type amidase in the presence of butyramide.

The final group of regulatory mutants which were resistant to catabolite repression were isolated by virtue of their ability to grow upon succinate/lactamide (S/L) plates (Smyth & Clarke, 1975). Lactamide is a poor substrate for \textit{Ps. aeruginosa} amidase, but a very good inducer, while succinate is a strong catabolite repressor. When wild-type cells were plated on this medium, large mutant colonies began to appear after a few days. Some of these mutants were found to produce amidase constitutively and some were inducible, but they were all resistant to catabolite repression. The constitutive catabolite-resistant mutants invariably carried mutations in the amidase regulator gene in addition to the mutations
which conferred resistance to catabolite repression which was not linked to the amidase structural gene (Smyth & Clarke, 1975; Clarke, 1984).

Amidase-negative mutants of *P. aeruginosa* have been isolated by a number of different methodologies. The first, and rather tedious, method was to treat the parent strain with chemical mutagens (ethylmethane sulphonate [EMS] or *N*-methyl-*N*-nitro-*N*-nitroso-guanidine [NMG]) and then plate out on medium containing acetamide as the major source of carbon supplemented with traces of succinate (Skinner & Clarke, 1968). The wild-type produced normal colonies on such medium and were thus able to hydrolyse acetamide to acetate, which subsequently leaked from the cell; this acetate, plus the trace amounts of succinate, allowed the growth of amidase-negative mutants as minute or shadowy colonies. This method isolated mutants which were defective in acetate metabolism in addition to amidase-negative mutants. A positive selection method using floroacetamide has also been used to isolate amidase-negative mutants (Clarke & Tata, 1973). When constitutive mutants were plated on pyruvate or lactate medium containing floroacetamide most of the resultant colonies demonstrated an amidase-negative phenotype. These isolates included both structural gene and regulatory gene mutants. A subsequent method for the positive selection of amidase-negative mutants using lactate/glycollamide medium has been described by Brown & Tata (1987).

The final type of mutants which were isolated were those producing altered enzymes which thus enabled the mutants to grow on novel substrates not used by the wild-type. Mutants which were able to utilise butyramide as a carbon source were isolated from a constitutive mutant (C11, isolated on S/F plates) by selection on butyramide plates, following NMG mutagenesis (Brown et al., 1969; Brown & Clarke, 1970). These mutants were found to produce an altered amidase (termed B amidase to distinguish it from the wild-type A amidase).

A butyramide-utilising mutant (B6) was used to isolate further mutants (either spontaneously or following treatment with EMS) which grew on valeramide (Brown et al., 1969). The amidase produced by these strains (V amidase) appeared to be very unstable in cell-free extracts and only showed partial cross-reactivity against antiserum raised to A and B amidases. This suggested that a further mutation in the *amiE* gene had occurred which altered not only the substrate profile, but also the antigenic specificity of the protein.
Phenylacetamide has a very bulky side chain and is therefore neither a substrate or an inducer of the wild-type amidase. This amide therefore represented a considerable challenge in isolating mutants which were able to utilise it. Phenylacetamide-utilising mutants were isolated (following mutagenesis by EMS, NMG or u.v. irradiation) from various parent strains by selection on phenylacetamide/succinate plates (Bentz & Clarke, 1972). The resultant mutants produced an altered amidase (Ph amidase) which was most active with longer-chain amides (>C₃). Ph amidase gave complete cross-reactivity with A and B amidases, which suggested that it had arisen by further mutation in the amiE gene.

Acetanilide (N-phenylacetamide)-utilising mutants were isolated from a catabolite repression resistant mutant (which was also resistant to amide analogue repression; L10), following mutagenesis with NMG or EMS and selection on acetanilide plates (Brown & Clarke, 1972). These mutants produced AI amidase which had a very different substrate profile compared with both the A and B amidase, suggesting that the mutation which had produced the acetanilide-utilising phenotype was different to that which had occurred in B and V mutants.

1.2.2.3 Purification and characterisation of wild-type and mutant amidases

The wild-type amidase (A amidase) was purified from the constitutive strain C11 and found to be a homohexamer with a native Mᵦ of approximately 200,000 (Clarke, 1984). Each subunit was estimated to have an Mᵦ of 35,000-41,000, but amino acid sequencing (Ambler et al., 1987) later showed this to be 38,400, thus producing a native Mᵦ of 230,400. Each subunit contains eight cysteines, a property which is consistent with the observation that the presence of thiol compounds (e.g. mercaptoethanol or dithiothreitol) was necessary to maintain enzyme activity during purification. The enzyme was found to be inhibited by iodoacetamide and urea, and the latter has subsequently been shown to be an active-site-directed inhibitor causing a conformational change in the enzyme molecule (Tata et al., 1994).

The mutant B, V, Ph and AI amidases have also been purified and, like the A amidase are homohexamers but show different electrophoretic mobilities to the A amidase (Clarke, 1984). Also, some of the V, AI and Ph enzymes are very thermolabile, some to both heat
and cold, whereas the B amidase demonstrates a similar thermal stability to the A amidase. The amino acid sequences of the amidases have been compared by “fingerprint” analysis of peptide fragments following proteolytic cleavage of pure enzymes (Brown & Clarke, 1972; Paterson & Clarke, 1979; Clarke, 1970; 1984). The AI3 enzyme was found to differ from the wild-type amidase by a single amino acid substitution (threonine substituted for an isoleucine) which allowed the utilisation of acetonilide. The family of amidase mutants derived from strain B6 (V9, PhB3 and PhV1) also exhibited a single amino acid substitution, from a serine in the A amidase to a phenylalanine in the mutants. This substitution was therefore conserved during subsequent evolution that extended the substrate specificity of the enzyme to include valeramide and phenylacetamide. It was suggested that this, and subsequent, changes in the enzyme caused slight changes in conformation which increased the flexibility of the molecule and produced the observed changes in substrate specificity.

1.2.2.4 Mechanism of regulation of amidase synthesis in Ps. aeruginosa

The regulation of expression of amidase in Ps. aeruginosa has been elucidated at the molecular level. This elucidation process began by the suggestion of Farin & Clarke (1978) that amidase was under positive rather than negative control, as described by the Jacob-Monod model of gene expression. It had previously been reported that some lac operon mutants were constitutive at higher growth temperatures (>37°C) and inducible at lower temperatures, reflecting the production of a mutant lac repressor protein which was thermostable. It was therefore hypothesised that if amidase was also under negative control, it should be possible to isolate similar temperature-sensitive mutants. However, when a large number of potential mutants were screened no such temperature-sensitive phenotype was found, but several mutants were isolated which were amidase-negative at a growth temperature of 41°C and constitutive at 28°C. As the amidase produced by these mutants was physico-chemically similar to the wild-type enzyme, it was concluded that the temperature-sensitive phenotype was caused by a mutation in a regulatory gene (amiR) and that amidase was regulated by positive control. This was later confirmed by Drew (1984), who also showed that it occurred via a transcriptional anti-termination mechanism (Drew
The nucleotide sequences of the amidase structural gene \textit{(amiE)} and the regulatory gene \textit{(amiR)} have been determined (Brammar et al., 1987; Low et al., 1989), and the amino acid sequences of both gene products deduced (Ambler et al., 1987; Low et al., 1989).

The control of amidase synthesis has been further dissected at the molecular level by Wilson and Drew, who have discovered a second regulatory gene \textit{(amiC)} which acts as a negative regulator (Wilson & Drew, 1991). AmiC has been shown to bind amides and to be a member of a structurally-related family of bacterial periplasmic binding proteins (see below), although located in the cytoplasm (Wilson et al., 1993). AmiC and AmiR are thought to form a two-component sensor-regulator system such that when amides are not present in the medium AmiC, by direct protein-protein interaction, prevents the anti-termination activity of AmiR and therefore blocks \textit{amiE} transcription. Conversely, when amides are present in the growth medium they bind to AmiC and prevent its interaction with AmiR (possibly by effecting a conformational change), thus allowing the transcriptional anti-termination activity of AmiR and hence potentiating \textit{amiE} transcription.

1.2.3 The amidases of \textit{Brevibacterium} sp. R312

\textit{Brevibacterium} sp. R312 is able to grow in minimal medium containing acetonitrile as the sole source of carbon and nitrogen, by the sequential action of a nitrile hydratase and an amidase. The nitrile hydratase converts acetonitrile to acetamide which is then hydrolysed to ammonia and acetate by the amidase. The amidase activities of \textit{Brevibacterium} sp. R312 have been extensively studied by Arnaud and his co-workers (reviewed by Maestracci et al., 1988). This organism is able to hydrolyse a very large number of amides; only internal amides (lactams) and amide analogues (e.g. thioacetamide and N-methylacetamide) were not hydrolysed. Several mutant strains were isolated which were unable to hydrolyse fluoroacetamide (in a similar manner to that of \textit{Ps. aeruginosa}). These strains exhibited a nitrile hydratase activity similar to that of the wild-type organism, but were able to hydrolyse only a limited number of amides (formamide, nicotinamide, \(\alpha\)-aminoamides and urea). This suggested that the wild-type organism possessed a wide-spectrum amidase in
addition to enzymes which specifically hydrolysed formamide, nicotinamide, α-aminoamides and urea. This organism has also recently been found to contain an enantiomer-selective amidase which is active with several 2-aryl and 2-aryloxy propionamides (Mayaux et al., 1990).

The wide-spectrum amidase from *Brevibacterium* sp. R312 is induced by acetamide and N-methylacetamide, and repressed by organic acids (but not by glucose or ammonia) (Maestracci et al., 1984; 1988). The enzyme has been purified and found to be a homotetramer with a native $M_r$ of approximately 180,000, each subunit having an $M_r$ of approximately 43,000 (Thiery et al., 1986a). It hydrolyses a wide range of amides, both aliphatic and aromatic, as well as urea (the highest activity being observed with acetamide) and also exhibits acyl transferase activity (Thiery et al., 1986b). The gene encoding the wide-spectrum amidase has recently been cloned and sequenced, and the deduced amino acid sequence shown to be more than 80% identical to that of the *Ps. aeruginosa* amidase (Soubrier et al., 1992). The evolutionary implications of this will be discussed in Chapter 7.

1.2.4 The amidase of *Rhodococcus rhodochrous* J1

*Rhodococcus rhodochrous* J1, like *Brevibacterium* sp. R312, is also able to grow at the expense of nitriles. However, this organism possesses two pathways of nitrile utilisation. The first, catalysed by nitrilase, hydrolyses nitriles directly to their corresponding carboxylic acid and ammonia; the second involves the sequential action of nitrile hydratase and amidase (Kobyashi et al., 1993). The physiological regulation of the amidase has not been reported. However, the gene encoding the enzyme has been cloned and sequenced, and the heterologously-expressed enzyme purified and characterised. The amidase is a homodimer with an $M_r$ of approximately 110,000, and a subunit $M_r$ determined from the gene sequence of 54,626. The enzyme hydrolyses a wide range of amides, both aliphatic and aromatic, but shows no activity to amino acid amides. Maximum activities were observed with short-chain aliphatic amides, the optimum substrate being isobutyramide. The enzyme also exhibited acyl transferase activity. The derived amino acid sequence of the *Rh. rhodochrous* J1 amidase is highly similar to that of some other bacterial amidases,
but not those from *Ps. aeruginosa* or *Brevibacterium* sp. R312. The evolutionary implications of this will be discussed in Chapter 7.

### 1.2.5 The amidases of *Mycobacterium smegmatis*

*M. smegmatis* can grow in minimal medium with acetamide as the sole carbon source (Draper, 1967). Washed cells and cell-free extracts were found to hydrolyse a number of amides, including benzamide, although the highest activity was observed with formamide. However, formamide was not able to support growth as *M. smegmatis* cannot assimilate C1-compounds. The next best substrate was butyramide, which was only hydrolysed at 2.5% of the rate with formamide; all other amides tested were hydrolysed at much lower rates. The enzyme, referred to as “acetamidase,” was also induced by acetate, butyramide and the non-substrate amide analogues N-methylacetamide and N-acetyl-acetamide. Moreover, the induction of benzamidase activity was independent of “acetamidase” activity, suggesting that a discrete aryl acylamidase was expressed by this organism. Bacterial extracts were also found to contain acyltransferase activity. A possible explanation for the large number of amides hydrolysed by crude-extracts of *M. smegmatis* was that it produced at least two amidases, one specific for formamide and one or more specific for butyramide and longer-chain amides.

The gene encoding the “acetamidase” from *M. smegmatis* has recently been cloned and sequenced (Mahenthiralingam et al., 1993). The gene was found to encode a protein with an *Mr* of 43,965, but the multimeric structure of the enzyme has not been reported. The derived amino acid sequence of the *M. smegmatis* enzyme did not show any notable homology to the *Ps. aeruginosa* amidase. The evolutionary implications of this will be discussed in Chapter 7.
1.2.6 The amidases of *Methylophilus methylotrophus*

1.2.6.1 Introduction

Previous studies have shown that *M. methylotrophus* contains a short-chain aliphatic amidase (EC 3.5.1.4; Silman, 1990; Silman *et al.*, 1989; 1991; Carver & Jones, 1993; Wyborn, 1994; hereafter referred to as acetamidase) which demonstrates high activity with acetamide, acrylamide and propionamide (the optimum substrate), but little or no activity with other short-chain amides and urea. *M. methylotrophus* also possesses a formamidase (formamide amidohydrolase EC 3.5.1.49; Carver & Jones, 1993; Wyborn, 1994; Wyborn *et al.*, 1994) which demonstrates optimum activity with formamide and little or no activity with other short-chain aliphatic amides or urea, and a urease which is specific to urea (Jenkins *et al.*, 1987; Silman *et al.*, 1989; Silman, 1990; Wyborn *et al.*, 1994; Carver & Jones 1993; H. Murphy, J. Mills & C. W. Jones, unpublished).

1.2.6.2 Regulation of acetamidase activity

The physiological regulation of *M. methylotrophus* acetamidase was first investigated at ZENECA Bio Products (formerly ICI Bio Products) during pilot studies concerned with the commercial production of the enzyme for bioremediation purposes (see section 1.3.6), but was subsequently investigated in greater detail in Leicester (Silman, 1990; Silman *et al.*, 1989; 1991). The enzyme is induced by acetamide and acrylamide and repressed by ammonia. Acetamidase is maximally expressed during growth under acetamide-limited/methanol-excess conditions. However, during growth under this regime large quantities of viscous exopolysaccharide were produced by the organism (see Southgate & Goodwin, 1989), causing problems during downstream processing and making production of acetamidase under this growth regime commercially unviable. The growth of *M. methylotrophus* under methanol-limited/acetamide-excess conditions eliminated polysaccharide production but caused repression of amidase synthesis due to the high concentration of ammonia in the culture supernatant, such that the enzyme was only present in these cells at a very low level. In an attempt to overcome these problems, the
organism was grown under dual acetamide/methanol limitation (dual limitation); the resultant culture produced little polysaccharide and exhibited maximal induction of acetamidase protein. Unfortunately, however, it exhibited an unexpectedly-low acetamidase activity. Subsequent analysis of the enzyme from these low-activity cells demonstrated that the low activity was due to the production of an enzyme with a significantly decreased $k_{cat}$ for acetamide ($<45s^{-1}$) compared with that synthesised by acetamide-limited cultures ($128s^{-1}$) (Carver & Jones, 1993). It was hypothesised that the low activity acetamidase may have been down-regulated by a cryptic biochemical process, possibly involving a post-translational modification. This down-regulation of acetamidase activity was termed “switch-off” by analogy with the process observed in nitrogenase (Zumft & Castillo, 1978). This nomenclature led to the low-activity acetamidase being colloquially referred to as switched-off, and the high-activity enzyme being referred to as switched-on. Fortuitously, it was found that dual-limited, switched-off cells exhibiting a low acetamidase activity demonstrated a significant increase in specific activity (up to 5000%) after a period of heating at 60°C, which was termed “heat-reactivation” (Carver & Jones, 1993). This ability to reanimate switched-off acetamidase allowed the development of a large-scale, high cell-density, continuous-culture process for the commercial production of acetamidase. The downstream processing incorporated a heating step following cell harvesting and breakage, which had the three-fold function of coagulating insoluble material, precipitating many soluble proteins and activating the acetamidase (Carver & Jones, 1993).

The products of acetamide hydrolysis by \textit{M. methylotrophus} acetamidase are acetate and ammonia. The ammonia is assimilated by the GS/GOGAT system (see section 1.1.4), but being a restricted facultative methylotroph \textit{M. methylotrophus} cannot utilise acetate as a sole source of carbon and energy. However, the organism has a limited ability to metabolise acetate, such that during growth at low cell density no acetate is spilled into the culture supernatant (see Chapter 3). This ability is conferred on \textit{M. methylotrophus} by the possession of the enzyme acetyl CoA synthetase, which synthesises acetyl CoA from acetate and CoA (Lloyd, 1990). However, the TCA cycle of \textit{M. methylotrophus} is incomplete (lacking 2-oxoglutarate, succinate and malate dehydrogenases) and the
glyoxylate cycle is absent (Lloyd et al., 1993). The metabolic fate of acetate therefore remains obscure.

This limited ability to metabolise acetate was utilised during the commercial production of acetamidase. During this process, high input concentrations of acetamide were required to maintain a high cell density, which led to very high acetate concentrations in the culture supernatant as the limited ability of \textit{M. methylotrophus} to metabolise acetate apparently became saturated. As 30-40mM acetate is toxic and causes the culture growth rate to decrease below the imposed growth rate, and hence the culture to wash-out (Silman et al., 1989; Silman, 1991; Carver & Jones, 1993), an alternating nitrogen source of acetamide and ammonia (or urea) was used. Cultures were maximally induced by growth under methanol/acetamide limitation until the acetate concentration in the culture supernatant had increased to a level just below that which had an adverse effect on the growth rate. At this point the acetamide feed was replaced by ammonia in such a manner as to maintain a constant input nitrogen concentration and to attain growth under methanol/ammonia limitation. The acetate concentration was then allowed to decrease as ammonia rather than acetamide was used as the nitrogen source. This decrease was found to be more rapid than that predicted for simple diluting out from the culture, confirming that \textit{M. methylotrophus} was able to metabolise acetate. Ammonia was used as nitrogen source for approximately 2h before the reintroduction of acetamide in place of ammonia. By using this pseudo steady-state, dual-limited growth regime with an alternating nitrogen source maximum induction of acetamidase was maintained (as long as the ammonia feed periods were approximately 2h) and acetate toxicity was avoided. In spite of this, however, acetamidase switch-off occurred in such cultures upon the replacement of acetamide with ammonia as the nitrogen source (Carver & Jones, 1993).

The physico-chemical mechanism of acetamidase switch-off in \textit{M. methylotrophus} has been investigated by Silman (1990) and Wyborn (1994). The former purified switched-off acetamidase from \textit{M. methylotrophus} grown at high cell-density under pseudo steady-state dual limitation with an alternating nitrogen source (supplied by ZENECA Bio Products) and compared its properties with that of switched-on acetamidase purified from cells grown at low cell density under acetamide limitation. The switched-off acetamidase was found to lose the ability to be heat-reactivated during purification. This was subsequently
found to be due to the absence of a cellular fraction (referred to as activator fraction) being required for heat reactivation, which was separated from acetamidase during purification. Activator fraction was also purified from *M. methylotrophus* and shown to facilitate heat-reactivation of pure switched-off acetamidase. The purified switched-off acetamidase was found to have a similar subunit and native Mr to that of switched-on enzyme. Isoelectric focusing suggested that the switched-off enzyme had a slightly lower pl (4.0 cf. 4.1) than the switched-on enzyme, but no difference in proteolytic fragment pattern was seen between the two forms of acetamidase following digestion with clostripain. Further examination of the switched-off acetamidase for the presence of a post-translational modifying group were unfruitful, and it was concluded that if a modification had occurred it was not the result of ADP-ribosylation, phosphorylation (of histidine, arginine, serine or threonine), N-terminal formylation or acetylation, or the formation of a disulphide bridge.

Wyborn (1994) also purified switched-off acetamidase and compared its properties to those of the switched-on enzyme. The possibility of a post-translational modification was further investigated by attempting to compare very accurately the Mr of the switched-on and switched-off enzymes using electrospray mass spectrometry. The results from this study, though not conclusive, suggested that any difference in Mr between the two forms was ≤52, thus eliminating most of the more common post-translational modifications. The mechanism of heat reactivation and the nature of activator fraction were also investigated further. It was discovered that activator fraction acted in a stoichiometric fashion during heat-reactivation of switched-off enzyme, and that it did not require to be heated in the presence of acetamidase to facilitate reactivation i.e. activator fraction could be heated, cooled and then mixed with switched-off enzyme and still produce reactivation. However, it was noted that heat-reactivated acetamidase tended to undergo spontaneous switch-off following activation. Activator fraction was found to be comprised of at least two components, one with a high Mr and one with a lower Mr, and was extremely thermostable even following incubation with acid at 100°C. It was therefore concluded that the activator fraction was probably not a protein, as suggested by Silman (1990). The majority of work carried out by Wyborn was hampered by the observation that switched-on acetamidase frequently underwent spontaneous switch-off during purification. The magnitude of this *in vitro* switch-off was highly variable and no correlation was found between the extent of
switch-off and factors such as culture growth regime or purification protocol. It was further found that pure switched-on acetamidase could undergo spontaneous switch-off during storage at -20°C. Switched-off acetamidase in contrast did not lose further activity, which intimated that this was the more stable form of the enzyme. These and other data suggested that there was not a distinct switched-on and switched-off form of the enzyme, but a spectrum of activities between the two extremes, representing relative levels of switch-off. The major conclusions drawn from the extensive work carried out by Wyborn were that the switched-on and switch-off forms of acetamidase probably exist in different conformational states and that the regulation of activity probably involves an allosteric effector.

1.2.6.3 Experimental evolution of *M. methylotrophus* acetamidase

The evolution of *M. methylotrophus* acetamidase has been investigated in continuous culture during prolonged growth under acetamide and acrylamide limitation at low dilution rate (Silman, 1990; Silman et al., 1989; 1991; Carver & Jones, 1993). It was initially found that during acetamide-limited growth at low dilution rate (D=0.025h⁻¹) a large increase in acetamidase activity was observed after 8-9 generations. This culture was termed culture A and a single colony isolated from it, mutant MM6, was taken for further investigation. MM6 was subjected to a further round of selection under acrylamide limitation which led to the isolation of a second spontaneous mutant MM8. A third mutant, MM15, was then isolated by chemical mutagenesis of MM8 with NTG, followed by a further round of acrylamide-limited continuous culture. The mutants were characterised during growth under acetamide limitation, and all were found to over-produce acetamidase (approximately 20-25% of cell protein cf. 5% in the wild-type organism). In contrast, the acetamidase specific activities of MM6 and MM8 whole cells were respectively four-fold and twelve-fold higher than the wild-type, whereas that of MM15 was similar to the wild-type. The amidases from the mutants were also purified and characterised. The kinetic properties of acetamidase from wild-type and MM6 were essentially identical, indicating that MM6 over-expressed the wild-type enzyme. However, the enzyme from MM8 exhibited a three-fold higher kcat than the wild-type and MM6 enzymes (310s⁻¹ cf. 28...
approximately 100s⁻¹), whereas the enzyme from MM15 exhibited similar $k_m$ and $k_{cat}$ values for acetamide as the wild-type enzyme (1.3mM cf. 1.1mM and 96s⁻¹ cf. 128s⁻¹, respectively), but had an eight-fold lower $k_m$ for acrylamide (2.1mM cf. 16.1). The observation that the MM15 whole cell specific activity was similar to the wild-type, although the cell contained approximately five-fold more enzyme of apparently similar $k_{cat}$, was investigated by further physico-chemical characterisation of the purified MM15 acetamidase. It was found to exhibit a five-fold higher purification factor than predicted, to react more extensively with the thiol group reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), to have a significantly lower sedimentation coefficient, to have a higher activity to longer chain amides and to exhibit a decreased thermal stability, all of which pointed to the MM15 enzyme having a much more open structure than the other enzymes. This much looser association of the MM15 acetamidase subunits led to the dissociation of approximately 80% of the enzyme into inactive monomers and dimers even at the normal growth temperature of 37°C, thus explaining the similar whole cell specific activities of MM15 and the wild-type, even though the cellular enzyme concentration was substantially higher in MM15. Furthermore, during purification the inactive monomers and dimers were separated from the active enzyme, thus producing the unexpectedly high purification factor. When MM15 was grown at the lower temperature of 25°C it exhibited a fifteen-fold higher specific activity than following growth at 37°C, and a similar $k_{cat}$ to MM8. Since MM15 rapidly outgrew MM8 in acrylamide-limited continuous culture at 37°C, it was clear that the decreased $k_m$ of MM15 for acrylamide offset the deleterious effects of its increased thermostability.

It was concluded that all four of the acetamidase phenotypes which may have been expected to be selected by this method were in fact isolated, viz. (i) over-production of wild-type enzyme (MM6), (ii) over-production of an altered enzyme with an increased $k_{cat}$ (MM8), (iii) over-production of an altered enzyme with a decreased $k_m$ (MM15) and (iv) over-production of an altered enzyme with both increased $k_{cat}$ and decreased $k_m$ (MM15 grown at 25°C).
1.2.6.3 The formamidase of \textit{M. methylotrophus}

\textit{M. methylotrophus} has been found to possess a formamidase in addition to the acetamidase described above (Wyborn, 1994; Wyborn \textit{et al.}, 1994). The regulation of the enzyme was briefly investigated in batch, fed-batch and continuous culture and found to be induced by acetamide and formamide, and repressed by ammonia. The enzyme was purified and found to have a subunit \textit{M}_r of approximately 51,000 and a native \textit{M}_r of approximately 123,000. It showed high activity with formamide, but very low activity with other short-chain aliphatic amides and no activity with urea. The gene encoding formamidase (\textit{fmdA}) was cloned and sequenced and the derived amino acid sequence was found to exhibit 57\% identity to the \textit{M. smegmatis} "acetamidase" (Wyborn, 1994). The protein has also been crystallised and preliminary structure determinations carried out (O'Hara \textit{et al.}, 1994).

1.2.7 Microbial ureases

A large number of microorganisms including; bacteria, yeasts, filamentous fungi and algae, are able to grow on urea as a source of nitrogen by virtue of having a urease (urea amidohydrolase EC 3.5.1.5). This enzyme catalyses the hydrolysis of urea (\(\text{NH}_2\text{CO.NH}_2\)) to two molecules of ammonia and one molecule of carbon dioxide. Large quantities of urea are found in nature due to the release of this nitrogenous compound by biological action. It is the major excretion product of all mammals, and human urine contains approximately 0.4-0.5M urea (Mobley & Hausinger, 1989). Birds, reptiles and most terrestrial insects excrete uric acid which, when further degraded, also yields urea. Urea is also an end product of purine catabolism and of the biodegradation of other nitrogenous compounds such as amino acids. However, it is generally short lived in the environment due to its hydrolysis by urease-producing organisms.

A large amount of research has been directed towards understand microbial ureases due to their involvement in a number of important processes, including the production of various microbially-induced human disease states. Urease is directly associated with the formation of infection stones, pyelonephritis, ammonia encephalopathy, hepatic
encephalopathy and hepatic coma, urinary catheter obstruction and peptic ulceration. This latter disease has recently received a large amount of attention due to the observation that peptic ulcers could be both treated and prevented by the administration of oral antibiotics rather than expensive antacid drugs, following this discovery it was found that peptic ulcers were caused by the presence of the bacterium Helicobacter pylori in the gut. The exact mechanism by which this occurs is still unclear, but it has been found that the highly-active urease of this organism is a virulence factor (for recent review see Telford et al., 1994). The action of urease-positive soil bacteria is also important in the utilisation of urea fertilisers. Urea is an excellent fertiliser due to its low cost, ease of handling and high nitrogen content. However, it does pose an inherent problem as uncontrolled urea hydrolysis can lead to plant damage from ammonia toxicity and elevated pH. Conversely, low soil urease activity can lead to inefficient use of applied fertiliser. Hence, methods of controlling soil urease activity have been investigated (see Mobley & Hausinger, 1989).

Ureas have been purified and characterised from a number of microorganisms, and can be extracellular, periplasmic, membrane associated or cytoplasmic. However, it must be noted that in the majority of bacteria investigated it is a cytoplasmic enzyme (Mobley & Hausinger, 1989). The physiological regulation of urease activity appears to vary from species to species and will be considered further in Chapter 3. Many ureases are large, multi-subunit enzymes (often containing α, β and γ subunits in varying stoichiometries) with a native Mr of 125,000-380,000. Most of them are unstable, and the presence of EDTA and a thiol compound such as mercaptoethanol or dithiothreitol are required to maintain activity during purification. The kinetic characteristics of microbial ureases vary from species to species such that the $k_M$ for urea can range from 0.1mM to >100mM. However, all are most active around neutral pH and, more importantly, contain nickel. Nickel is generally not considered to be an important metal in biology; however, it was shown in 1975 to be an essential component of jack bean urease (Dixon et al., 1975); since then all ureases have been found to contain nickel (see Hausinger, 1987; Mobley & Hausinger, 1989) and the possession of a nickel metallocenter is essential for urease activity. M. methylotrophus possesses a urease, the physiological regulation of which will be discussed in Chapter 3.
1.3 Biotechnology of amidases

1.3.1 Introduction

The potential biotechnological applications of amidases have centred around their participation in a number of biotransformation reactions due to their ability to hydrolyse amides, frequently with stereospecificity, to the corresponding organic acid. The use of enzymes in the traditional realm of the organic chemist potentially has many advantages over more established synthetic chemical processes; these include stereospecificity, mild reaction conditions and the ability, in whole-cell systems, to carry out sequential reactions (e.g. hydration of a nitrile to an amide by nitrile hydratase followed by hydrolysis of the amide to the corresponding acid by amidase) (see Turner, 1995). It is not the intention here to give an exhaustive review of the potential applications of amidases, but rather to give a general outline of this field of research.

1.3.2 Production of aliphatic organic acids

The production of aliphatic organic acids from their corresponding amides has elicited much interest in recent years due to the potential use of these acids themselves or as synthons for the formation of other compounds. For example lactic acid, which can be produced from lactamide by amidase, is used as an additive to soft drinks, fruit juices and other food products; it is also used in hide tanning and the production of plastics (polylactate being used in plastic foils) and lactate salt therapeutics (Higgins et al., 1988). A process for the production of lactate from lactonitrile (also requiring the action of nitrile hydratase) by Brevibacterium sp. R312 has been proposed by Jallagease et al. (1980) and Maestracci et al. (1988). However, this process is not commercially viable as lactate can be produced much more cheaply by conventional fermentations. Acrylic acid is also an industrially important acid (Bernet et al., 1987; Nagasawa & Yamada, 1989; Nawaz et al., 1994) which can be used in the production of plastics. Acrylic acid can be produced from acrylamide by the action of amidase. It is interesting to note that acrylamide itself is currently being produced by an enzymatic biotransformation process from acrylonitrile by the enzyme nitrile hydratase to the order of 6000 tons per year (Kobayashi et al., 1992).
This process is the first successful application of biotechnology in the petrochemical and commodity chemical industries.

1.3.3 Production of aromatic organic acids

The enantiomer-selective hydrolysis of racemic aromatic amides by amidases has attracted a lot of interest due to their use as pharmaceuticals. The 2-arylpropionic acids (such as ibuprofen and naproxen) are nonsteroidal anti-inflammatory drugs which contain a chiral centre (Layh et al., 1994; Ciskanik et al., 1995). The activity of such drugs resides almost exclusively with the S-isomer and therefore methods of synthesis which are stereospecific are very attractive. Enantioselective amidases have been isolated from various bacterial sources which can specifically hydrolyse the S-amide to the pharmaceutically active S-acid (Mayaux et al., 1990, 1991; Yoshioka et al., 1991; Layh et al., 1994; Ciskanik et al., 1995). But it remains to be seen as to whether these potential processes have a commercial impact.

1.3.4 Production of amino acids

Amino acids have numerous commercial applications, being used as precursors in the chemical industry for the manufacture of detergents, polyamino acids (used in synthetic fibres and films), polyurethane and agricultural chemicals (such as pesticides and herbicides). Amino acids are also used as synthons in the pharmaceutical industry for the production of semisynthetic antibiotics, immunosuppressants, antitumour agents and anti-hypertension drugs (Van der Tweel et al., 1993). They are also used in the food industry as nutritional supplements (e.g. to enrich vegetable protein), flavour enhancers (e.g. monosodium glutamate), sweeteners (e.g. NutraSweet), bacteriostatic agents and antioxidants (Higgins et al., 1988). Hence, amino acid amidases could be used in the synthesis of amino acids from the corresponding amino amide. The enzymatic racemization of chemically synthesised amino acids is already carried out by a number of enzyme systems. However, amidases represent a group of enzymes which have not yet been exploited for this function. Optically-pure amino acids could also be produced by the
action of stereospecific amidases on the corresponding amino amide (see Jallagease et al., 1980; Maestracci et al., 1988).

1.3.5 Penicillin amidase

Penicillin amidase is a very important enzyme industrially due to its use in the production of semisynthetic β-lactam antibiotics. This enzyme is used to hydrolyse either penicillin G (benzyl-penicillin) or penicillin V (phenoxymethylpenicillin) to 6-aminopenicillanic acid (6-APA) which is the precursor of semisynthetic penicillins (see Valle et al., 1991). These penicillin derivatives (e.g. ampicillin, methicillin and oxacillin) have enhanced properties over the naturally produced penicillins such as increased stability, easier absorption and fewer side-effects. In addition to these the semisynthetics also represent a solution to the problem of adaptive microbial resistance to antibiotics, as new molecules can be synthesised with relative ease by chemical means, rather than the time consuming and expensive process of isolating new naturally occurring antibiotics.

1.3.6 The acetamidase of M. methylotrophus for bioremediation purposes

Acrylamide is used extensively in a number of industrial processes and current global production is estimated to be over 200,000 tonnes per annum (Nagasawa & Yamada, 1989; Nawaz et al., 1994). The widespread use and indiscriminate discharge of acrylamide has lead to significant environmental contamination. A major use of acrylamide is in the production of polyelectrolyte flocculants which are used in the treatment of potable water, sewage sludge conditioning, paper making, mining and secondary oil recovery (Carver & Jones, 1993). The acrylamide monomer (of which residual amounts are present in polyacrylamide flocculants) is a potent cumulative neurotoxin and a suspected human carcinogen. Hence, its concentrations in flocculants is tightly regulated by legislative control, particularly in the USA. Current methodologies for the removal of residual acrylamide from polymers have associated problems of polymer damage and increased reactor residence times. Hence, in the late 1980’s ZENECA Bio Products developed an enzyme-based process for the removal of residual acrylamide from polyacrylamide.
polymers (see Carver & Jones, 1994), which could also be applied to environmental contamination. The developed process utilised the acetamidase from *M. methylotrophus* to hydrolyse acrylamide to ammonia and the non-neurotoxic acrylic acid. The choice of *M. methylotrophus* for the production of this enzyme reflected the large amount of experience which ZENECA Bio Product had gained in growing this organism in large-scale continuous culture during the production of *M. methylotrophus* biomass for the single-cell protein product Pruteen (see Vasey & Powell, 1984; Sharp, 1989). Although the Pruteen process was ultimately unsuccessful commercially, it did initiate the design and operation of the largest, sterile continuous culture fermentation technology to date (a novel pressure cycle reactor). The acetamidase production process represented a means of drawing upon the knowledge gained, and the technology developed, during Pruteen production and returning some of the costs of the original research and development. However, it must be noted that the acetamidase production process no longer operates due to the shelving of predicted US legislation which would have further decreased the acceptable concentrations of acrylamide in polyacrylamide flocculants.

1.4 Solute transport in Gram-negative bacteria

1.4.1 Introduction

The first stage in the utilisation of any nutrient by bacteria is its uptake from the growth medium into the cell. Waste products of cellular metabolism must also be able to exit the cell to prevent toxic build-up (excretion; which will not be considered further here). These processes work in tandem to maintain the optimum cellular concentration for each nutrient. Transport occurs by way of four general processes: (i) passive diffusion, (ii) facilitated diffusion, (iii) active transport and (iv) group translocation. The distinction between passive or facilitated diffusion and active transport or group translocation is made upon the basis of energy requirement. Passive and facilitated diffusion processes do not require energy input, whereas active transport and group translocation do (Antonucci & Oxender, 1986). The uptake of nutrients into Gram-negative bacteria is a complex process, which is further complicated by the nature of the cell wall structure present in these bacteria. The
cell wall of Gram-negative bacteria consists of an outer membrane, the cell wall proper (consisting of a layer of peptidoglycan) and a cytoplasmic membrane (Nikaido & Vaara, 1985). The compartment between the inner (cytoplasmic) membrane and the outer membrane is known as the periplasmic space. Hence, for a nutrient molecule to enter the cytoplasm of a Gram-negative bacterium it must first traverse the outer membrane, cross the periplasm and finally be transported into the cytoplasm by passage through the inner membrane. These processes will be considered individually in relation to the proteins involved and the type of transport process they carry out.

1.4.2 Transport across the outer membrane

The outer membrane is composed of a phospholipid bilayer (in common with all other biological membranes), lipopolysaccharides (LPS; composed of six or seven fatty acids connected to a glucosamine disaccharide backbone) and other polysaccharide components anchored to phospholipids and proteins (Nikaido & Vaara, 1985). The major function of the outer membrane is to act as a permeability barrier and a "coarse sieve" that excludes many of the noxious molecules that can exist in the external medium, but at the same time allow the passage of nutrient molecules into the cell (Nikaido, 1993; 1994). The lipid bilayer of the outer membrane is relatively impermeable to hydrophilic solutes, and the presence of LPS diminishes permeability to lipophilic (hydrophobic) solutes. Most nutrients therefore enter through protein channels, and indeed most of the proteins found in the outer membrane appear to be involved with channel formation (Nikaido & Vaara, 1985; Nikaido, 1993; 1994). There are, in general, three types of protein channel present in the outer membrane; viz. porins, specific channels and high-affinity receptors.

Porins are large, open water-filled channels which traverse the outer membrane and allow the influx of small, hydrophilic nutrients by passive diffusion, but exclude noxious molecules such as antibiotics. The "classical" porins are homotrimers of subunits with $M_r$ 36,000-38,000 that produce very high permeability (e.g. OmpF of *E. coli*) (Nikaido, 1994). The exclusion limit of porins is thought to be an $M_r$ of approximately 600, which has been estimated to correspond to a pore size of approximately 11Å. Porins tend to show charge preference, e.g. OmpF and OmpC from *E. coli* prefer neutral molecules and cations,
whereas PhoE shows a preference for anions, but all three tend to exclude lipophilic molecules. The production of porins can be physiologically regulated, e.g. *E. coli* PhoE which allows the rapid influx of phosphate and phosphorylated compounds is only expressed when the organism is starved of phosphate.

Porins are not hydrophobic proteins and do not contain long stretches of hydrophobic amino acids of the type seen in cytoplasmic membrane proteins (see below; Nikaido, 1993; 1994). The polypeptide chain of each subunit traverses the membrane 16 times as antiparallel β-strands, forming a β-barrel structure surrounding a large channel. Each subunit forms a channel and therefore each trimer possesses three channels. The polypeptide chain is folded in such a way as to form a constriction in the lumen of the β-barrel. Hence, the channel has a wide entrance, a wide exit and a short central constriction; it is probably the latter that confers the solute-discriminating property of the porin, rather than the overall dimensions of the β-barrel.

In addition to the classical trimeric porins, there are also monomeric porins which allow slow, nonspecific diffusion of small solutes across the outer membrane (e.g. OprF from *P.s. aeruginosa*). The striking characteristics of such porins are that they form wider pores (approximately 20 Å) but demonstrate much slower rates of solute diffusion, approximately two orders of magnitude slower, than trimeric porins. The reason why these monomeric porins exhibit such slow rates of penetration has not been elucidated, but it seems possible that only a small fraction of the protein population folds in the correct conformation to produce functional channels (Nikaido, 1993).

Some nutrient molecules are either too large to enter the nonspecific porin channels, or demonstrate such low rates of diffusion through these porins as to be unusable as physiological substrates. To overcome these limitations, specific channels are used which accelerate their diffusion across the outer membrane. Specific channels can be thought of as porins which contain specific ligand-binding sites (Nikaido, 1993; 1994) e.g. LamB from *E. coli* (facilitating the diffusion of maltose and maltodextrins) and OprB from *P.s. aeruginosa* (facilitating the diffusion of glucose). Although these outer-membrane transport proteins contain specific binding sites they also produce open channels which can allow the passive diffusion of solutes unrelated to the binding site-specific ligand. Due to the presence of a specific binding site, Michalis-Menten saturation kinetics are observed.
during the transport of solutes by specific channels (*i.e.* the rate of diffusion is rapid at low substrate concentrations, but becomes saturated at high concentrations). These channels are therefore highly efficient under nutrient-limited conditions.

High-affinity receptors catalyse the uptake of very large molecules such as Vitamin B$_{12}$ and Fe$^{3+}$-siderophore complexes (Kadner, 1990). These transporters bind their ligands specifically and with high affinity, but do not appear to form open channels (Nikaido, 1994). Unusually, this is thought to be an active process; the energy coupling mechanism remains obscure, but may require the involvement of an inner membrane energy transducing protein such as TonB.

1.4.3 Periplasmic transport systems

Once solutes have crossed the outer membrane and entered the periplasm they can either be metabolised immediately by periplasmic enzymes (*e.g.* Mdh) and then the product(s) transported into the cytoplasm for utilisation, or they can be directly transported into the cytoplasm to be metabolised by cytoplasmic enzymes. Both of these fates require transport across the inner, cytoplasmic membrane. This process can occur by passive or facilitated diffusion but, as the cytoplasmic membrane is impermeable to most solutes (Ames, 1986), occurs mainly by active transport or group translocation. Active transport systems are generally of two types, which differ in their energy transduction mechanisms; *viz.* ion-linked transport systems, and ATP-linked transport systems involving a periplasmic binding protein. Many sugars and other nutrients are transported into bacteria, with a concomitant influx of protons (or less-frequently other ions such as Na$^+$), via a solute-proton symport system which is driven by the protonmotive force (*e.g.* the lactose permease of *E. coli*) (see Henderson, 1986; 1990). Many sugars and other nutrients are also transported into bacteria via periplasmic binding protein transport systems. These consist of a soluble, periplasmic substrate-binding protein and an inner membrane complex containing one or two transmembrane proteins and an ATP-binding protein. These systems are energised by the hydrolysis of ATP, and have been extensively reviewed (see Ames, 1986; Ames *et al.*, 1990; Higgins *et al.*, 1990). Binding protein-dependent systems were originally referred to as osmotic shock-sensitive permeases to distinguish them from the
ion-linked permeases which are unaffected by osmotic shock (binding protein-dependent systems are inactivated by osmotic shock due to the loss of the binding protein). These systems can accumulate substrates against very large concentration gradients (of the order of $10^5$-fold) and are able to scavenge solutes from very low concentrations ($k_m$ for transport is in the range 0.01-1µM cf. 190µM for the lac permease) (Ames et al., 1990). The genes encoding such systems are frequently encoded in a single operon, but may also be found in two divergent operons. These operons may also contain genes which encode proteins involved in the metabolism of the transported solute (Ames, 1986; see also Antonucci & Oxender, 1986). These binding protein-dependent transport systems, and their individual components, are considered in greater detail below. Group translocation, where the solute is chemically modified during transport, is used by numerous anaerobic and facultatively-anaerobic bacteria for the uptake of various sugars. During transport into the cell the sugar is phosphorylated (e.g. the transport of glucose by the phosphotransferase system [PTS] which phosphorylates glucose to glucose 6-phosphate during transport) (Postma et al., 1993). Group translocation will not be considered further.

1.4.3.1 Periplasmic substrate-binding proteins

The role of the periplasmic binding protein is to bind its ligand with high affinity, thus sequestering the solute for later passage into the cytoplasm. Binding proteins are the most thoroughly-analysed components of these systems, since their soluble nature facilitates easy purification and characterisation. All binding proteins so far described are monomeric proteins with $M_r$ values of 25,000-59,000 (Higgins et al., 1990). Although the solutes bound by these proteins have diverse chemical structures, all binding proteins have similar three-dimensional structures, i.e. they are ellipsoid proteins consisting of two similar globular domains with a cleft between them which forms the substrate binding site (Sack et al., 1989; Ames et al., 1990; Higgins et al., 1990). The protein is flexible and undergoes a conformational change after binding the substrate (Boos et al., 1972), thus narrowing the cleft and trapping the substrate deep within the molecule (this mechanism has been referred to as a "venus flytrap"). The substrate is primarily bound via hydrogen bonds. The affinity of binding proteins for their substrate is very high, with $K_D$ values between 0.1 and 1µM.
for sugar substrates and approximately 0.1μM for amino acids (Ames et al., 1990). The conformational change which is induced in the binding protein upon substrate binding is thought to be important during the second stage of transport i.e. the interaction with the membrane bound components. It is commonly thought that unliganded binding proteins cannot interact with these components. The affinity of the liganded binding protein for the membrane components of the histidine uptake system has been found to be approximately 10μM (Prossnitz et al., 1989; Ames et al., 1990), and it is therefore this complex which probably interacts with the membrane components of the uptake system.

1.4.3.2 Membrane-bound components

The membrane-bound components of binding protein-dependent transport systems are of two types; viz. hydrophobic integral membrane proteins and hydrophilic peripheral membrane proteins containing ATP-binding domains. Both type of protein are required for transport to occur. The concentration of these membrane proteins in the cell is very low compared with the concentration of the binding protein (at least 30-fold lower) and a single membrane bound complex can be used by more than one binding protein (Ames et al., 1990).

Two hydrophobic membrane proteins are required for transport activity and they may be either the same or different proteins, forming a homodimer or a heterodimer respectively. Irrespective of this, all of the hydrophobic membrane proteins have similar three dimensional structures. Each consists of a core structure of six membrane spanning α-helices separated by short stretches of hydrophilic amino acids. Hence the two hydrophobic proteins together form twelve membrane-spanning helices, which appears to be a recurrent feature of active transporters (Maloney, 1990 quoted in Higgins et al., 1990). One of the hydrophilic domains of the membrane protein is highly conserved and exposed to the cytoplasmic side of the membrane, enabling it interact with the peripherally associated ATP-binding protein (Higgins et al., 1990). The hydrophobic membrane-spanning proteins are therefore thought to interact with the binding proteins on the periplasmic side of the cytoplasmic membrane and the ATP-binding proteins on the cytoplasmic face of the
membrane. It is this interaction which is thought to facilitate the transport of solute from the periplasm to the cytoplasm (see below).

Finally, each binding protein-dependent transport system requires one or two hydrophilic proteins which are associated with the cytoplasmic membrane. These proteins contain ATP-binding sites and are thought to catalyse ATP hydrolysis (Higgins et al., 1990). Like the hydrophobic proteins, two ATP-binding proteins (or a single protein containing two ATP-binding sites; see Higgins et al., 1990) are required for transport activity. These may be identical or different proteins, forming a homodimer or a heterodimer respectively.

The possession of these ATP-binding proteins, and the observation that similar transport proteins can be found in the cytoplasmic membranes of numerous and diverse organisms (see Ames et al., 1990), has led to them being referred to as “traffic ATPases” or “ABC (ATP-binding cassette) transporters.”

1.4.3.3 Transport model

Models of transport for binding protein-dependent transport systems have been proposed by Ames & Higgins (1983), Ames (1986) and Ames et al. (1990). These models draw upon all of the information gained from various systems and attempt to unify them in a general mechanism. It is envisaged that the substrate enters the periplasm by passage through the outer membrane and binds reversibly to the binding protein. This causes the total concentration of substrate (bound plus free) to be higher in the periplasm (the free concentration of substrate being the same in the periplasm and the surrounding medium), and this difference is dependent upon the concentration of binding protein in the periplasm and its affinity for the substrate. Upon liganding the binding protein undergoes a conformational change which increases its affinity for the membrane-bound complex. The binding protein then interacts with the membrane-bound apparatus and triggers a conformational change within this protein complex. The substrate is then released from the binding protein and enters the cytoplasm. The exact mechanism by which this occurs has not been conclusively determined, but may occur by one of two proposed routes; (i) the conformational change induced in the membrane bound-complex by interaction with the liganded binding protein forms a pore in the membrane components via which the substrate
enters the cell, or (ii) interaction with liganded binding protein forms, or activates, binding sites for the substrate on the components of the membrane complex and the substrate then enters the cytoplasm by being passed from protein to protein within the membrane-bound complex in a cascade-like manner. This second mechanism of passage through the cytoplasmic membrane requires the binding sites to be juxtaposed to each other to prevent release of the substrate back into the periplasm, which would negate any specific function of the binding protein. ATP is hydrolysed concomitantly during substrate passage, possibly as a consequence of the liganded binding protein interacting with the membrane complex. Once the binding protein has passed its substrate through the membrane complex it dissociates due to its decreased affinity for the complex. ATP hydrolysis may alternatively be required to release the binding protein and/or to close the putative pore formed in the membrane complex. The cycle then repeats itself.

1.4.3.4 Evolutionary relationship between binding proteins

The relative complexity of periplasmic binding protein-dependent uptake systems and their widespread occurrence in different genera of bacteria has been suggested to be indicative of a single ancestral system. Based upon this hypothesis, the numerous systems present in contemporary bacteria which transport diverse solutes have evolved from a progenitor system by divergence to different substrate specificities, while retaining the same basic architecture (Ames & Higgins, 1983). It has also been noted that the ABC-type proteins which are involved in bacterial binding protein-dependent systems show significant homology to other proteins demonstrating similar roles in organisms as diverse as other bacteria and humans (see Ames et al., 1990; Saier, 1994). However, only the interrelatedness of periplasmic binding proteins will be considered here.

The structural, functional and evolutionary relationships between periplasmic binding proteins of bacteria has recently been investigated by Tam & Saier (1993). This was carried out by the computer-aided comparison of the amino acid sequences of approximately 50 bacterial binding proteins to define their relatedness. These comparisons established that some of the proteins were clearly (or probably) related by descent, whereas others demonstrated too great a degree of sequence divergence to allow the establishment of a
common evolutionary origin on the basis of amino acid sequence similarities alone. What became clear during the study was that the vast majority of binding proteins could be grouped into eight families or clusters (clusters 1-8), which share similar molecular size and substrate specificities. It was also found that, in general, proteins which bind the same or similar substrates, but are from evolutionary divergent bacteria, have more similar amino acid sequences than do binding proteins from the same organism which bind vastly different substrates. This suggested that solute binding imposes a severe constraint on the amino acid sequence and structure of proteins.

Cluster 1 proteins bind oligosaccharides, glycerol 3-phosphate and iron. Cluster 2 proteins bind pentoses and hexoses; this cluster also demonstrates homology to a group of bacterial transcription factors, which are known to bind sugars. Cluster 3 proteins bind polar amino acids and opines. Cluster 4 proteins bind aliphatic and aromatic amino acids; this family of binding proteins are of particular pertinence to this study as they also show homology to AmiC from *Ps. aeruginosa* (these homologies will be further discussed in Chapter 5). Cluster 5 proteins bind peptides and nickel. Cluster 6 proteins bind multivalent-inorganic-anions. Cluster 7 contains two divergent proteins which bind multivalent-organic-polyanions. Finally, cluster 8 proteins bind organic-iron complexes and may also include vitamin B₁₂-binding proteins. As more binding proteins are sequenced, additional members of each cluster will no doubt be discovered, as probably will additional clusters.

1.5 Objectives

The objectives of this work were generally five-fold; (i) to determine the physiological regulation of acetamidase, formamidase and urease activity in *M. methylotrophus*, (ii) to determine the physiological and biochemical mechanisms of acetamidase switch-off, (iii) to investigate amide transport into *M. methylotrophus*, (iv) to isolate mutants of *M. methylotrophus* by experimental evolution using formamide-limited continuous culture, and (v) to determine the interrelatedness of microbial amidases using comparative immunology.
CHAPTER 2

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MATERIALS AND METHODS

2.1 Growth and maintenance of *Methylophilus methylotrophus*

2.1.1 Maintenance of *M. methylotrophus*

Stock cultures of *M. methylotrophus* (NCIB 10515; ZENECA ref. G37 (5m) vial 126), kindly provided by ZENECA Bio Products, Billingham, Cleveland, TS23 1YN (formerly known as ICI Biological Products) were maintained in 20% (v/v) sterile glycerol at -70°C and on mineral salts (SEII) agar plates and slopes. SEII-agar contained in 1 l: K$_2$HPO$_4$, 1.90g; NaH$_2$P0$_4$, 1.56g; MgSO$_4$, 20mg; (NH$_4$)$_2$SO$_4$, 1.80g; FeCl$_3$6H$_2$O, 9.70mg; Fisons trace elements solution (Cu, 5ppm; Mn, 24-25ppm; Zn, 22-23ppm; Ca, 720ppm, supplied by ZENECA), 1ml; Oxoid purified agar, 15g. The medium was autoclaved at 121°C (15psi) for 15 min and cooled to approximately 40°C before the aseptic addition of methanol to a final concentration of 4g l$^{-1}$. Stock cultures were regularly maintained on SEII-agar plates which were subcultured every 2-4 weeks and stored at 4°C when not in use.

2.1.2 Growth of *M. methylotrophus* in batch culture

*M. methylotrophus* was grown in batch culture as an inoculum for continuous culture and also for the identification of mutant strains. When used for this purpose, *M. methylotrophus* was inoculated into 100ml of sterile SEII liquid medium (as described in section 2.1.1 but minus agar and plus 4g l$^{-1}$ methanol) contained in a 250ml conical side-arm flask. The culture was incubated at 37°C and agitated at about 150rpm in an orbital shaker (Gallenkamp) for about 16h.

When used to inoculate cultures for the subsequent identification of mutant strains of *M. methylotrophus*, a similar procedure as described above was used except that randomly-selected single colonies were inoculated into a 50ml conical flask containing 5ml of sterile SEII medium (without (NH$_4$)$_2$SO$_4$) supplemented with 0.32g l$^{-1}$ filter sterile formamide.
and 5.76g l⁻¹ methanol. The cultures were incubated under the same physical conditions as described above.

2.1.3 Growth of *M. methylotrophus* in chemostat continuous culture

*M. methylotrophus* was grown in chemostat continuous culture in a 1l laboratory fermenter (working volume 980ml, height to diameter ratio 1.5 with 4 baffles radially situated at 90º, two disc-turbine impellers and a bottom magnetic stirrer; Series 500, LH Engineering) at a dilution rate of 0.1h⁻¹. Culture was initiated by inoculating 700ml of sterile SEII medium contained in a fermenter with 100ml of overnight *M. methylotrophus* batch culture. The inoculated culture was allowed to grow in batch for approximately 6h before initiating continuous cultivation. The continuous culture medium contained in 1l: MgSO₄·7H₂O, 0.27g; K₂SO₄, 35mg; K₂HPO₄, 1.12g; 1.1M-H₃PO₄, 3.75ml; Fisons trace elements solution, 6ml. The medium was sterilised by autoclaving (121°C, 15psi for 60min) before the aseptic addition of the appropriate amount of methanol and filter-sterilised nitrogen source (see Table 2.1 for input concentrations). Iron was introduced into the culture separately as a solution containing in 1l: FeSO₄·7H₂O, 0.16g and H₂SO₄, 2ml; pumped in at approximately 2ml hr⁻¹.

The pH of the medium was controlled at pH 7.0±0.1 by the addition of 10% (v/v) ammonia solution when growing under ammonia-excess conditions, and by the addition of 2M-KOH under all other growth regimes. The culture was grown at 37°C, an agitation rate of approximately 800rpm and an aeration rate of 0.5v v⁻¹(min⁻¹).

Culture samples were checked to ensure that steady state carbon and/or nitrogen limitation had been reached after approximately six changes in culture volume. The nitrogen status of the culture was determined by measuring the ammonia concentration (section 2.6.1) in the culture supernatant (section 2.4); no detectable ammonia was taken to indicate nitrogen limitation. The carbon status of the culture was determined by placing 1ml of fresh culture immediately (<1min) into an oxygen electrode (see section 2.6.6) containing 2ml of 20mM-glycylglycine buffer pH 7.0 at 37°C. Any endogenous rate of respiration was followed for several min before the addition of methanol to a final
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Table 2: Nutrient Input Concentrations for Growth of M. methylotrophus in Continuous Culture

M. methylotrophus was grown in continuous culture (D=0.1 h⁻¹) under various nutrient-limited conditions with the carbon and nitrogen inputs below.
concentration of 1.3 g l$^{-1}$. Carbon limitation was inferred if no endogenous rate of respiration was seen, together with a high rate of respiration after the addition of methanol.

Purity checks were performed on the culture at regular intervals by plating culture samples on to SEII-agar plates, and also on to nutrient-agar plates containing in 1: Oxoid nutrient broth, 15g and Oxoid nutrient agar, 15g; sterilised by autoclaving (section 2.1.1). As M. methylotrophus does not grow on nutrient agar, overnight growth was taken to indicate culture contamination.

2.2 Determination of cell density

The cell density of M. methylotrophus suspensions was determined spectrophotometrically from the OD$_{600nm}$ measured in a Pye-Unicam SP600 UV spectrophotometer (4ml disposable cuvettes, 1cm pathlength) against a water blank. The OD$_{600nm}$ was found to be a linear function of the cell density up to an optical density ≤1.0 with the following relationship:

$$\text{Cell density (mg dry wt. ml}^{-1}) = \text{OD}_{600nm} \times 0.53$$

2.3 Preparation of cell suspensions

Cells were harvested by centrifugation in an MSE High Speed 18 centrifuge at 10,000rpm (12,200 x g) for 15 min at 4°C. Cell pellets were then washed in 20mM-Bis/Tris buffer pH 6.8, re-centrifuged and finally resuspended to approximately 5mg dry wt. ml$^{-1}$ in the same buffer.

2.4 Preparation of culture supernatants

Culture supernatants were obtained using two methods; (i) a culture sample (approximately 20ml) was taken from the chemostat and immediately (<1min) filtered through a 0.2µ pore size Acrodisc (Gelman Sciences) into a 20ml sterile plastic universal bottle (Sterilin), and (ii) A culture sample (1.5ml) was immediately placed into an
Eppendorf microfuge tube and the cells removed using an MSE MicroCentaur bench-top centrifuge operating at full speed (13,000 rpm; 13,400 x g) for 1 min; the culture supernatant was then carefully removed using a Gilson pipette.

2.5 Preparation of sub-cellular fractions

2.5.1 High speed supernatant and crude-membrane fractions

Cells were harvested and washed as above but resuspended to 40-50 mg dry wt. ml⁻¹. Cell suspensions were disrupted by passage three times through an Aminco French pressure cell at 15,000 psi. Unbroken cells and cell debris were removed by centrifugation at 18,000 rpm (40,000 x g) for 20 min in an MSE High Speed 18 centrifuge to produce a cell-free extract. Cell membranes were removed by centrifuging the cell-free extract in a Sorval OTB 65B centrifuge at 44,000 rpm (175,200 x g) using a TFT 50.38 fixed angle rotor for 75 min. The resultant high speed supernatant was removed carefully with a Pasteur pipette and, if not used immediately, was stored frozen at -20°C until required. The high speed membrane fraction was resuspended in 20 mM Bis/Tris buffer pH 6.8 using a hand held glass homogeniser to the same volume as the original washed-cell suspension.

2.5.2 Purified membrane fractions

Purified membrane fractions were prepared using a modification of the method described by Hancock & Carey (1979) by sucrose density-gradient centrifugation of the crude membrane fractions prepared as described in section 2.5.1. Several different sucrose gradients were used. The initial gradient was prepared by pipetting 2 ml 70% (w/v) sucrose into the bottom of a 12 ml plastic centrifuge tube (Polyallomer; Beckman) then very carefully layering 3 ml 60% (w/v) sucrose on top of it, and finally 3 ml 20% (w/v) sucrose was layered on top to produce a stepped-gradient of 70, 60 and 20% (w/v) sucrose. The interfaces between the different layers were marked on the tube for later reference. Crude membranes to be fractionated (2 ml) were then carefully layered on the top of the gradient. The separation was carried out at 33,000 rpm (183,000 x g) in a Sorval OTB 65B ultra...
centrifuge using a Sorval TH 641 swing-bucket rotor. After the gradients had reached equilibrium (approximately 16h) each layer was removed using the markers on the tube as a guide and put into separate centrifuge tubes. The fractions were then diluted with approximately 5ml Elga water before being recentrifuged at 55,000rpm (100,000 x g) for 60min using a TFT 65.13 fixed angle rotor. The resultant pellets were resuspended to a final volume of 2ml in 20mM-Bis/Tris buffer pH 6.8 using a hand held glass homogeniser. In subsequent experiments, crude membranes were also purified using gradients of 70, 64, 58, 52, 20% and 70, 60, 58, 55 and 20% (w/v) sucrose.

2.5.3 Shock fluid (periplasm)

Shock fluids were prepared from formamide-limited cultures of *M. methylotrophus* by several different methods. The initial method was that of Silman (1990). Approximately 200ml of culture was harvested by centrifugation (section 2.3) and washed twice in 30mM-Tris/HCl buffer pH 8.0. The washed cells were then resuspended to a cell density of 10-15mg dry wt. ml\(^{-1}\) in 30mM-Tris/HCl buffer pH 8.0 + 10% (w/v) sucrose and the volume noted. The cells were plasmolysed by this buffer and the integrity of the outer membrane disrupted by adding aliquots of 100mM-EDTA (50μl of per ml cells) over 10min whilst shaking in a water bath at 30°C. The plasmolysed cells were then harvested by centrifugation at 10,000rpm (12,200 x g) for 15min and subjected to osmotic shock by resuspension in the noted volume of 20mM-HEPES buffer pH 7.0. The shocked cells were then stirred on ice for 10min before being centrifuged at 18,000rpm (40,000 x g) for 20min. The shock fluid was then carefully removed and the shocked cells resuspended to the noted volume in 20mM-HEPES buffer pH 7.0. The shocked cells were then disrupted by sonication (4 x 30s bursts in a Soniprep 150 ultrasonicator (MSE) with alternate 1min cooling periods in an ice/salt/water bath). High-speed supernatant and crude membrane fractions were then prepared from the broken cells as detailed in section 2.5.1.

The second method was that of Eftekhar & Schiller (1994). Approximately 200ml of culture was harvested, washed and resuspended to a cell density of 10-15mg dry wt. ml\(^{-1}\) in 20mM-Bis/Tris buffer pH 6.8 and the volume noted. The washed cells were made 0.2M with respect to MgCl\(_2\) by adding an appropriate amount of stock 2M-MgCl\(_2\). The cells
were then incubated at 37°C for 10 min before being subjected to a temperature shock by incubation at 0°C for 15 min. This temperature cycle was repeated four times before the shocked cells were recovered by centrifugation at 18,000 rpm (40,000 x g) for 20 min. The shock fluid was carefully removed with a Pasteur pipette and the shocked cells resuspended to the noted volume in 20 mM Bis/Tris buffer pH 6.8. The shocked cells were then disrupted by sonication and the high-speed supernatant and crude membrane fractions produced as detailed in section 2.5.1.

The third method was a modification of that described by Cheng et al. (1970). Approximately 200 ml of culture was harvested, washed twice in 10 mM Tris/HCl buffer pH 8.4 and then resuspended to a cell density of 10-15 mg dry wt ml⁻¹ and the volume noted. Enough stock 2M MgCl₂ was then added to make the cell suspension 0.2M MgCl₂ and also enough stock 1 mg ml⁻¹ lysozyme (Sigma) to make the suspension 0.1 mg ml⁻¹ lysozyme. The suspension was then mixed gently at room temperature for 30 min before removing the shocked cells by centrifugation at 18,000 rpm (40,000 x g) for 20 min. The shock fluid was removed carefully with a Pasteur pipette and the shocked cells were resuspended to the noted volume in 10 mM Tris/HCl buffer pH 8.4 before being disrupted by sonication and the high-speed supernatant and crude membrane fractions prepared as described above.

2.6 Measurement of enzyme activities and kinetic characteristics

2.6.1 Amidohydrolase activity

Amidohydrolase (acetamidase and formamidase) activity was measured by following the initial rate of production of ammonia from the appropriate amide at 37°C. The reaction can be summarised by the following general equation:

\[
R.C{O.NH}_2 + H_2O \rightarrow R.COO^- + NH_4^+
\]

Amidase activities were calculated from a linear plot of the A₆₃₀ versus time (min) as described below. The ammonia concentration was measured colorimetrically using a
phenol/hypochlorite/nitroprusside assay (M. A. Carver, unpublished) modified from that of Muftic (1964).

Assay mixtures varied with respect to their absolute composition depending upon the nature of the sample being assayed, but the final volume of the reaction mixture after the addition of substrate was always 1ml. For washed cells, a volume corresponding to about 0.4mg dry cell wt. was added to an appropriate volume of prewarmed 0.1M-Citric acid/0.2M-Na$_2$HPO$_4$ buffer pH 6.0. For pure or partially-pure acetamidase or formamidase a volume corresponding to 1-4µg protein was treated in a similar manner. The assay mix was allowed to equilibrate at 37°C for several min before the reaction was started by the addition of 50µl of 1.0M-amide (final concentration 50mM; acetamide: Sigma; formamide: PHlqBIO/Fisons). Aliquots (100µl) from the assay mix were withdrawn at time intervals of 1min, over a 5min time course, into 1ml of Fisons sodium phenoxide/acetone reagent (0.675M-NaOH; 0.064M-phenol; 0.136M-acetone in water) to stop the reaction immediately. The colour was developed by the addition of 1.5ml of 0.01% (w/v) sodium nitroprusside (Fisons; AR grade) in 0.1M-sodium acetate/0.08M-HCl buffer pH 6.0, followed by 1.5ml of 0.5%(v/v) sodium hypochlorite (Spectrosol 12% 'available chlorine' sodium hypochlorite solution; BDH) in the same buffer. The mixture was stored in the dark at room temperature for 30min prior to measuring the A$_{630}$nm in a Pye-Unicam SP600 spectrophotometer (4ml disposable cuvettes, 1cm pathlength) against a water blank. The amount of ammonia released was calculated by extrapolation from a standard curve which was produced daily; aliquots (100µl) of a standard NH$_4$Cl solution in 0.1M-citric acid/0.2M-Na$_2$HPO$_4$ buffer pH 6.0 containing 0.00, 0.05, 0.10 and 0.15µmol ammonia were pipetted into 1ml of sodium phenoxide/acetone reagent and the colour developed as described above. The A$_{630}$nm of the "blank" standard was subtracted from all of the absorbances of the standards and assay results before calculating enzyme activity.

Occasionally, a discontinuous amidase assay was used which was the same as that described above except that only one aliquot of reaction mixture was taken after 5 min. This modified method was used to screen FPLC fractions collected during enzyme purification.

Ammonia concentrations of culture supernatants were also measured using the same spectrophotometric method. Aliquots (100µl) of culture supernatant (section 2.4) were
pipetted into 1ml phenoxide/acetone reagent and the colour developed. The ammonia concentration was then calculated by reference to the standard curve.

2.6.2 Urease activity

Urease activity was measured by following the initial rate of ammonia production from urea at 37°C. The reaction can be summarised by the following equation:

\[
\text{NH}_2\text{CO.NH}_2 + \text{H}_2\text{O} + 2\text{H}^+ \rightarrow \text{CO}_2 + 2\text{NH}_4^+
\]

Urease activity was measured by a modification of the method described above (section 2.6.1) with 0.1M-MES pH 7.0 as reaction buffer and 50mM (final concentration) urea (USB; Ultra pure) as substrate. Enzyme activities were expressed as the amount of ammonia produced from urea hydrolysis.

2.6.3 NADH oxidase activity

NADH oxidase activity of cell extracts was measured spectrophotometrically at 37°C by following the oxidation of NADH at 340nm. This activity was used as a “marker” for inner membranes. Reaction mixtures (1ml) were prepared in 1ml disposable plastic cuvettes (1cm pathlength). The reaction mixture contained 0.83ml prewarmed 0.1M-Tris/HCl buffer pH 8.0, 50μl 0.1M-MgCl₂ and 100μl neat extract. A blank cuvette was set up as above, but using 0.85ml of buffer, and put in to the reference cell of a Pye-Unicam SP1800 spectrophotometer with temperature control at 37°C. The test cuvette was inserted into the spectrophotometer and the chart recorder set to read zero. Any endogenous rate was followed for several min before the reaction was started by adding 20μl of 5.5mM-NADH (final concentration 110μM; Boehringer) to the test cuvette and mixing. The linear change in A340nm was followed for several min, and the activity calculated using the mM absorbance coefficient for NADH of 6.22.
2.6.4 Glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase activity of cell extracts was measured at 37°C by following the reduction of NADP+ at 340 nm in the presence of glucose-6-phosphate. This activity was used as a cytoplasmic "marker". Reaction mixtures (3ml) were prepared in 4ml disposable plastic cuvettes (1cm pathlength). The reaction mixture contained 2.73ml of prewarmed 0.1M-Tris/HCl buffer pH 8.0, 100μl 0.1M-MgCl2, 20μl 50mM NADP+ (Sigma) and 100μl of suitably-diluted cell extract. A blank cuvette was set up as above but containing 2.78ml of buffer. The reaction mixture and blank were inserted into a Pye-Unicam SP1800 spectrophotometer with the temperature controlled at 37°C. The chart recorder was set to zero and any endogenous rate followed for several min. The reaction was started by adding 50μl of 100mM-glucose-6-phosphate (final concentration 1.7mM; Sigma) and mixing. The linear change in A340nm was followed for several min and the activity was calculated using the mM absorbance coefficient for NADP+ of 6.22.

2.6.5 2 Keto-3-deoxyheptonic acid (KDO) concentration

KDO concentration was determined by the method of Weissback & Hurwitz (1959) and was used as an outer membrane "marker". Appropriately-diluted samples of membrane fraction were prepared to 0.2ml in clean, dry test tubes to which an equal volume of 0.025M-HIO4 in 0.125N-H2SO4 was added. The solution was mixed and left at room temperature for 20min, after which time 0.5ml of 2% (w/v) sodium arsenite in 0.5N-HCl was added and thoroughly mixed. The mixture was then allowed to stand at room temperature for a further 2min before the addition of 2ml of 0.3% (w/v) thiobarbituric acid pH 2.0 (Sigma). The solution was then incubated in a boiling water bath for 10min, after which the solution was allowed to cool and the A548nm measured in a Pye-Unicam SP1800 spectrophotometer (4ml disposable cuvettes, 1cm light path) against a reagent blank. The A548nm of each fraction was expressed as a percentage of the total.
2.6.6 Measurement of methanol and formate oxidation by washed cells

Methanol and formate oxidation by washed cells of *M. methylotrophus* was measured at 40°C by following the decrease in dissolved oxygen concentration in a polarographic oxygen electrode (Rank Bros., Cambridge) fitted with a thin Teflon membrane and attached to a chart recorder. Reaction mixtures were set up so that the total volume after all additions was 4ml. Reactions were carried out in prewarmed 20mM-glycylglycine buffer pH 7.0 which was allowed to equilibrate in the oxygen electrode with the top on until a steady base line was seen on the chart recorder. Washed cells (1mg dry wt.) were then introduced into the oxygen electrode chamber using a glass Hamilton syringe. The height of the electrode top was raised to ensure that nearly all the liquid was present in the chamber, being careful to making sure that no air bubbles were introduced into the chamber during this operation. The endogenous respiration rate was followed for several min. The reaction was then initiated by the addition of 40μl of methanol (final concentration 250mM; Fisons HPLC grade) or 1.5M-potassium formate (pH 7.0; final concentration 15mM), and the resultant linear decrease in dissolved oxygen concentration was followed for several minutes. Rates of methanol and formate oxidation (ng atom oxygen min⁻¹ [mg dry wt. cells]⁻¹) were calculated using a solubility for oxygen at 40°C of 420ng ml⁻¹. Final corrected rates were obtained by subtraction of any endogenous rate.

2.6.7 Determination of amidase k_cat

The k_cat (s⁻¹) of acetamidase and formamidase in whole cells was determined from the amidase specific activity (μmol min⁻¹[mg cell protein]⁻¹), the amidase concentration (% cell protein) as determined by analytical SDS-PAGE and gel scanning (section 2.7.1) and the native Mr (acetamidase 155,000 (Silman, 1990); formamidase 130,000 (J.A. Greenwood, personal communication) using the following relationship:

\[
\text{k_cat (s}^{-1}) = \frac{\text{Specific activity} \times \text{Mr} \times 100}{60 \times [\text{enzyme (%cell protein)}] \times 0.65}
\]
The $k_{\text{cat}}$ ($s^{-1}$) of purified acetamidase and formamidase was determined from the specific activity and the native $M_r$, taking the purified enzymes to be essentially homogeneous, by the following relationship:

$$k_{\text{cat}} (s^{-1}) = \frac{\text{Specific activity} \times M_r}{60}$$

2.6.8 Determination of Michaelis constant ($K_m$)

The Michaelis constant ($K_m$) of formamidase with respect to formamide was determined by using a modification of the standard amidohydrolase assay (section 2.6.1). Formamidase activity was measured over a wide range of formamide concentrations (0.1-50mM) using an appropriate constant amount of washed cells (80µg dry wt. cells) or pure enzyme (3 µg protein). The $K_m$ was then determined from a hyperbolic plot of formamidase specific activity ($v$) against formamide concentration ($s$) by an Apple-Macintosh microcomputer with KFitSim computer software (kindly lent by C.R. Bagshaw).

2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.7.1 Analytical SDS-PAGE

Discontinuous electrophoresis of proteins was carried out using two different slab gel systems; large (1.5mm thick, Hoefer Scientific Instruments) and small (minigel) (Mini-Protean II dual slab cell, Bio-Rad; minigel, 0.75mm thick). Large gels consisted of a stacking gel (about 10ml) containing 2.5% (w/v) acrylamide (PROTOGEL Protein and sequencing electrophoresis grade; National Diagnostics) and 0.1% (w/v) SDS (Sodium dodecyl sulphate) in 125mM-Tris/HCl buffer pH 6.8, and a resolving gel (28ml) containing 12.5% (w/v) acrylamide and 0.1% (w/v) SDS in 375mM-Tris/HCl buffer pH 8.8. Gel solutions were mixed and degassed under vacuum for about five min prior to polymerisation to create microaerobic conditions. Large stacking and separating gels were polymerised by the addition of 0.05% (v/v) TEMED (Sigma) and either 0.075% (v/v) or
0.075% (w/v) ammonium persulphate respectively. Minigels consisted of a stacking gel (approximately 2ml) containing 4% (w/v) acrylamide and 0.1% (w/v) SDS in 125mM-Tris/HCl buffer pH 6.8, and a resolving gel (3.5ml) containing 12% (w/v) acrylamide and 0.1% (w/v) SDS in 375mM-Tris/HCl buffer pH 8.8. Minigel stacking and separating gels were polymerised by the addition of 0.2% (v/v) and 0.05% (v/v) TEMED respectively and 0.1% (w/v) and 0.05% (w/v) ammonium persulphate respectively. For separation of both sizes of gel the reservoir buffers contained 25mM-Tris (base), 192mM-glycine and 0.1% (w/v) SDS.

Whole cells of *M. methylotrophus* and pure and partially-pure amidases were mixed with dissociating buffer (60mM-Tris/HCl pH 6.8, 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 5% (v/v) glycerol and 1% (w/v) bromophenol blue) in the proportion 3:1 (sample:buffer) and heated in a boiling water bath for 2 min in capped Eppendorf tubes. A volume containing about 30μg dry wt. cells for large gels and 15μg dry wt. cells for minigels was then loaded on to the gel. When partially-purified or pure enzymes were separated volumes of sample were loaded to produce discrete bands on the gel; about 1-2μg per band for a large gel and about 0.5μg per band on a minigel. Electrophoresis was carried out at 40mA constant current for large gels and 200V constant voltage for minigels until the dye marker reached at the bottom of the gel. The gel was then removed from the apparatus and one corner marked for later orientation identification. The gel was washed briefly in water before staining. Large gels were stained overnight and minigels for 1h with Kenacid blue R (10% (w/v) Kenacid blue R, 45% (v/v) methanol and 10% (v/v) glacial acetic acid in Elga water). The gel was then destained by washing in 7.5% (v/v) glacial acetic acid and 5% (v/v) methanol in Elga water, until all background staining had been removed.

Dalton Mark VII-L M₇ markers (Sigma) were also run on the gel to allow the calculation of M₇. The standard proteins were: α-lactalbumin (M₇ 14,200), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000) and bovine serum albumin monomer (66,000).

Destained SDS-polyacrylamide gels were photographed and, when required, were also quantitatively analysed using a GDS2000 gel documentation system (UVP International).
2.7.2 Preparative SDS-PAGE

This technique was used to prepare completely-pure acetamidase and formamidase for raising antibodies. A superose-6 fraction containing almost-pure enzyme was separated on a large SDS-polyacrylamide gel, and two small strips were removed from each side of the gel prior to being stained for 5 min in Kenacid blue R. The strips were then carefully lined up with the unstained gel and the area of gel containing the required protein was excised using a scalpel. This piece of gel was then cut into small strips and put into dialysis tubing containing a small volume of electroelution buffer (62.5 mM-Tris, 48 mM-glycine pH 8.3). The tubing was sealed at both ends and put into a flat-bed electrophoresis tank containing the same buffer. The gel was moved close to the side of the tubing which faced the positive electrode, and the protein was eluted overnight at 4°C using a constant current of 40 mA. The eluted protein was removed from the side of the dialysis tubing by reversing the polarity of the electrodes for 2 min before the sample was carefully removed from the dialysis tubing. The approximate protein concentration of the sample was determined by measuring the A280 and A260 of a 1 ml aliquot (section 2.9.2).

2.8 Protein purification

2.8.1 Purification of acetamidase by Fast Protein Liquid Chromatography (FPLC)

Acetamidase was purified from M. methylotrophus high-speed supernatants prepared from cells grown in continuous culture under acetamide and dual acetamide/methanol limitation (D=0.1 h⁻¹) by the method of Wyborn (1994) using anion-exchange and gel-filtration FPLC. Frozen high-speed supernatant was thawed and aliquots containing approximately 200 mg protein were filtered by passage through a 0.2 µm acrodisc prior to being loaded on to a Mono-Q 10/10 anion exchange column (bed volume 8 ml; Pharmacia) pre-equilibrated with 20 mM-Bis/Tris buffer pH 6.8. All FPLC buffers were filtered (0.2 µ
pore size, cellulose nitrate membrane filter; Whatman) and degassed before use. Under these conditions the majority of soluble proteins bound to the column, and were sequentially eluted using a linear KCl gradient (0-1M-KCl in 20min at a flow rate of 4ml min⁻¹) formed by differential pumping (Pump P-500; Pharmacia) of a high salt buffer, 20mM-Bis/Tris buffer pH 6.8 + 1M-KCl (controlled by a GP-250 gradient controller; Pharmacia) on to the column. The absorbance (A₂₈₀nm) of the column eluent was continuously monitored using a UV-detector flow cell connected to a chart recorder (Pharmacia) and plotted against elution volume/fraction number to give an elution profile. Fractions (4ml) were collected throughout the elution process using an automatic fraction collector (FRAC-100; Pharmacia), and were assayed discontinuously for acetamidase activity (section 2.6.1). Acetamidase eluted from the Mono-Q column at a salt concentration of 270-290mM-KCl. Fractions containing peak acetamidase activity were pooled, taking care to avoid neighbouring fractions with high formamidase activity, then desalted using PD-10 desalting columns equilibrated with 20mM-Bis/Tris buffer pH 6.8, according to the manufacturer's instructions (Pharmacia). The desalted pool was then re-loaded on to the Mono-Q 10/10 anion-exchange column pre-equilibrated with 20mM-Bis/Tris buffer pH 6.8, and bound proteins were eluted using a shallower salt gradient (0-350mM-KCl in 24min at 4ml min⁻¹). Fractions (4ml) were collected, assayed and the peak acetamidase fractions pooled as described above.

The pool was concentrated using a stirred ultra-filtration cell (Amicon) at 4°C with a 10,000 (M₀) exclusion limit membrane (YM-10; Diaflo) to a final volume of 200-300μl. Depending on the protein concentration, the concentrated sample was then loaded on to a Superose-6 HR 10/30 or 16/50 column (25ml and 100ml bed volume respectively; Pharmacia) pre-equilibrated with 20mM-Bis/Tris buffer pH 6.8 + 100mM-KCl. Proteins were eluted using the same buffer at a flow rate of either 0.3ml min⁻¹ (Superose-6 HR 10/30 column) or 0.25ml min⁻¹ (Superose-6 HR 16/50 column). Fractions (0.25 or 1ml) were collected and A₂₈₀nm was continuously measured as described above. Fractions corresponding to protein peaks were assayed for acetamidase activity using the discontinuous assay. Acetamidase eluted from the gel-filtration columns with an elution volume (Vₑ) of approximately 14.5ml (Superose-6 HR 10/30 column) or 63ml (Superose-6 HR 16/50 column). Fractions containing peak acetamidase activity were pooled and
adjudged to be ≥ 90% pure by SDS-PAGE and gel analysis (section 2.7.1). The acetamidase was stored at -20°C until required.

During the purification procedure column fractions and pools were regularly stored at 4°C overnight or stored frozen for longer periods of time.

2.8.2 Purification of formamidase by FPLC

Formamidase was purified from *M. methylotrophus* high speed supernatants prepared from cells grown in continuous culture under formamide or dual acetamide/methanol limitation (D=0.1h⁻¹) essentially as described by Wyborn et al. (1994) and Wyborn (1994). Fractions (4ml) eluting from the first Mono-Q column (section 2.8.1) were assayed discontinuously for formamidase activity (Section 2.6.1). Formamidase eluted from the Mono-Q column at a salt concentration of approximately 340mM-KCl, and fractions containing peak formamidase activity were pooled, taking care to avoid neighbouring fractions containing acetamidase. A second anion-exchange chromatography step followed by Superose-6 gel-filtration chromatography were carried out as described above for acetamidase. Formamidase eluted from the Superose-6 HR 10/30 column with an elution volume (Ve) of approximately 15ml at a flow rate of 0.3ml min⁻¹. Fractions containing peak formamidase activity were pooled and adjudged to be ≥ 90% pure by SDS-PAGE and gel analysis (section 2.7.1). This purification procedure gave a yield of formamidase of approximately 28%. The formamidase was stored at -20°C until required.

Formamidase was also purified from *M. methylotrophus* strain MM25 by a modification of this method as described below for purification of the urea/formamide-binding protein (section 2.8.3).

2.8.3 Purification of urea/formamide-binding protein by ammonium sulphate precipitation and FPLC

Urea/formamide-binding protein was purified from *M. methylotrophus* MM25 high-speed supernatant prepared from cells growing in continuous culture under formamide limitation (D=0.1h⁻¹). Frozen high-speed supernatant was thawed and a volume equivalent
to about 260mg protein was made 65% saturated with respect to (NH₄)₂SO₄ (Dawson et al., 1989). This was carried out at 0°C in an ice-water bath by slowly adding the appropriate amount of solid (NH₄)₂SO₄ whilst the solution was gently stirring. Once all the (NH₄)₂SO₄ had been added, the suspension was stirred on ice for 30min. The precipitate was then removed by centrifugation at 18,000rpm (40,000 x g) for 20min, and the supernatant was placed into a dialysis sack and dialysed for approximately 18h against several litres of 20mM-Bis/Tris buffer pH 6.8 which was changed at least twice. After dialysis the sample was tested to ensure that no residual (NH₄)₂SO₄ was present (section 2.6.1).

Aliquots of dialysed 65% (NH₄)₂SO₄ supernatant were filtered through a 0.2μm pore size acrodisc and then loaded on to a Mono Q 10/10 anion-exchange column pre-equilibrated with 20mM-Bis/Tris buffer pH 6.8. Bound proteins were eluted by the application of a linear salt gradient (0-1M-KCl in 20min at a flow rate of 4ml min⁻¹) and fractions (4ml) were collected throughout the elution. As the urea/formamide-binding protein exhibited no easily-assayable activity, small amounts of fractions which corresponded to protein peaks were analysed by minigel SDS-PAGE (section 2.7.1). The urea/formamide-binding protein eluted from the Mono-Q anion-exchange column at a salt concentration of approximately 115mM-KCl; two peak fractions were pooled and shown to be ≥95% pure by SDS-PAGE and gel analysis.

The above method also proved very convenient for co-purifying formamidase from newly-isolated M. methylotrophus strain MM25, as the majority of the formamidase was also present in the 65% (NH₄)₂SO₄ supernatant. Furthermore, formamidase subsequently eluted from the Mono-Q anion-exchange column well separated from the urea/formamide-binding protein (with most of it located in a single fraction which eluted at a salt concentration of 340mM-KCl). Formamidase purified by this simplified method without the use of gel-filtration chromatography was ≥95% pure as determined by SDS-PAGE and gel analysis.
2.8.4 Determination of native M_r of urea/formamide-binding protein

The native M_r of the urea/formamide-binding protein was determined by chromatographing a small amount of essentially-pure protein on a Superose-12 HR 10/30 gel-filtration column (bed volume, 25ml) pre-equilibrated with 20mM-Bis/Tris buffer pH 6.8 + 100mM-KCl (flow rate 0.5ml min^{-1}). The column was first calibrated with the following M_r standards: bovine serum albumin (monomer), 66,000; ovalbumin, 45,000; carbonic anhydrase, 29,000 and cytochrome c, 12,400 (Sigma). A standard curve was constructed using the M_r standards by plotting log M_r against elution volume (ml). The native M_r of the urea/formamide-binding protein was then calculated by extrapolation from the standard curve.

2.9 Determination of protein concentration

2.9.1 Bio-Rad (Bradford) protein assay

The concentration of soluble protein present in preparations was determined using a dye reagent kit (Protein assay kit II; Bio-Rad) which works by the method of Bradford (1976). Protein solutions, 0.8ml containing 0-20μg protein, were placed in clean dry test tubes to which was added 0.2ml of neat dye reagent. The solution was mixed and incubated at room temperature for 15min. The A_{595nm} of the mixture was measured in a Perkin Elmer Lambda 5 UV/VIS split-beam recording spectrophotometer using 1ml plastic cuvettes (1cm light path) against a reagent blank. A standard curve of 0-20μg protein was prepared as described above, using bovine serum albumin as standard (Sigma). The protein concentration of samples was then determined by extrapolation from the standard curve of A_{595nm} plotted against protein concentration (μg). A new standard curve was prepared each time the assay was carried out.
2.9.2 Estimation of protein from A\textsubscript{280}/A\textsubscript{260} measurement

Protein concentration was sometimes determined by direct measurement of A\textsubscript{280} and A\textsubscript{260}. This method was used especially during the preparation of pure enzymes for the raising of antibodies and other purposes. Samples of unknown protein concentration (1ml) and buffer blank (1ml buffer in which the sample was suspended) were placed in matched 1ml quartz cuvettes (Perspec Services, Leicestershire) and the A\textsubscript{280} and A\textsubscript{260} measured in a Perkin Elmer Lambda 5 UV/VIS split-beam recording spectrophotometer. The protein concentration of the sample was then calculated from the following relationship (Dawson et al., 1989):

\[
\text{Protein concentration (mg ml}^{-1} = (1.55 \times \text{A}\textsubscript{280}) - (0.76 \times \text{A}\textsubscript{260})
\]

2.10 N-terminal amino acid sequencing

The N-terminal amino acid sequences of various proteins of interest, were determined by separating the protein(s) by SDS-PAGE (1-3\textmu g protein; section 2.7.1) and then blotting on to a polyvinylidene difluoride (PVDF) membrane. The blot was visualised by staining with Kenacid blue R for about 5min, then destained for about the same period of time before the required protein was cut out from the membrane. Membrane samples were loaded into an Applied Biosystems model 470 gas-phase sequencer and the N-terminal amino acids were separated and analysed by HPLC after sequential Edman degradation cycles. N-terminal amino acid sequencing was carried out by Miss E. Cavanagh or Dr K. Lilley.

2.11 Analysis of culture supernatants by High Pressure Liquid Chromatography (HPLC)

Culture supernatant samples (section 2.4) prepared from continuous cultures of \textit{M. methylotrophus} grown under various conditions were analysed for the presence of acetamide, formamide, acetate and formate by HPLC. Samples (200\mu l) were separated on a Fast Acid column (100 x 7.8mm; Bio-Rad) at room temperature with 0.005M-H\textsubscript{2}SO\textsubscript{4} as
the mobile phase at a flow rate of 0.5 ml min⁻¹ using a Gilson HPLC system fitted with an autosampler. The system was controlled using an Apple-Macintosh computer. The A₁₀₈nm and refractive index of the column effluent was monitored continuously using a Dynomax absorbance detector (model UV-1; Rainin Instruments Co.) and a refractive index monitor (model 1755; Bio-Rad) respectively. The elution profiles were recorded and saved to computer disc. Under the above conditions standard solutions of acetamide, formamide, potassium acetate and potassium formate eluted from the column after retention times of 72.3 min, 32.4 min, 20.3 min and 18.6 min respectively. Culture supernatant samples were analysed for the above by comparison of peak retention times using a Dynomax R data reprocessing computer program (Rainin Instruments Co.).

2.12 Immunological techniques

2.12.1 Preparation of antiserum

Pure acetamidase (250 μl of 1.35 mg protein ml⁻¹) or formamidase (200 μl of 1.50 mg protein ml⁻¹, section 2.7.2) prepared by FPLC and electroelution was mixed with Freund's complete adjuvant and, after a 1-2 ml pre-immune bleed had been taken, injected into a rabbit. Two further injections were given at 2 week intervals but in Freund's incomplete adjuvant, after a small 1-2 ml bleed had been taken to test for antibody production. The final bleed (about 200 ml) was taken 2 weeks after the last injection.

The blood samples were left to stand at room temperature to allow clot formation, the clot retracted and then red blood cells were removed by centrifugation in an MSE MicroCentaur bench-top centrifuge operating at full speed (13,000 rpm; 13,400 x g) for 5 min. The final bleed was centrifuged in 50 ml disposable plastic centrifuge tubes (Costar) in a Minor (MSE) benchtop centrifuge at full speed (10,000 rpm; 17,000 x g) for 15 min. The serum was carefully removed using a Gilson pipette, then aliquoted and stored at -20°C until required.
2.12.2 Western blotting

Proteins which had been separated by minigel SDS-PAGE (section 2.7.1) were electrophoretically transferred from the polyacrylamide gel on to a nitrocellulose membrane (0.45μm pore size; Sartorius) using a semi-dry “Multiphor II” transblot system (LKB). The transfer buffer used was 48mM-Tris/39mM-glycine pH 8.3 containing 0.0375% (w/v) SDS and 5% (v/v) methanol. 3MM paper was cut to the same size as the gel to be blotted and soaked in transblot buffer. A sheet of nitrocellulose was also cut to size and soaked in buffer. Nine sheets of pre-soaked 3MM paper were placed on the wetted, bottom graphite electrode of the transblot apparatus. The sheet of wet nitrocellulose was then placed on top of the 3MM layer, and the gel on top of the nitrocellulose. The “sandwich” was completed by placing another nine sheets of wetted 3MM over the gel. During this process it was ensured that no air bubbles were trapped between any layers of the “sandwich”. Finally the top pre-wetted graphite electrode was placed on top of the “sandwich”. Transfer was carried out at room temperature with a current of 0.8mA (cm²)⁻¹ of gel for 1.5h, with the gel towards the negative electrode and the nitrocellulose towards the positive electrode.

Transferred proteins were temporarily stained on the membrane using Ponceau's red stain solution (5% (w/v) in trichloro acetic acid; Sigma) and the position of Mr standards marked before removing the stain with reverse-osmosed water. The blot was then incubated overnight at 4°C with agitation in block buffer (10mM-Tris/0.9% (w/v) NaCl/0.1% (v/v) Tween 20 pH 7.4 and 3% (w/v) “Marvel” milk powder). The blot was washed for 3 x 10min in Tris buffered saline (TBS)/Tween (the above buffer without Marvel) on a gyroshaker. After washing the blot was incubated for 1.5h in amidase antiserum diluted 1000-fold in block buffer. The blot was then washed with 4 changes of TBS/Tween for 5min each, and then for 2 x 5min in 50mM-Tris/150mM-NaCl buffer pH 7.5. A secondary antibody conjugated to alkaline phosphatase was then coupled to the bound amidase antibody by incubating the blot for 1h in swine anti-rabbit alkaline phosphatase (DacoPatts) diluted 1000-fold in block buffer. The blot was then washed 4 x 5min in TBS and then developed. Developing solution was made fresh each time and consisted of 20ml buffer (0.1M-Tris/0.1M-NaCl, 5mM-MgCl₂ pH 9.5), 132μl nitroblue tetrazolium (50mg
mL⁻¹ in 70% (v/v) dimethyl formamide) and 66μL 5-bromo-4-chloro-3-indolyl phosphate (50mg mL⁻¹ in dimethyl formamide). The blot was incubated in this solution until it had developed to the desired intensity (usually a few minutes). The reaction was then stopped by incubation in stop buffer (PBS + 20mM-EDTA), following which the blot was dried and photographed.

2.13 Nutrient pulse experiments

2.13.1 Nutrient pulses into growing culture

Nutrient pulse experiments were carried out into dual acetamide/methanol-limited continuous cultures of *M. methylotrophus* (D=0.1h⁻¹). Pulses of ammonia (2mM) were made by the aseptic addition of (NH₄)₂SO₄ solution to give a final ammonia concentration of 2mM. Preliminary experiments showed that the culture took up 2.3% of its cell density as nitrogen therefore reducing the expected supernatant ammonia concentration at t=0; appropriate correction was therefore made for this in the amount of ammonium sulphate added. The required weight of (NH₄)₂SO₄ was dissolved in a small volume (<10ml) of Elga water and filter sterilised by passage through a 0.2μ pore size acrodisc into a sterile plastic universal bottle. The (NH₄)₂SO₄ solution was then aseptically transferred into a sterile 10ml plastic syringe and introduced into the culture by way of a sterile hypodermic needle via a subaseal on the chemostat top plate. Culture samples were taken directly before the addition of the nutrient pulse (t=0h) and at hourly intervals thereafter. Acetamidase activity, culture supernatant ammonia concentration (section 2.6.1) and cell density (section 2.2) were measured. Pulses of acetamide (3mM), formamide (2mM), neutralised potassium acetate (3mM, 10mM and 20mM), neutralised potassium formate (2mM) and methanol (50% of the input concentration; 1.25g L⁻¹) were carried out in a similar manner to that described above.
2.13.2 Nutrient pulses into non-growing culture samples

Nutrient pulse experiments into non-growing culture samples were carried out at 37°C with stirring to maintain aerobic conditions. Reactions (20ml) were carried out in 65ml water jacketed perspex vessels fitted with a magnetic stirrer. A sample of dual acetamide/methanol-limited \textit{M. methylotrophus} culture was taken from the chemostat and the required volume immediately pipetted into the reaction vessel. The pH of the culture sample was measured and adjusted, if required, to pH 7.0. Culture samples were taken directly before the addition of 2mM (final concentration) nutrient pulses (t=0h) and at hourly intervals thereafter. Acetamidase activity, culture supernatant ammonia concentration and cell density were measured as above. The pH of culture samples was also measured hourly and re-adjusted to 7.0 if required. A control reaction where no additions were made, but the pH controlled, was always run in-tandem with each set of experiments.

Modifications of the above methodology were used to find the effect upon acetamidase activity of pH. The same procedure as described above was carried out except that at t=0 the culture pH was altered to the required value with dilute acid or alkali and no nutrient pulse was added.

2.14 Equilibrium dialysis

2.14.1 Substrate-binding specificity of urea/formamide-binding protein

The substrate specificity of the urea/formamide-binding protein for urea, amides, organic acids and ammonia was measured by competitive equilibrium dialysis. The reactions were carried out at 4°C in two eight-cell rotating modules (Hofer Scientific Instruments). Each cell was divided into two chambers of 0.5ml volume separated by a dialysis membrane (6-10,000 Mₜ cut-off). Pure urea/formamide-binding protein (0.5nmol) was added to one chamber of each cell, the volume being made up to 300µl with filter-sterilised 20mM-Bis/Tris buffer pH 6.8. To the other chamber of the cell was added 0.8nmol [¹⁴C]urea (48.9mCi mmol⁻¹; Sigma) together with either 0.6 or 25x (i.e. 0.48 or 20nmol) the
concentration of unlabelled competing "substrate", the volume again being made up to 300μl with filter-sterilised 20mM-Bis/Tris buffer pH 6.8. Controls, in which either no urea/formamide-binding protein or no competing "substrate" was present, were always included in a set of experiments. These controls demonstrated that equilibrium was reached within the period of the experiment and also indicated the extent of \([^{14}C]\text{urea} \text{ binding in the absence of competing "substrate". The module was rotated at 10rpm for 24h to attain equilibrium, after which time samples (50μl) were taken in duplicate from each chamber, added to 4ml of OptiPhase "Hi Safe" scintillation fluid (Wallac Scintillation Products) and analysed using a 2000 CA Tri-Carb liquid scintillation counter (United Technologies; Packard). The amount of binding (nmol urea nmol protein\(^{-1}\)) was expressed as a percentage of that seen when no competing "substrate" was present. Other control experiments were carried out in which the urea/formamide-binding protein was replaced with boiled binding protein (10min at 100°C) or bovine serum albumin.

2.14.2 Binding constant of urea/formamide-binding protein for urea

The binding constant (K\(_D\), μM) of the urea/formamide-binding protein for urea was determined using a range of urea concentrations by a modification of the equilibrium dialysis method (section 2.14.1). Reactions were set up as described above with 0.5nmol urea/formamide-binding protein in one chamber and \([^{14}C]\text{urea} \text{ concentrations ranging from 13.3μM (8nmol) to 261.7μM (157nmol) in the other chamber. Samples were taken from each chamber after 24h and analysed for \([^{14}C]\text{urea binding as described above. The amount of free and bound }^{14}\text{C} \text{urea was then calculated and used to construct a Scatchard plot of bound urea/free urea versus free urea (μM). The linear plot gave a gradient of } -1/K_D \text{ with an intercept on the } x \text{ axis equal to the stoichiometry of binding (mol urea/mol protein).}

2.15 Preparation of dialysis tubing

Dialysis tubing was boiled in 2% (w/v) sodium bicarbonate + 1mM-EDTA, then thoroughly rinsed in Elga water before being boiled for a further 10min in 1mM-EDTA.
and rinsed again. Dialysis tubing prepared in this way was routinely stored at 4°C in either Elga water or 10% (v/v) ethanol until required. The tubing was always thoroughly rinsed in Elga water before use.

**Presentation of results**

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

PHYSIOLOGICAL REGULATION IN CONTINUOUS CULTURE OF ACETAMIDASE, FORMAMIDASE & UREASE
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PHYSIOLOGICAL REGULATION IN CONTINUOUS CULTURE OF ACETAMIDASE, FORMAMIDASE & UREASE

3.1 Introduction

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3.3 Acetamidase, formamidase and urease activities of *M. methyloptrophus* following growth in continuous culture under different growth regimes

3.4 Analysis of *M. methyloptrophus* whole cells grown in continuous culture under various nutrient limitations by SDS-PAGE and Western blotting

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3.1 Introduction

Bacterial cells, ecologically, exist in a changing, dynamic environment in which the type and concentration of nutrients as well as physical environmental factors (such as temperature and pH) can vary widely with time. Bacteria have only a limited ability to control their environment and hence must respond to changes in that environment if they are to survive. Microbial cells in general have the ability to utilise a wide range of substrates for growth, this ability ultimately being imparted by the nature of the genome. However, at no given time is the entire bacterial genome expressed. The production of proteins that are of no immediate use to the cell represent an inefficient use of cell resources and would be a disadvantage to the cell evolutionarily. Hence, bacteria have evolved an "energy-efficient" system whereby only proteins essential to the survival of the cell under a given set of environmental conditions are synthesised. These proteins can be of two types; constitutive and inducible. Proteins which are present in the cell under all growth conditions are termed constitutive and those which are only present under certain culture conditions are referred to as inducible.

Inducible enzymes are produced only in the presence of an inducer (often, but not always, the substrate of the enzyme) and down regulated, i.e. repressed, in the presence of a repressor (frequently, but not always, the product of the enzyme reaction) (Clarke & Brammar; 1964). Amidases represent such a group of inducible enzymes and have been widely studied in *Pseudomonas aeruginosa* (see reviews by Clarke, 1970, 1984; Clarke & Drew, 1988; Brammar & Clarke, 1964; Boddy *et al.*, 1967), *Aspergillus nidulans* (Haynes, 1970; Haynes & Pateman, 1970), *Brevibacterium* sp. (Maestracci *et al.*, 1984; 1988), *Alcaligenes eutrophus* (Friedrich & Mitrenga, 1981) and *Methylophilus methylotrophus* (Silman *et al.*, 1989, 1990; Wyborn *et al.*, 1994). The amidases of these organisms are all induced by amides and repressed by ammonia and/or products of carbon metabolism depending on whether the amide is used as a source of carbon and nitrogen or nitrogen alone. Repression of amidase by products of carbon metabolism appears to occur only in the cases where amide hydrolysis supplies carbon as well as nitrogen for growth.
Control of nitrogen-metabolising enzymes has been studied mostly in the enteric bacteria and is governed by an elaborate system known as the \textit{ntr} system (Magashanik, 1982; Merrick, 1988). An \textit{ntr}-like system also appears to operate in other genera of bacteria. The system is reliant on three regulatory proteins; NtrA, NtrB and NtrC (products of the \textit{ntrA}, \textit{ntrB} and \textit{ntrC} genes respectively) which act both positively and negatively. The site of action of these proteins is thought to be at \textit{ntr} promoter sites situated at -24 and -12 from the transcriptional start-site of \textit{ntr} regulated genes. NtrB and NtrC form a two-component sensor-regulator system which is sensitive to the intracellular nitrogen status of the cell. NtrA is an alternative sigma factor which alters the recognition of RNA polymerase to \textit{ntr} promoter sites. However, NtrA alone is insufficient to initiate transcription of \textit{ntr} genes, NtrC is also required as an activator protein. The "activation" of NtrC, to allow it to interact with NtrA and thence initiate transcription, is reliant upon phosphorylation of NtrC by NtrB. Under conditions of nitrogen-limitation NtrB phosphorylates NtrC to make it "active", thus potentiating transcription of \textit{ntr} genes by way of NtrA. Under conditions of nitrogen-excess NtrB acts as a phosphatase, thus "deactivating" NtrC and producing a down regulation of \textit{ntr} gene expression. Hence, it is the ability of NtrB to sense the nitrogen status of the cell which regulates the expression of \textit{ntr} genes. The kinase/phosphatase activity of NtrB is controlled by the small regulatory protein P_{II}, involved also in the control of glutamine synthetase activity (see Chapter 4). In nitrogen-excess conditions P_{II} is deuridylylated by the \textit{glnD} product (UTase) and in \textit{E. coli} this form has been shown to promote dephosphorylation of NtrC by NtrB. Conversely under nitrogen-limitation P_{II} is uridylylated and this form promotes phosphorylation of NtrC by NtrB. The uridylylated/deuridylylated state of P_{II} is dependent upon the intracellular glutamine:α-ketoglutarate ratio in such a way as to produce deuridylylated P_{II} at high ratios and uridylylated P_{II} at low ratios. This demonstrates how nitrogen assimilating enzymes can be regulated by the level of available nitrogen in the cell, and also the central role which glutamine plays in the nitrogen budget of the cell.

The physiological regulation of microbial enzymes has been studied under a number of culture conditions including batch, fed-batch and continuous culture. The method of continuous culture is particularly suited to this application as it allows the growth of microbial cells under closely-regulated conditions (see Tempest, 1970). One method of
continuous culture which has been widely applied to the study of microbial physiology is the chemostat (Novick & Szilard, 1950a). A chemostat culture consists of a well-mixed microbial culture of fixed volume (V) into which medium is constantly added at a constant flow rate (F) and all physical parameters such as temperature, pH and DO\(_2\) are controlled within desired ranges to allow the culture to grow. Spent culture is constantly removed by way of a suitable overflow device at the same rate as fresh medium is added to maintain a fixed volume. The in-flowing medium contains all of the nutrients required for growth of the culture in "nutrient-sufficient" amounts except for one, which is termed the growth-limiting nutrient. If a batch culture was allowed to grow in such a medium the limiting nutrient would be the first to become depleted. It therefore follows that the biomass concentration of the continuous culture (\(x\)) is determined by the concentration of the growth-limiting nutrient in the in-flowing medium (s). The dilution rate of such a culture (D, h\(^{-1}\)) is defined by the relationship:

$$D = \frac{F}{V} \quad (1)$$

In the chemostat culture cells are constantly growing and dividing but at the same time they are also leaving the vessel. The change in biomass concentration (dx/dt) is determined by the net effect of each process such that:

$$\frac{dx}{dt} = \mu x - Dx \quad (2)$$

Where \(\mu\) is the specific growth rate of the culture (h\(^{-1}\)). Eventually, if \(D<\mu_{\text{max}}\) (the maximum specific growth rate) a steady-state is reached where:

$$\frac{dx}{dt} = 0 \quad (3)$$

It therefore follows that at steady-state:

$$\mu = D \quad (4)$$
Hence, in chemostat culture the growth rate of the culture can be manipulated to any values, as long as $0<D<\mu_{\text{max}}$, simply by changing the dilution rate. The relationship between the specific growth rate and the growth-limiting substrate concentration in chemostat culture is governed by classical Michaelis-Menten kinetics (Monod, 1942; quoted in Tempest, 1970) by the following relationship:

$$\mu = D = \frac{\mu_{\text{max}} S}{K_S + S}$$  \hspace{1cm} (5)

Where $K_S$ is the saturation constant of the organism for the growth-limiting substrate (numerically equal to the growth-limiting substrate concentration which gives a $\mu$ of 0.5 $\mu_{\text{max}}$). From the above it can clearly be seen that chemostat culture provides a method whereby the growth of a culture can be carried out under clearly-defined physiological conditions.

Under chemostat culture the bacterial cell is subjected to nutrient limitation. Such a situation is found frequently in natural microbial ecosystems as a consequence of the metabolic activities of the indigenous microbial populations (Harder & Dijkhuizen, 1983). Microbial cells have hence evolved a number of strategies to enable survival and evolutionary competition within such nutrient-limited environments. Changes in the cell to accommodate the restrictive nature of nutrient limitation can be brought about in the relative short term by regulation of existing gene expression, and in the long term by altering the genotype. Only the former will be considered here. The strategies evolved by microbes in response to nutrient limitation to allow them to grow at the maximum possible growth rate under nutrient limitation, thus imparting a selective advantage, are generally of three types; (i) to increase the rate of transport of the limiting nutrient into the cell, (ii) to increase the rate of initial metabolism of the nutrient once it has entered the cell and, (iii) to rearrange the chemical composition of the cell in such a way as to redirect the use of the limiting nutrient to allow the maximum amount of cell material to be produced (Harder & Dijkhuizen, 1983). As a result of these strategies, the composition and activities of microbial cells can change dramatically depending upon the nutrient status of the cell.

It has previously been shown that a single microorganism may possess a number of amidases with different substrate profiles. The data supporting the presence of more than
One amidase in a particular microorganism has come mostly from often rather-limited physiological and genetic studies. It has been concluded that *A. nidulans* contains at least three amidases (Haynes, 1975), one specific to formamide (formamidase), one specific to short-chain aliphatic amides (acetamidase) and one with a wider substrate profile (termed the general amidase). A fourth amidase active towards valeramide and hexanamide may also be present as well as a urease. *Brevibacterium sp.* R312 has been shown to contain a broad spectrum amidase as well as several enzymes specific for the hydrolysis of urea, formamide, nicotinamide, L-α-amino amides and L-glutamine (Jallageas et al., 1980; Maestracci et al., 1984). In *A. eutrophus* both formamidase and acetamidase enzymes are present (Friedrich & Mitrenga, 1981). However, the purification and characterisation of amidases from the above sources has not been reported. *M. methylotrophus* has been reported to have a short-chain aliphatic amidase (acetamidase), a formamidase and a urease (Jenkins et al., 1987; Silman et al., 1989; Silman, 1990; Wyborn et al., 1994; M.A. Carver, unpublished; H. Murphy, J. Mills & C.W. Jones, unpublished) each of these enzymes having been purified and at least partially characterised.

Investigations of the physiological regulation of amidase activities in several species of bacteria have been carried out using batch, fed-batch and continuous culture. The regulation of acetamidase activity in wild-type *M. methylotrophus* has previously been reported in batch, fed-batch and acetamide-limited continuous cultures (Silman et al., 1989; Silman, 1990). The regulation of *M. methylotrophus* formamidase has similarly been reported in batch, formamide-limited fed-batch and formamide-limited continuous culture (Wyborn et al., 1994). The regulation of *M. methylotrophus* urease has not been investigated. Therefore, a detailed study of the physiological regulation of all three enzymes of *M. methylotrophus* in continuous culture has not previously been reported. This chapter describes a detailed study of the physiological regulation of *M. methylotrophus* acetamidase, formamidase and urease in continuous culture. The results are discussed with reference to previous studies and to the regulation of other bacterial amidases and ureases.
3.2 Raising of specific antibodies to acetamidase and formamidase

Antibodies were raised against purified acetamidase and formamidase from *M. methylotrophus* to enable the accurate and sensitive determination of enzyme expression by SDS-PAGE and Western blotting (Materials & Methods). Acetamidase and formamidase were purified from acetamide-limited continuous cultures (D=0.1h<sup>−1</sup>) of *M. methylotrophus* using FPLC and preparative-PAGE as described in Materials & Methods. Aliquots of pure protein were then inoculated into a rabbit at fortnightly intervals. Samples of antiserum taken during the immunisation procedure were checked for antibody titre by carrying out “spot blots” whereby 1-2μl of pure antigen was spotted on to a piece of nitrocellulose and then treated as for the development of Western blots (Materials & Methods). The titre of antiserum to acetamidase was found to be high after three inoculations, whereas the titre of antiserum to formamidase did not reach a similar level until four inoculations had been administered (data not shown). This demonstrated that acetamidase is more antigenic than formamidase, but the reason for this remains obscure.

The specificity of the antibodies was investigated by carrying out SDS-PAGE and Western blotting with samples of pure acetamidase (switched-on and switched-off forms, both M<sub>r</sub> 38,000; see chapters 1 and 4), formamidase (M<sub>r</sub> 51,000) and urease (M<sub>r</sub> 61,000 & 14,000; kindly donated by H. Murphy). Two Western blots were prepared, one developed using acetamidase antiserum and the other developed with formamidase antiserum. Each antiserum was found to be specific to the enzyme to which it was raised (Fig. 3.1), no cross-reactivity was seen between acetamidase and formamidase antiserum and proteins. The two forms of acetamidase were not immunologically distinct. No cross-reactivity was observed between either acetamidase or formamidase antiserum and urease. Interestingly, the Western blot showed that formamidase was contaminated by a very low concentration of acetamidase (Fig. 3.1 (b)) and that urease was contaminated by a very low concentration of formamidase (Fig. 3.1. (c)), whereas neither form of acetamidase was contaminated with formamidase. The concentrations of both contaminants were too low to be visualised on gels stained for protein with Kenacid blue R, thus demonstrating the high sensitivity of Western blotting with these antibodies.
Figure 3.1 The specificity of anti-acetamidase and anti-formamidase antibodies

Purified acetamidase (switched-on and switched-off forms), formamidase and urease were subjected to SDS-PAGE and Western blotting as described in Materials & Methods. Tracks: 1, $M_r$ standards; 2, acetamidase (switched-on); 3, acetamidase (switched-off); 4, formamidase; 5, urease; 6, $M_r$ standards. (a), SDS-PAGE; (b), Western blot developed with acetamidase antiserum and (c), Western blot developed with formamidase antiserum.
The formamidase antiserum was also tested against samples of *E. coli* expressing the cloned *fmd* gene, and against the subsequently purified enzyme, in order to demonstrate that the product of the cloned *fmd* gene was immunologically indistinguishable from the native enzyme. Samples of *E. coli* JM109::pUC19 and JM109::pNW3 (pUC19 containing a 3.2kbp insert carrying the *M. methyloptrophus* formamidase structural gene:*fmdA*) were separated by SDS-PAGE, together with samples of pure formamidase from JM109::pNW3 and *M. methyloptrophus*. The formamidase antiserum cross-reacted with the formamidase expressed in JM109::pNW3 and both of the purified enzymes (Fig. 3.2); no cross-reaction was seen between the formamidase antiserum and any other proteins in JM109::pUC19.

The above results demonstrated that specific antisera had been raised to acetamidase and formamidase from *M. methyloptrophus*.

### 3.3 Acetamidase, formamidase and urease activities of *M. methyloptrophus* following growth in continuous culture under different growth regimes

Wild-type *M. methyloptrophus* was grown in continuous culture (D=0.1h⁻¹) with ammonia, acetamide, formamide or urea as nitrogen source as described in Materials & Methods. Culture was carried out under both nitrogen-limited/methanol-excess and nitrogen-excess/methanol-limited conditions. Washed cells were prepared and assayed for acetamidase, formamidase and urease activity (Materials & Methods). Acetamidase activity was induced by acetamide, formamide and urea, with maximum activity being observed during culture under acetamide-limitation (Table 3.1). The induction of acetamidase activity by amides and urea being in the order acetamide>formamide>urea. Acetamidase activity was also subject to repression by ammonia, either added to the culture as excess ammonia or produced by the hydrolysis of excess amide or urea. The acetamidase activity under any growth regime therefore appeared to be a balance between induction by amide or urea and repression by ammonia, as most-clearly demonstrated during growth under acetamide-excess conditions. Very little constitutive expression of acetamidase activity was seen, as demonstrated by very low activity (<1% of the maximally-induced activity) following growth under ammonia-limitation.
Figure 3.2 Immunological identification of formamidase in *E. coli* JM109::pNW3

Washed cells of *E. coli* JM109::pUC19 and *E. coli* JM109::pNW3, purified formamidase from *E. coli* JM109::pNW3 and purified formamidase from *M. methylotrophus* were obtained from N.R. Wyborn. The samples were subjected to SDS-PAGE and Western blotting with formamidase antiserum as described in Materials & Methods. Tracks: 1, Mr standards; 2, *E. coli* JM109::pUC19; 3, *E. coli* JM109::pNW3; 4, formamidase purified from *E. coli* JM109::pNW3; 5, formamidase purified from *M. methylotrophus*; 6, Mr standards. (a), SDS-PAGE and (b), Western blot.
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Methanol</th>
<th>Formamide</th>
<th>Formamide concentration (µM)</th>
<th>Ammonia concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.08</td>
<td>0.02</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
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<td>0.03</td>
<td>0.09</td>
<td>0.03</td>
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<td>5.0</td>
<td>0.17</td>
<td>0.08</td>
<td>0.17</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Supporting experiments were also conducted as described in Materials & Methods. Ammonia concentrations in cultures were measured and assayed for ammonia formamidase and urease activity as described in Materials & Methods. Ammonia concentrations in culture (mg/l) under various nutrient limitations (see Table 2) cells were harvested, washed, and dried. M. methylotrophus was grown in continuous culture (D=0.13) under various nutrient limitations (see Table 2) cells following growth in continuous culture under various nutrient limitations.

Table 3.1 Ammonidamidase activities of M. methylotrophus washed cells following growth in continuous culture under various nutrient limitations.
Formamidase activity was also induced by amides and urea, with maximum activity being observed during growth under urea-limitation. The induction of formamidase activity by amides and urea being in the order urea>formamide>acetamide. Formamidase activity, like acetamidase activity, was also subject to repression by ammonia. Hence, formamidase activity under any growth regime was also a balance between induction by amides or urea and repression by ammonia. However, formamidase demonstrated significantly higher constitutive expression (approximately 10% of the maximally-induced activity) than acetamidase during growth under ammonia limitation.

Urease activity was also induced by amides and urea, with maximum activity being observed during growth under urea or formamide limitation. The induction of urease activity by amides and urea was therefore in the order urea/formamide>acetamide. Urease activity, like both acetamidase and formamidase activity, was subject to repression by ammonia. Therefore, the urease activity observed under any growth regime was also a balance between induction by amides or urea and repression by ammonia. However, urease demonstrated even higher constitutive expression (approximately 15% of the maximally-induced activity) during growth under ammonia limitation, this level of expression being higher than that seen with formamidase.

The effect upon urease activity of adding nickel to urea-limited medium was also investigated. The rationale behind this being that all ureases studied to date have been nickel-containing enzymes (see Chapter 1), and as the continuous culture medium contained no added nickel salts it was hypothesised that urease activity may be enhanced by the addition of Ni^{2+}. However, upon the addition of 200μM-NiCl₂ to the medium no increase in urease activity was seen, demonstrating that either the culture was not deficient in this element, or that the enzyme was novel and did not require nickel.

3.4 Analysis of M. methylotrophus whole cells grown in continuous culture under various nutrient limitations by SDS-PAGE and Western blotting

Whole cells of M. methylotrophus grown in continuous culture (D=0.1h⁻¹) under methanol-limited and methanol-excess conditions with ammonia, acetamide, formamide or urea as nitrogen source were subjected to SDS-PAGE, gel analysis and Western blotting as...
described in Materials and Methods (Fig. 3.3). During growth under methanol limitation, the methanol dehydrogenase-α subunit (Mr 60,000; Mdh-α) was found to constitute 16-24% of cell protein, whereas under methanol-excess conditions it constituted only 5-7% of cell protein (Fig. 3.3 (a)). A similar pattern was seen with the concentration of the Mdh-β subunit (Mr 8,500), but accurate quantification of its concentration was not possible. These observations confirmed that methanol dehydrogenase was strongly repressed under methanol-excess conditions, as previously shown by Greenwood and Jones (1986); probably by a catabolite-repression-like mechanism (Goodwin & Anthony, 1995). It was concluded, therefore, that Mdh-α could be used as a "marker" of the methanol status of the culture.

During growth with ammonia as the nitrogen source no acetamidase (Mr 38,000) was detectable, and this was confirmed by the absence of bands on the Western blot developed with acetamidase antiserum (Fig. 3.3 (b)). During growth with ammonia as the nitrogen source the concentration of formamidase (Mr 51,000) was ≤1% cell protein, rendering accurate determination of concentration very difficult, and this low concentration was confirmed by the presence of very faint bands on the Western blot developed with formamidase antiserum (Fig. 3.3 (c)).

During growth under acetamide limitation acetamidase was found to constitute approximately 4.8% of cell protein, similar to that reported previously by Silman et al. (1989; 1991). From this concentration and the measured acetamidase activity of washed cells (Table 3.1), together with a native Mr of 155,000, a whole-cell kcat for acetamidase was calculated of 236s⁻¹. This value is higher than that previously reported for wild-type acetamidase by Silman et al. (1989) of 148s⁻¹ (recalculated from the previously miscalculated value of 96s⁻¹, taking into account that protein constitutes approximately 65% dry cell weight). Acetamidase mutant strains are known to be selected very rapidly during growth under acetamide limitation (Silman et al., 1989). This difference was therefore possibly due to the rapid selection during these studies of a mutant strain with an enhanced kcat for acetamide, as the maximum acetamidase activity during acetamide-limited continuous culture (D=0.15h⁻¹) previously quoted was approximately half that described here for approximately the same acetamidase concentration. During growth under acetamide-excess conditions acetamidase was repressed to a level below detection by
Figure 3.3 SDS-PAGE and Western blotting of *M. methylotrophus* whole cells grown in continuous culture (D=0.1h⁻¹) under various nutrient limitations.

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under various nutrient limitations, washed cells prepared and subjected to SDS-PAGE and Western blotting as described in Materials & Methods. Tracks: 1, M₇ standards; 2, ammonia limitation/methanol excess; 3, ammonia excess/methanol limitation; 4, acetamide limitation/methanol excess; 5, acetamide excess/methanol limitation; 6, formamide limitation/methanol excess; 7, formamide excess/methanol limitation; 8, urea limitation/methanol excess; 9, urea excess/methanol limitation; 10, M₇ standards. (a), SDS-PAGE; (b), Western blot developed with acetamidase antiserum; (c), Western blot developed with formamidase antiserum.
SDS-PAGE and gel analysis, although it was still detectable by Western blotting, demonstrating the high sensitivity of this methodology. During growth under acetamide limitation formamidase was found in the cell at a concentration of approximately 3.8% of cell protein. From this concentration and whole-cell activity (Table 3.1), together with a native Mr of 130,000, a whole cell $k_{cat}$ for formamidase of $120 s^{-1}$ was calculated. This $k_{cat}$ is higher than the value of $64 s^{-1}$ reported by Wyborn et al. (1994) for purified enzyme, but similar to that calculated for purified wild-type formamidase in this study (see section 4.9). Under acetamide-excess conditions the formamidase was repressed to a concentration of $\leq 1\%$ of cell protein.

During growth with formamide as the nitrogen source acetamidase was undetectable by SDS-PAGE and gel analysis, and only just detectable by Western blotting, confirming that formamidase was only a very poor inducer of acetamidase. Conversely formamidase was induced to a concentration of approximately 4.1% of cell protein during growth under formamide-limited conditions, giving rise to a whole cell $k_{cat}$ of $215 s^{-1}$. During growth under formamide-excess conditions formamidase was repressed to a concentration of approximately 1% of cell protein.

During growth with urea as nitrogen source acetamidase was undetectable by SDS-PAGE and gel analysis, and was only just detectable by Western blotting, confirming that urea (like formamide) was only a very poor inducer of acetamidase. Conversely, formamidase constituted approximately 4.3% of cell protein during growth under urea-limited conditions giving rise to a whole cell $k_{cat}$ of $263 s^{-1}$. During growth under urea-excess conditions formamidase was repressed to a concentration of approximately 1% of cell protein.

During growth under formamide and urea limitation a protein was found in high concentration (16-18% of cell protein) with a subunit Mr of approximately 40,000, which showed no cross-reactivity to acetamidase antiserum (Fig. 3.3). This protein was not present during growth with ammonia as nitrogen source, and was repressed to an undetectable level during growth under formamide- or urea-excess conditions (Table 3.2). It was present however, during growth under acetamide limitation as a discernible band just above acetamidase (constituting approximately 6% of the cell protein), which was repressed to an undetectable level during acetamide-excess conditions. The identity of this
protein was investigated by protein sequencing (see Materials & Methods). Cellular proteins of cells grown under acetamide, formamide and urea limitation were separated by SDS-PAGE, blotted on to a PVDF membrane and the N-terminal amino acid sequence of the 40,000 Mr protein determined. Following growth under acetamide limitation the sequence reflected a mixture of an unknown protein (GATI-) and a smaller quantity of contaminating acetamidase (MIHG-) (Silman et al., 1991; Silman, 1990) (Table 3.3). Following growth under formamide and urea limitation a mixed sequence was again obtained comprising approximately equal amounts of the unknown protein (GATI-) and a second unknown protein (ADYP-). The identity and subcellular location of these proteins will be discussed in detail later (Chapters 5 & 6).

Urease (Mr 61,000 and 14,000) was undetectable by SDS-PAGE and gel analysis following growth under any of the growth regimes. Preliminary purification data (Murphy, Mills & Jones, unpublished) indicated that urease exists in the cell at a very low concentration. The subunit MrS of urease are also very similar to those of Mdh-α and β, this and its low cellular concentration made it undetectable even under formamide and urea limitation where its activity is very high.

3.5 Analysis of *M. methylotrophus* culture supernatants by HPLC

Culture supernatant samples were collected during the growth of *M. methylotrophus* in continuous culture with acetamide or formamide as nitrogen source. The supernatants were analysed for the presence of amides and organic acids by HPLC. During growth under acetamide-limited or acetamide-excess conditions no residual acetamide or acetate was detectable (<0.5 and 4mM, respectively) in the culture supernatant. This suggested that during growth under both of these regimes the *in vivo* acetamidase activity, as predicted from the acetamidase activity and cell density, was sufficient to hydrolyse eventually all of the input acetamide to acetate and ammonia within the retention time of the culture (10h). Furthermore, as no acetate was detected (though it must be noted that the assay was not very sensitive to acetate), *M. methylotrophus* must have been able to metabolise acetate at a rate commensurate with the rate of acetamide hydrolysis. This is in keeping with the previous suggestion (Carver & Jones, 1993) that excess acetate does not build up to
Table 3.2 Physiological regulation of 40,000 Mr protein(s) following the growth of *M. methylotrophus* in continuous culture under various nutrient limitations

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under various nutrient limitations, washed cells prepared and subjected to SDS-PAGE and N-terminal amino acid sequencing of the 40,000 Mr proteins (ADYP- and GATI-) as described in Materials & Methods.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Limiting nutrient</th>
<th>40,000 Mr protein(s)</th>
<th>GATI-</th>
<th>ADYP-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>Ammonia</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acetamide</td>
<td>Acetamide</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Formamide</td>
<td>Formamide</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Urea</td>
<td>Urea</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND=Not detectable
Table 3.3 N-terminal amino acid sequences of Mr 40,000 protein(s) following the growth of *M. methylotrophus* in continuous culture under various nutrient limitations

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under acetamide, formamide and urea limitation, whole cells subjected to SDS-PAGE and the Mr 40,000 protein(s) subjected to N-terminal amino acid sequencing as described in Materials & Methods. Figures in brackets are those observed under urea limitation.

<table>
<thead>
<tr>
<th>Limiting nitrogen source</th>
<th>N-terminal amino acid sequence of Mr 40,000 protein(s)</th>
<th>Amount of PTH amino acid (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>4.97</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>5.33</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>5.19</td>
</tr>
<tr>
<td>Formamide or Urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>11.40 (17.44)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>12.63 (14.89)</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>7.00 (15.98)</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>11.04 (10.63)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>9.99 (15.60)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10.41 (13.60)</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>5.97 (7.97)</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>6.03 (17.47)</td>
</tr>
</tbody>
</table>
detectable levels in the culture supernatant of acetamide-limited cultures unless *M. methylotrophus* is grown at high cell density (with a concomitantly high input concentration of acetamide), when the limited ability of *M. methylotrophus* to metabolise acetate apparently becomes saturated.

During growth under formamide-limited or formamide-excess conditions no residual formamide or formate was detectable (<0.5 and 4mM, respectively) in the culture supernatant. This suggested that during growth under both of these regimes the *in vivo* formamidase activity, as predicted from the formamidase activity and cell density, was sufficient to hydrolyse all of the input formamide to ammonia and formate. The formate oxidation rate of washed cells prepared from cultures grown under formamide limitation was $58.6 \pm 3.9(\text{SEM})$ nmol formate min$^{-1}$ (mg cells)$^{-1}$, which increased to approximately $100$ nmol formate min$^{-1}$ (mg cells)$^{-1}$ in cells grown under formamide-excess conditions. Hence, in both cultures the formate oxidation capacities were far in excess of those required to oxidise all of the formate produced from formamide hydrolysis, thus supporting the observation that no formate was spilled into the culture.

### 3.6 Biomass yields of *M. methylotrophus* during growth in continuous culture

*M. methylotrophus* was grown in continuous culture ($D=0.1h^{-1}$) under various nutrient limitations, the cell density measured and biomass yields of limiting nutrients ($Y_{X/S}$) calculated. During growth under methanol limitation with ammonia or urea as nitrogen source approximately similar biomass yields (g cells g carbon$^{-1}$) were seen; with acetamide and formamide as nitrogen sources the biomass yields were again similar, but substantially higher that those obtained with ammonia or urea (Table 3.4). Although *M. methylotrophus* is a restricted facultative methylotroph it has a limited ability to utilise the acetate produced by hydrolysis of acetamide (Carver & Jones, 1993; Lloyd, 1990), but only in conjunction with methanol as a primary carbon source. Conversely, formate produced by hydrolysis of formamide is readily oxidised to carbon dioxide via a soluble NAD$^+$-linked formate dehydrogenase and the resultant NADH is available for oxidation by the respiratory chain with the concomitant production of ATP (Patchett *et al.*, 1985; Jones *et al.*, 1987; Wyborn *et al.*, 1994). Hence, the higher biomass yields obtained during growth
Table 3.4 Biomass yields of M. methylotrophus grown in continuous culture under various nutrient limitations

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under various nutrient limitations, steady-state cell densities were measured and biomass yields calculated as described in Materials & Methods.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Limiting nutrient</th>
<th>[Cell] (\bar{x}; \text{g l}^{-1})</th>
<th>[Limiting nutrient] (s; \text{g l}^{-1} \text{ C or N})</th>
<th>(Y_{X/s}) (\text{g g}^{-1} \text{ C or N})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>Methanol</td>
<td>0.52±0.04(15)</td>
<td>0.56</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Ammonia</td>
<td>0.38±0.02(14)</td>
<td>0.04</td>
<td>9.50</td>
</tr>
<tr>
<td>Acetamide</td>
<td>Methanol</td>
<td>0.82±0.01(7)</td>
<td>0.56</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>Acetamide</td>
<td>0.24±0.05(30)</td>
<td>0.04</td>
<td>6.00</td>
</tr>
<tr>
<td>Formamide</td>
<td>Methanol</td>
<td>0.81(2)</td>
<td>0.56</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>Formamide</td>
<td>0.63±0.03(21)</td>
<td>0.10</td>
<td>6.30</td>
</tr>
<tr>
<td>Urea</td>
<td>Methanol</td>
<td>0.85±0.01(3)</td>
<td>1.11</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>0.51±0.03(6)</td>
<td>0.09</td>
<td>5.67</td>
</tr>
</tbody>
</table>
under methanol limitation with acetamide or formamide as the nitrogen source may have been due to the organic acid products of amide hydrolysis being able to contribute to the overall carbon economy of the cell. Biomass yields of 1.333 g cells g carbon\(^{-1}\) (Vasey & Powell; 1984) and 0.858-0.992 g cells g carbon\(^{-1}\) (Southgate & Goodwin; 1989) have previously been reported for \textit{M. methylotrophus} during growth with ammonia as nitrogen source in methanol-limited continuous culture. Hence, there may appear to be some variability in biomass yield seen under similar culture conditions.

When \textit{M. methylotrophus} was grown in continuous culture under ammonia limitation a much higher biomass yield (g cells g nitrogen\(^{-1}\)) was observed than during growth under acetamide, formamide and urea limitation (Table 3.4). Biomass yields of 6.4-6.7 g cells g nitrogen\(^{-1}\) have previously been reported for \textit{M. methylotrophus} grown in ammonia-limited continuous culture (Southgate & Goodwin, 1989). These yields were lower than those seen here under ammonia limitation, but approximately similar to those when acetamide, formamide or urea were limiting. This discrepancy may be a reflection of variability in biomass yields on nitrogen, in a similar manner to those on carbon. During growth of \textit{M. methylotrophus} in nitrogen-limited continuous culture a "nitrogen deficit" was seen. \textit{M. methylotrophus} biomass was assumed to be approximately 10% (w/w) nitrogen under these conditions (M.A. Carver, personal communication) and as no excess ammonia (<0.01 mM) or amide was detectable in the culture supernatant, essentially all of the input nitrogen should have been assimilated into biomass. However, a nitrogen deficit of approximately 15-35 mg nitrogen l\(^{-1}\) (equivalent to 1-2.5 mM ammonia) which was not present as biomass, free ammonia or free amide was observed. During growth under nitrogen limitation, bacterial cells utilise nitrogen efficiently (Harder & Dijkhuizen, 1983) and therefore it would be predicted that all of the input nitrogen would be assimilated into biomass. A possible explanation for this "nitrogen deficit" was that gaseous ammonia may have been vented-off from the culture due to high localised pH upon the addition of KOH used to control the culture pH (Materials & Methods). However, when this was investigated by lowering the KOH addition port under the surface of the culture to ensure immediate mixing, and thus to decrease the possibility of a localised high pH, the "nitrogen deficit" remained unchanged. An alternate explanation for the "nitrogen deficit" was that \textit{M.}
methylotrophus was excreting nitrogenous compounds into the culture medium and therefore not assimilating all of the available nitrogen into biomass.

3.7 Investigation of amide transport in M. methylotrophus

In the absence of radiolabelled acetamide or acrylamide, investigation of amide transport into M. methylotrophus was carried out via an indirect approach using cells grown in continuous culture (D=0.1h⁻¹) under acetamide limitation. Amidase assays were carried out with 400mM-K₂HPO₄/KH₂PO₄ pH 7.0 as buffer and acrylamide as substrate at final concentrations of 250μM and 50mM. Acrylamide, rather than acetamide, was used as the substrate since acetamidase has a much higher kₘ for acrylamide than acetamide (16.1 cf. 1.1mM; Silman, 1990; Silman et al., 1991). Therefore, assuming Michaelis-Menten kinetics (i.e. \( v = \frac{V_{\text{max}} \cdot S}{k_{\text{m}} + S} \)) it would be predicted that the amidase activity at 250μM-acrylamide would be 2.0% of that at 50mM-acrylamide, whereas the predicted amidase activity at 250μM-acetamide would be 18.9% of that at 50mM-acetamide. The rationale behind this approach is therefore that if no accumulative uptake system is operating the predicted and observed activities at 250μM-acrylamide would be similar, whereas if accumulation is occurring the measured rate would be greater than the predicted rate.

Assays were carried out at 250μM and 50mM acrylamide on whole cells and broken cells (disrupted by sonication) in the presence and absence of 20μM-FCCP dissolved in methanol. FCCP is a potent uncoupler of oxidative phosphorylation in M. methylotrophus and rapidly diminishes both the proton-motive force and the phosphorylation potential (Dawson & Jones, 1981); energy-dependent acrylamide transport at the expense of the proton-motive force or ATP would therefore be inhibited in the presence of FCCP.

The amidase activities at 250μM and 50mM acrylamide were not significantly decreased by cell breakage or FCCP, both of which would abolish energy-linked transport (Table 3.5). Furthermore, amidase activity at 250μM-acrylamide was very similar to that predicted (0.8% cf. 2.0%), indicating no accumulation of substrate in the cell. It is therefore likely that acrylamide is taken up via either simple or facilitated diffusion, rather than by active transport. Uptake of short-chain amides into bacterial cells by simple or facilitated diffusion has previously been suggested in M. methylotrophus (Silman, 1990) and in
Table 3.5 Investigation of amide transport in *M. methylotrophus*

*M. methylotrophus* was grown in acetamide-limited continuous culture (D=0.1 h⁻¹), and cells were harvested and disrupted as described in Materials & Methods. Amidase activity was measured in whole and broken cells using acrylamide as substrate at concentrations of 250µM and 50mM in the presence and absence of FCCP (20µM) dissolved in methanol. Methanol was included as a control. Activities are expressed as a percentage of the activity in whole cells in the absence of FCCP. Amidase activities were 0.04 (2) and 5.10 (2) µmol min⁻¹ (mg cells)⁻¹ at 250µM and 50mM acrylamide respectively (i.e. 0.8% at 250µM).

<table>
<thead>
<tr>
<th>Cells used in assay</th>
<th>Additions</th>
<th>Activity at 250µM-acrylamide (%)</th>
<th>Activity at 50mM-acrylamide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>FCCP</td>
<td>107</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>102</td>
<td>91</td>
</tr>
<tr>
<td>Broken cells</td>
<td>-</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>FCCP</td>
<td>104</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>109</td>
<td>91</td>
</tr>
</tbody>
</table>
Brevibacterium sp. R312 (Miller & Knowles, 1984). The suggestion that Ps. aeruginosa contains a constitutive amide permease (Brammar et al., 1966) has previously been discounted (see Miller & Knowles, 1984; W. J. Brammar, personal communication).
3.8 Discussion

The acetamidase of *M. methylotrophus* is strongly induced by acetamide, only poorly induced by formamide or urea, and repressed by ammonia. The acetamidase activity observed during growth with formamide and urea as nitrogen sources can largely be explained by formamidases ability to hydrolyse acetamide at 5% of the rate of formamide. Previous work has also shown that acetamidase is not repressed by methanol or acetate (Silman *et al.*, 1989; Silman, 1990). These observations are consistent with acetamide and other short-chain amides supplying mainly nitrogen for the growth of *M. methylotrophus*. The physiological regulation of acetamidase has previously been reported in a number of other microorganisms, viz. *Ps. aeruginosa* (Kelly & Clarke, 1962; Brammar & Clarke, 1964; Boddy *et al.*, 1967; Clarke *et al.*, 1968; Clarke, 1970; Potts & Clarke, 1976), *Brevibacterium* sp. R312 (Maestracci *et al.*, 1984; 1988), *Rhodococcus* sp. (Miller & Gray, 1982), *Arthrobacter* sp. J-1 *(Asano et al.*, 1982), *A. nidulans* (Haynes, 1970; Haynes & Pateman, 1970; Davis *et al.*, 1993) and a thermophilic bacillus (Thalenfeld & Grossowicz, 1976), which are summarised in Table 3.6. These studies were generally carried out in batch cultures, and were not as detailed as that reported here for *M. methylotrophus*. In general all are strongly induced by amides, in similarity to the acetamidase of *M. methylotrophus*, with inducer specificities being species specific. The acetamidases of *Rhodococcus* sp., *Arthrobacter* sp. J-1 and *A. nidulans* are also induced by acetate, and the enzyme from the thermophilic bacillus was strongly induced by urea, in contrast to *M. methylotrophus*. Acetamidases from *Ps. aeruginosa, Brevibacterium* sp. R312 and *Rhodococcus* sp. are subject to repression by organic acids, with the latter also being sensitive to repression by ammonia. The enzymes from *A. nidulans* and the thermophilic bacillus both being sensitive to repression by glucose, with the former also subject to repression by ammonia and acetate. Therefore, it can be seen that the physiological regulation of bacterial acetamidases can vary significantly from species to species. In general, however, if both carbon and nitrogen for growth are supplied by amide hydrolysis the acetamidase is subject to induction by amides and repression by organic acids and/or ammonia. Conversely, if amide hydrolysis supplies only nitrogen for growth the acetamidase is subject to induction by amides and repression only by ammonia.
Table 3.6 Physiological regulation of acetamidase activity in various microorganisms

See text for references.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Induced by</th>
<th>Repressed by</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. methylotrophus</em></td>
<td>acetamide</td>
<td>ammonia</td>
</tr>
<tr>
<td><em>Ps. aeruginosa</em></td>
<td>acetamide</td>
<td>organic acids</td>
</tr>
<tr>
<td><em>Brevibacterium</em> sp. R312</td>
<td>acetamide</td>
<td>organic acids</td>
</tr>
<tr>
<td><em>Rhodococcus</em> sp.</td>
<td>acetamide, acetate</td>
<td>organic acids, ammonia</td>
</tr>
<tr>
<td><em>Arthrobacter</em> sp. J-1</td>
<td>acetamide, acetate</td>
<td>NR</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>acetamide, formamide, acetate</td>
<td>ammonia, glucose, acetate</td>
</tr>
<tr>
<td>Thermophilic bacillus</td>
<td>acetamide, formamide, urea</td>
<td>glucose</td>
</tr>
</tbody>
</table>

NR=Not reported
The formamidase of *M. methylotrophus* is strongly induced by formamide, urea and acetamide, and repressed by ammonia. Higher constitutive levels of formamidase appear under general nitrogen limitation, in the absence of inducer, compared with acetamidase. *M. methylotrophus* possesses a soluble NAD⁺-linked formate dehydrogenase which readily oxidises formate to carbon dioxide and NADH, which can then be oxidised by the respiratory chain with the concomitant production of ATP. Hence, formamide not only acts as a nitrogen source in *M. methylotrophus* but also as an additional energy source (but not as a carbon source). The effect upon formamidase activity of formate was not investigated. The formamidase activity of cells grown under formamide limitation (D=0.1h⁻¹) was higher than that previously reported during the growth of *M. methylotrophus* under similar conditions but at a lower dilution rate (0.05h⁻¹) (Wyborn *et al.*, 1994). The reason for this difference in activity may have been due to the standing substrate concentration at the lower dilution rate being insufficient for maximum induction of formamidase. However, it may also have been due to difficulties in attaining steady-state growth under formamide limitation at the lower dilution rate of 0.05h⁻¹ where *M. methylotrophus* appears, within approximately 5 generations, to attain the ability to oxidise excess methanol very rapidly (Sherr & Jones, unpublished), thus shifting from formamide limitation/methanol excess to dual formamide/methanol limitation before the attainment of steady-state growth. To maintain high formamidase activities the input methanol concentration had to be progressively increased. At D=0.1h⁻¹ this inability to attain steady-state growth under formamide limitation was not encountered and very high formamidase activities were seen. The reason for the increase in *M. methylotrophus* methanol oxidation at low dilution rates when grown under formamide-limitation remains obscure.

The physiological regulation of formamidases has also been reported in a number of other microorganisms, viz. *A. nidulans* (Haynes, 1970), *A. eutrophus* (Fredrich & Mitrenga, 1981), *My. smegmatis* (Draper, 1967) and *Brevibacterium* sp. R312 (Jallageas *et al.*, 1980), which are summarised in Table 3.7. These studies, in similarity to those of acetamidases from organisms other than *M. methylotrophus*, were carried out in batch cultures and were not as detailed as those described here. In general, in similarity to acetamidases, all microbial formamidases are induced by amides, with inducer specificity being species specific. None of the other formamidases were reported to be strongly
Table 3.7 Physiological regulation of formamidase activity in various microorganisms

See text for references.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Induced by</th>
<th>Repressed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. methylotrophus</td>
<td>formamide, urea, acetamide</td>
<td>ammonia</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>acetamide, formamide</td>
<td>ammonia</td>
</tr>
<tr>
<td>A. eutrophus</td>
<td>acetamide, formamide</td>
<td>ammonia, succinate</td>
</tr>
<tr>
<td>My. smegmatis</td>
<td>acetamide, acetate</td>
<td>NR</td>
</tr>
<tr>
<td>Brevibacterium sp. R312</td>
<td>acetamide</td>
<td>NR</td>
</tr>
</tbody>
</table>

NR=Not reported
induced by urea, in contrast to that seen with *M. methylotrophus*. *M. smegmatis* possesses a formamidase which is structurally homologous to the formamidase of *M. methylotrophus* (Wyborn, 1994). However, the *M. smegmatis* formamidase is induced by acetamide and also acetate. Hence, it would appear that the activity of this structurally-similar formamidase is controlled in a different manner to that of *M. methylotrophus*. In all cases formamidase is subject to repression by ammonia, in similarity to *M. methylotrophus*. However, it has also been reported that formamidase from *A. eutrophus* is sensitive to repression by succinate as well as ammonia (Table 3.7; Fredrich & Mitrenga, 1981). In *A. eutrophus* acetamide and formamide are reportedly only used as nitrogen sources, even though the organism has the ability to utilise acetate and formate as carbon sources (Fredrich et al., 1979; Fredrich & Mitrenga, 1981). Hence, this repression of formamidase synthesis by succinate is probably not similar to the repression of acetamidases by organic acids when amide hydrolysis supplies both carbon and nitrogen for growth.

The urease of *M. methylotrophus* is strongly induced by formamide and urea and repressed by ammonia. However, higher urease activities were observed during growth under general nitrogen limitation, in the absence of inducer, compared with acetamidase or formamidase, suggesting that urease has a higher constitutive level than the other two enzymes. The urease activity during urea-limited continuous culture was not enhanced by the supplementation of the medium with NiCl$_2$ (200µM). This suggested that either the urease of *M. methylotrophus* is novel and does not contain nickel, or that the culture was able to "scavenge" enough nickel for urease synthesis from contaminated medium components or from nickel-containing components of the fermenter vessel. On further investigation, it was discovered that the addition ports for growth medium and iron solution (both acidic) into the fermenter were nickel/chromium plated (G. Nash, LH Engineering, personal communication). It is likely, therefore, that the acidic growth medium and iron solution was probably leaching sufficient nickel from the addition ports to allow the synthesis of active urease.

Physiological regulation of urease has previously been studied in a number of species of aerobic bacteria in batch culture by Kaltwasser et al. (1972), *Selenomonas ruminantium* (Smith et al., 1981), *Rhizobium meliloti* (Miksch & Eberhardt, 1994), *Klebsiella aerogenes* (Friedrich & Magasanik, 1977; Mulrooney et al., 1989; Miksch & Eberhardt, 1994),
Table 3.8 Physiological regulation of urease activity in various microorganisms

See text for references.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Subject to derepression by</th>
<th>Induced by</th>
<th>Repressed by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>urea/formamide</td>
<td>ammonia</td>
</tr>
<tr>
<td>M. methylophilus</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>+</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>+</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>A. europus H16</td>
<td>+</td>
<td>urea</td>
<td>ammonia</td>
</tr>
<tr>
<td>M. denitrificans</td>
<td>+</td>
<td>urea</td>
<td>ammonia</td>
</tr>
<tr>
<td>M. cereviscans</td>
<td>+</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>+</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Ps. acidovorans</td>
<td>+</td>
<td>urea</td>
<td>ammonia</td>
</tr>
<tr>
<td>A. aerogenes</td>
<td>+</td>
<td>urea</td>
<td>ammonia</td>
</tr>
<tr>
<td>S. ruminum</td>
<td>+</td>
<td>NR</td>
<td>ammonia</td>
</tr>
<tr>
<td>Rh. meliloti</td>
<td>NR</td>
<td>Ni</td>
<td>ammonia</td>
</tr>
<tr>
<td>K. aerogenes</td>
<td>+</td>
<td>NR</td>
<td>excess N</td>
</tr>
<tr>
<td>P. stuartii</td>
<td>NR</td>
<td>urea</td>
<td>ammonia</td>
</tr>
<tr>
<td>P. mirobiilis</td>
<td>NR</td>
<td>urea</td>
<td>ammonia</td>
</tr>
</tbody>
</table>

NR=Not reported
Providencia sturarii and Proteus mirabilis (Mulrooney et al., 1988; Jones & Mobley, 1988), which are summarised in Table 3.8. In general, microbial ureases are subject to repression by ammonia and derepression in response to general nitrogen limitation, with induction by urea being species specific. This is in contrast to that seen with the urease of M. methylotrophus, which only demonstrated low activity in response to general nitrogen limitation, in comparison to that seen under full induction (albeit higher than that seen with acetamidase and formamidase). A. nidulans and Brevibacterium sp. R321 both possess ureases, as well as amidases (Haynes, 1970; Haynes & Pateman, 1970; Jallageas et al., 1980; Maestracci et al., 1984; 1988), but the regulation of urease by acetamide, formamide and urea has not been reported in these organisms. A. eutrophus also possesses a formamidase as well as a urease (Table 3.8), however, the regulation of this enzyme by acetamide and formamide has not been reported. It would therefore appear that this is the first report of a comprehensive investigation of urease regulation in continuous culture, and the first report of the gratuitous induction of urease by formamide (purified urease shows no activity with formamide).

The acetamidase and formamidase activities of M. methylotrophus following growth under different nutrient limitations was approximately commensurate with the concentration of each enzyme as determined by SDS-PAGE and gel analysis, and confirmed semi-quantitatively by Western blotting. The calculated whole cell \( k_{cat} \) for acetamidase grown under acetamide limitation was higher than that previously reported (Silman et al., 1989), possibly reflecting the rapid selection of acetamidase mutants under this growth regime. Similarly, the calculated whole cell \( k_{cat} \) for formamidase grown under acetamide, formamide or urea limitation, although variable, was again substantially higher than that previously reported (Wyborn et al., 1994), but in this case it was probably not due to the rapid selection of formamidase mutants (see Chapter 5).

Acrylamide appears to enter M. methylotrophus by simple or facilitated diffusion and not by active transport in agreement with the conclusions of Silman (1990) (see also Miller & Knowles, 1984). It has recently been shown that the AmiC protein of Ps. aeruginosa binds acetamide, propionamide and the induction inhibitor butyramide (Wilson et al., 1993). This protein has also been shown to be a member of the amino acid-binding protein family of periplasmic binding-proteins by amino acid sequence alignment techniques (Tam & Saier,
1993). However, AmiC is a cytoplasmic protein involved in the control of amidase gene expression. In the amidase operon amIB and amIS appear to code for an ATP-binding protein and an integral membrane protein respectively. Therefore, it would appear that Ps. aeruginosa possesses all of the components required to constitute an active transport system for amides, except for the binding-protein which has relocated to the cytoplasm and become involved in gene expression. It is possible that the AmiB and AmiS proteins could constitute a low-affinity binding-protein-independent active transport system for amides. However, no evidence for active transport of acrylamide was seen in M. methylotrophus. The high acetamidase activity seen under acetamide limitation is a physiological response to the low extracellular acetamide concentration (Harder & Dijkhuizen, 1983). This high activity probably creates and maintains a significant diffusion gradient across the cell membrane, thus facilitating diffusion of amide into the cell at a rate sufficient to maintain the imposed growth rate. High formamidase and urease activities were also seen during growth under formamide and urea limitation, but formamide and urea transport were not investigated. It was noted, however, that proteins with a subunit Mr of approximately 40,000 were induced to high concentration during growth under either formamide or urea limitation, suggesting that these proteins were important during the growth of M. methylotrophus under these regimes (see Chapters 5 & 6).
CHAPTER 4

PHYSIOLOGY & BIOCHEMISTRY OF ACETAMIDASE
SWITCH-OFF
CHAPTER 4

PHYSIOLOGY & BIOCHEMISTRY OF ACETAMIDASE SWITCH-OFF

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4.1 Introduction

Enzyme activity can be regulated at the level of gene expression by controlling the cellular enzyme concentration by induction and repression, and also by controlling the specific activity of the enzyme following translation. Control at the gene expression level represents a long-term effect upon enzyme activity (in the absence of rapid protein degradation pathways), whereas control of enzyme activity at the protein level represents a potentially short lived, fine tuning mechanism. Control of enzyme activity at the protein level has been widely reported and is generally of two types; post-translational covalent modification and allosteric control. Many post-translational protein modifications have been reported (for review see Wold, 1981); it is not the intention here to give an exhaustive review of these, but to describe a few of the more common modifications involved in controlling bacterial metabolic enzyme activity.

Phosphorylation is a relatively common post-translational modification and involves the addition of a phosphoryl group to an amino acid side chain. The amino acids most commonly modified are serine, threonine and tyrosine. However, phosphorylation may also be carried out through an acyl group or a thiol group (phospho-cysteine) rather than via a hydroxyl group. These reactions are catalysed by protein kinases with ATP acting as the phosphoryl group donor. The reverse of this reactions, i.e. dephosphorylation, are catalysed by phosphoprotein phosphatases. Probably the best documented case of enzyme activity control by phosphorylation in bacteria is that of isocitrate dehydrogenase in the enteric bacteria E. coli and S. typhimurium (Wang & Koshland, 1978; LaPorte & Koshland, 1982). The control of isocitrate dehydrogenase activity is important during growth with acetate as the sole carbon and energy source. Under these conditions the enzymes of the glyoxylate bypass (isocitrate lyase and malate synthase) are induced as a means to convert C2 acetyl units into C4 succinate units thus allowing the subsequent utilisation of the TCA cycle for both dissimilatory and assimilatory purposes. Isocitrate dehydrogenase controls the branch point between the TCA cycle and the glyoxylate bypass, its activity in E. coli being
controlled by reversible phosphorylation of serine-133 (Thorsness & Koshland, 1987). When phosphorylated the enzyme becomes inactive, which is thought to be due to the negative charge of the phosphate, and carbon is assimilated via the glyoxylate bypass; when dephosphorylated, for example upon the addition of glucose to the medium, the enzyme is reactivated and carbon is dissimilated via the TCA cycle. This phosphorylation cycle is catalysed by a bifunctional protein, isocitrate dehydrogenase kinase/phosphatase (LaPorte & Koshland, 1982). Hence, phosphorylation and dephosphorylation of isocitrate dehydrogenase acts as a metabolic “switch” that determines whether carbon is assimilated or dissimilated in enteric bacteria grown on acetate.

Probably the best-documented regulation system in bacterial nitrogen metabolism is that of glutamine synthetase (GS) in enteric bacteria. The transcription of GS is regulated by the ntr system in relation to the nitrogen status of the cell. However, the activity of GS is also regulated, post-translationally, by the reversible covalent addition of an adenylyl moiety to a specific tyrosyl residue on each of the twelve identical subunits which constitute the GS molecule (Merric, 1988). Such adenylylation causes a decrease in enzyme activity under conditions of nitrogen-excess. Adenylylation of GS is carried out by an adenylyl transferase (ATase) which can also act in reverse to deadenylylate GS under conditions of nitrogen-limitation. The activity of ATase is controlled by interaction with a small regulatory protein P11, the regulatory activity of which is also determined by post-translational modification. P11 in its unmodified form stimulates the adenylylation of GS by ATase. However, P11 can be uridylylated by a uridylyl transferase (UTase) and in this form stimulates the deadenylylation of GS. UTase is also responsible for removing the uridylyl group from P11. The activity of UTase is controlled by the cellular pool glutamine:α-ketoglutarate ratio. Hence, the activity of GS is controlled by a complex cascade of effectors which ultimately respond to the nitrogen status of the cell. It is note-worthy that P11 also has a regulatory role in ntr gene expression (see introduction to Chapter 3; Merric, 1988).

Another nitrogen metabolising enzyme which has been extensively studied in bacteria and is regulated by post-translational modification is nitrogenase. This enzyme is found in bacteria capable of fixing atmospheric nitrogen and has been extensively studied in the purple nonsulphur photosynthetic bacterium *Rhodospirillum rubrum*. The fixation of
atmospheric nitrogen is a very “energy expensive” process and it is therefore tightly regulated. The activity of nitrogenase in *R. rubrum* and other organisms is controlled post-translationally by reversible covalent ADP-ribosylation (Ludden & Roberts, 1989). This modification is carried-out on a specific arginine residue of one of the dinitrogenase reductase subunits (this enzyme being dimeric). During growth under nitrogen-fixing conditions dinitrogenase reductase is unmodified and nitrogenase activity is concomitantly high. However, nitrogenase activity is rapidly down regulated by a number of physiological signals, this down regulation being termed switch-off (Zumft & Castillo, 1978). Signals which produce switch-off of nitrogenase activity in *Rhodospirillum rubrum* include the addition of pulses of fixed nitrogen such as ammonia, glutamine and asparagine, together with other signals such as darkness, oxygen and respiratory uncouplers (Ludden & Roberts, 1989). Switch-off is mediated by the covalent addition of an ADP-ribosyl group to dinitrogenase reductase by dinitrogenase reductase ADP-ribosyl transferase (DRAT). When exogenous ammonia (the most notable of the above physiological signals) is added to a nitrogen-fixing culture the nitrogenase is rapidly switched-off by the above mechanism (Sweet & Burris, 1981). This inhibition is reversed once the ammonia has been depleted from the culture. The reactivation of nitrogenase *in vivo* being carried out by the removal of the ADP-ribosyl group from dinitrogenase reductase by dinitrogenase reductase-activating glycohydrolase (DRAG). This reaction is dependent upon Mg-ATP and divalent cations. A similar reactivation of inactive nitrogenase can also be achieved *in vitro* by heating the enzyme at 60°C (Pope et al., 1985). The exact biochemical pathway by which nitrogenase activity is post-translationally regulated is not fully understood. However, the activities of DRAT and DRAG are also thought to be post-translationally regulated, as during nitrogen fixation DRAG is active and DRAT is inactive (Zhang et al., 1994). Upon the addition of exogenous ammonia this situation is reversed with DRAG becoming inactive and DRAT becoming active, resulting in the modification of dinitrogenase reductase and switch-off of nitrogenase activity. Once the added ammonia has been exhausted DRAG becomes active again and nitrogenase is reactivated. Switch-off of nitrogenase activity by ammonia has been shown to be prevented by inhibitors of GS (Sweet & Burris, 1981; Zhang et al., 1994), suggesting that the effector for DRAT/DRAG activity is not ammonia directly, but a product of ammonia assimilation.
The cellular NAD(P)+/NAD(P)H ratio has also been suggested as being a determinant in the regulation of DRAT activity since the enzyme is NAD+ dependent (Norén & Nordlund, 1994), but the role of these potential effectors in nitrogenase switch-off are not understood to date. Nitrogenase activity in *Azospirillum brasilense, A. lipoferum* and *A. amazonense* (Zhang et al., 1994; Hartmann et al., 1986), *Rhizobium* strain ORS571 (Kush et al., 1985), *Rhodobacter capsulatus* (Hallenbeck, 1992; Masepohl et al., 1993), *Anabaena variabilis* (Reich et al., 1986), *Azotobacter chroococcum* (Ruiz et al., 1990), *Rhodopseudomonas palustris* (Zumft & Castillo, 1978), *Rhodobacter sphaeroides* and *Methylosinus trichosporium* OB3b (Yoch et al., 1988) also appear to be physiologically controlled by a switch-off system similar to that of *R. rubrum* in response to ammonia. However, in contrast to *R. rubrum* the purified dinitrogenase reductases from the latter two organisms were not modified by ADP-ribosylation, suggesting that either the modification to these proteins is labile to purification or the mechanism of switch-off is different to that of *R. rubrum*. Hence, in bacteria capable of fixing atmospheric nitrogen this energetically-expensive pathway is regulated closely, not only at the genetic level but also at the level of activity. Physiologically, the role of switch-off is to rapidly down-regulate nitrogenase activity when sources of fixed nitrogen are available in order to prevent the unnecessary utilisation of ATP and reductant. Such a switch-off mechanism is much faster than the alternative of simply repressing nitrogenase synthesis and allowing the enzyme to "dilute out" as the culture grows.

Allosteric control of enzyme activity is observed in numerous enzymes. This form of control is reliant upon the enzyme containing two separate and distinct binding sites, *i.e.* the active site (which is responsible for the biological activity of the protein) and the allosteric site (which reversibly binds molecules other than the substrate of the enzyme, termed allosteric effectors) (Monod et al., 1963; Engel, 1981). When an allosteric effector binds to the allosteric site it brings about a reversible conformational change within the protein which alters the kinetic characteristics of the enzyme. This alteration can be either positive or negative (*i.e.* increasing or decreasing enzyme activity respectively). Probably the best understood allosteric enzyme is aspartate transcarbamoylase (ATCase) form *E. coli* (Stryer, 1988). This enzyme catalyses the first reaction in pyrimidine biosynthesis, the formation of N-carbamoyl-aspartate from aspartate and carbamyl phosphate. ATCase
consists of six catalytic subunits ($M_r$ 34,000) and six regulatory subunits ($M_r$ 17,000) in the conformation ($\alpha\beta)_6$; the regulatory subunits being essential for allosteric control. The binding of aspartate and carbamoyl phosphate to ATCase is cooperative and as such demonstrates a sigmoidal saturation curve. The enzyme is feedback-inhibited by CTP, the final product of the biosynthetic pathway (by decreasing the enzyme's affinity for the substrates), and activated by ATP (by increasing the enzyme's affinity for the substrates). The site of action of the activator ATP and the inhibitor CTP is at the allosteric binding sites on the regulatory subunits of ATCase. These two allosteric effectors compete for the allosteric sites on the regulatory subunits and, once bound, bring about conformational changes within the ATCase molecule which affect the affinity of the enzyme for its substrates, depending upon which effector has bound. Hence, the activity of ATCase is controlled in relation to the cellular CTP and ATP concentrations, so that CTP is synthesised when the CTP level is low and ATP is available for DNA replication, but not when pyrimidines are abundant and little ATP is available. This mechanism therefore prevents the "wasteful" utilisation of cell resources.

Acetamidase activity in *Methylophilus methylotrophus* has previously been shown to be regulated, not only by induction by amides and repression by ammonia, but also by a further down regulation of activity at the post-translational level (Silman 1990; Carver & Jones, 1993; Wyborn, 1994). The high-activity and low-activity forms of acetamidase have been termed the switched-on and switched-off forms by analogy with the regulation of nitrogenase. It is interesting to note that no other bacterial acetamidases have been reported to be controlled by post-translational switch-off in a manner similar to that of *M. methylotrophus*. Comparison of the N-terminal amino acid sequences of the acetamidases from *Brevibacterium* sp. R312 (Sourbrier et al., 1992) and *Ps. aeruginosa* (Ambler et al., 1987; Brammar et al., 1987) with that from *M. methylotrophus* (Silman, 1990; Silman et al., 1991) shows a remarkable homology. The former two organisms show an exact identity over the first 19 amino acids (>80% strict identity over the entire sequence) and exhibit only two amino acid changes from *M. methylotrophus* over the same region. Furthermore, the relative amino acid compositions of the enzyme from the latter two organisms are very similar and, even though the primary sequence of the *M. methylotrophus* protein is not known, a high level of homology is implied by this and
immunological evidence (see Chapter 7). It is therefore interesting that *M. methylotrophus* is the only organism in this apparently-related group of amidases that appears to control its amidase activity at the protein level. However, it has recently been reported that the activity of an amidase from an acrylamide-degrading *Rhodococcus* sp., with an N-terminal amino acid sequence 88% identical to that of *Brevibacterium* sp. R312 over the first 17 residues, and therefore highly similar to that of *M. methylotrophus*, is enhanced by (but not dependent on) the presence of Fe$^{2+}$, Ba$^{2+}$ and Cr$^{2+}$ (Nawaz *et al.*, 1994). Other amidases unrelated to that of *M. methylotrophus* have been shown to be enhanced by the presence of cations, including amidase activity in human α-thrombin (Cera *et al.*, 1991), N-acetylmuramyl-L-alanine amidase in *Staphylococcus simulans* 22 and *Bacillus subtilis* (Bierbaum & Sahl, 1987; Foster, 1991), and bovine des-1-41 light chain activated protein C amidase activity (Hill & Castellino, 1986). But none of these reports suggest a similar regulation to that of *M. methylotrophus* acetamidase. Extensive studies have been carried out to determine whether switched-off acetamidase is modified by any common post-translational modification (see Chapter 1; Silman 1990; Wyborn, 1994). However, no modification has been identified. The general, tentative, conclusion drawn from comprehensive physio-chemical comparisons of switched-on and switched-off acetamidase is that the mechanism of switch-off may be due not to a covalent post-translational modification, but to a conformational change brought about by an allosteric and/or hysteretic effector (Wyborn, 1994).

This chapter describes the investigation of the physiological determinants of switch-off in *M. methylotrophus* grown in continuous culture. This was carried out in an attempt to give some insight into the possible modification of the enzyme that potentiates switch-off. The signals producing switch-off of acetamidase activity in vivo are compared with those of other post-translationally regulated enzymes and the growth regime employed by ZENECA Bio Products for the commercial production of acetamidase. Attempts to define the biochemistry of the switch-off mechanism in vitro are also described, together with other events in vitro which cause switch-off of acetamidase activity. Switch-off of formamidase activity was also investigated to determine whether this post-translational down regulation of activity is a general control mechanism for *M. methylotrophus* amidases.
4.2 Growth of M. methylotrophus in continuous culture under dual acetamide/methanol limitation

Wild-type M. methylotrophus was grown in continuous culture (D=0.1h⁻¹) under dual limitation (Materials & Methods). Acetamidase activity was found to be of a similar order to that seen during growth under acetamide-limitation (1.78±0.11(7) cf. 2.85±0.42(4) µmol min⁻¹ [mg cells]⁻¹). Washed cells were prepared from dual-limited culture, subjected to SDS-PAGE and gel analysis (see Fig. 4.2). Acetamidase was found to constitute approximately 4.8% cell protein, again very similar to that seen during growth under acetamide limitation. The acetamidase activity and cellular concentration yielded a calculated whole cell kcat of 147s⁻¹. This value is very similar to that previously reported for fully active, switched-on wild-type acetamidase (Silman et al., 1989, 1991; Silman, 1990; section 3.4). Also, upon heating culture samples from the dual-limited culture, or washed cells prepared from it, at 60°C for 1h no increase in activity was seen (this treatment elicited a decrease in activity of approximately 50%). The high kcat and absence of heat-reactivation suggested that no switch-off of acetamidase activity was occurring during steady-state growth under dual limitation.

4.3 Growth in continuous culture at different carbon to nitrogen ratios

As growth under dual limitation did not produce switch-off, the relationship between input methanol and acetamide concentrations and acetamidase activity was further investigated by growing M. methylotrophus in continuous culture (D=0.1h⁻¹) at different carbon to nitrogen ratios. The medium input concentration of acetamide was maintained at 0.53 g l⁻¹, whereas the input concentration of methanol was varied over the range 1.0-6.5 g l⁻¹ to give input C:N ratios of 1.8-12.3. During growth at C:N ratios ≤2.8 the culture was acetamide-excess/methanol-limited, with a significant concentration of ammonia detectable in the culture supernatant resulting from hydrolysis of excess acetamide (Table 4.1). At C:N ratios in the range 4.7-10.4 dual acetamide/methanol-limitation was seen with no detectable methanol or ammonia in the culture supernatant. At C:N ratios ≥12.3 the culture was acetamide-limited/methanol-excess with no detectable methanol in the culture.
Supernatant. Culture acetamidase activities were measured ± heat (60°C, 1h) under all growth regimes. Washed cells were prepared following growth at each C:N ratio, and cellular proteins were then separated by SDS-PAGE and subjected to gel analysis or Western blotting with antiserum to acetamidase.

Dual-limited and acetamide-limited cultures exhibited high acetamidase activities (≥3.28 μmol min⁻¹ [mg cells]⁻¹) and concentrations (10.4-13.6% of cell protein) which were confirmed by Western blotting (Fig. 4.1; Table 4.2). During growth under acetamide-excess/methanol-limited conditions, the cultures exhibited lower acetamidase activities (<1.07 μmol min⁻¹ [mg cells]⁻¹) and concentrations (<2.93% of cell protein) due to repression by the high ammonia concentrations. Acetamidase activities and concentrations were significantly higher than observed earlier (see Chapter 3) during acetamide limitation, probably due to the culture having been grown under acetamide limitation for an extended period prior to the start of the experiment and thus having selected an over-producing strain similar to culture A (i.e. predominantly MM6; Silman et al., 1989; Silman, 1990). This was confirmed by calculated whole cell kcat values for each growth regime; values of 112-152 s⁻¹ were calculated for all C:N ratios and were thus characteristic of wild-type, switched-on acetamidase (Table 4.2). This, together with the complete absence of heat reactivation, indicated that acetamidase was not significantly switched-off under any of the growth regimes employed.

The concentration of Mdh-α was also determined (Table 4.2). During growth under acetamide-excess/methanol-limited conditions the Mdh-α concentration was high (approximately 17% of cell protein). However, during dual limitation, as the C:N ratio increased, the Mdh-α concentration gradually decreased (from 12.3 to 5.2% of cell protein), and remained at this low concentration under acetamide-limited/methanol-excess conditions. The concentration of Mdh present therefore reflected the carbon status of the culture as suggested previously (Greenwood & Jones, 1986).

4.4 Nutrient pulses into dual-limited culture

Due to the inability to demonstrate switch-off of acetamidase activity during steady-state growth of M. methylotrophus in continuous culture, nutrient pulse experiments were
<table>
<thead>
<tr>
<th>10'0</th>
<th>2.78 (2)</th>
<th>4.26 (2)</th>
<th>0.75</th>
<th>12.3</th>
<th>6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>10'0</td>
<td>1.39 (1)</td>
<td>1.43 (1)</td>
<td>0.82</td>
<td>10.4</td>
<td>5.5</td>
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<tr>
<td>10'0</td>
<td>1.46 (2)</td>
<td>3.28 (2)</td>
<td>0.76</td>
<td>8.5</td>
<td>4.5</td>
</tr>
<tr>
<td>10'0</td>
<td>1.16 (2)</td>
<td>3.07 (2)</td>
<td>0.75</td>
<td>6.6</td>
<td>3.5</td>
</tr>
<tr>
<td>10'0</td>
<td>1.91 (2)</td>
<td>3.07 (2)</td>
<td>0.75</td>
<td>4.7</td>
<td>2.5</td>
</tr>
<tr>
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<td>1.07 (2)</td>
<td>0.74</td>
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</tr>
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<td>1.5</td>
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<td></td>
<td>1.8</td>
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</tr>
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</table>

### Table 4: Growth of *M. methylotrophus* in continuous culture with acetaldehyde as nitrogen source at different carbon to nitrogen ratios

Each regime: methanol concentration as indicated. Acetamide: activity × heat (60°C, 1 h) and culture supernatant ammonia concentrations were measured under methanol concentrations *in vitro* in continuous culture (D=0.1 h⁻¹) with a constant acetamide input concentration of 0.5 g L⁻¹ and a variable input of "_". M. methylotrophus was grown in continuous culture (D=0.1 h⁻¹) with a constant acetamide input concentration of 0.5 g L⁻¹ and a variable input of "_".
Table 4.2 Calculated whole cell $k_{\text{cat}}$ values of acetamidase from *M. methylotrophus* grown in continuous culture at different C:N ratios

*M. methylotrophus* was grown in continuous culture (D=0.1 h$^{-1}$) with acetamide as nitrogen source at various C:N ratios. Washed cell samples were subjected to SDS-PAGE, stained for protein with Kenacid blue R, the gel analysed and whole cell $k_{\text{cat}}$ values calculated as described in Materials & Methods.

<table>
<thead>
<tr>
<th>C:N ratio</th>
<th>[Mdh-α] (% cell protein)</th>
<th>Acetamidase activity (µmol min$^{-1}$ [mg cells]$^{-1}$)</th>
<th>[Acetamidase] (% cell protein)</th>
<th>Calculated $k_{\text{cat}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>17.5</td>
<td>0.45</td>
<td>≤1.0</td>
<td>ND</td>
</tr>
<tr>
<td>2.8</td>
<td>17.2</td>
<td>1.07</td>
<td>2.9</td>
<td>145</td>
</tr>
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<td>4.7</td>
<td>12.3</td>
<td>3.91</td>
<td>13.2</td>
<td>117</td>
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</tr>
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<td>12.3</td>
<td>5.7</td>
<td>4.26</td>
<td>11.1</td>
<td>152</td>
</tr>
</tbody>
</table>

ND=Not determined
Figure 4.1 SDS-PAGE and Western blotting of *M. methylotrophus* grown in continuous culture with acetamide as nitrogen source at different C:N ratios.

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) with acetamide as nitrogen source at various C:N ratios. Washed cell samples were subjected to SDS-PAGE and stained for protein with Kenacid blue R or Western blotted and developed with acetamidase antiserum as described in Materials & Methods. Tracks 1 Mr standards; 2, C:N=1.8; 3, C:N=2.8; 4, C:N=4.7; 5, C:N=6.6; 6, C:N=8.5; 7, C:N=10.4; 8, C:N=12.2; 9, pure acetamidase; 10, Mr standards. (a), SDS-PAGE; (b), Western blot developed with acetamidase antiserum.
carried out with dual-limited cultures (Materials & Methods). The reasons for using this type of culture were two-fold; (i) when a nitrogen or carbon source was added to a dual-limited culture only one of the limitations was relieved, and hence the cell density of the culture did not change appreciably (≤20%), and (ii) switch-off of acetamidase activity had previously been observed during growth under dual limitation at high cell density at ZENECA Bio Products.

4.4.1 Ammonia pulses

When enough sterile ammonium sulphate solution to produce a culture supernatant ammonia concentration of 2mM was added to a dual-limited culture, a decrease in acetamidase activity of 71% in 4h was seen (Table 4.3). This decrease was much greater than expected simply by repression of acetamidase synthesis and subsequent dilution, suggesting that an additional mechanism (e.g. switch-off) was involved. However, no heat-reactivation of acetamidase activity was seen upon heating samples of ammonia-pulsed culture (60°C, 1h) or of washed cells prepared from it, suggesting that the large decrease in whole cell activity could have been due either to degradation of acetamidase or to heat-irreversible switch-off. These hypotheses were investigated by preparing washed cells before the addition of ammonia and at 2 and 4h after an ammonia pulse, then subjecting cell samples to SDS-PAGE and gel analysis (Fig. 4.2 (a)). The acetamidase concentration decreased by 35% over 4h (compared to an expected decrease of 24% by repression and dilution, and the 71% decrease in acetamidase activity), indicating that a small amount of degradation of acetamidase had occurred, but not sufficient to account for the large decrease in activity. Western blotting with antiserum to acetamidase (Fig. 4.2 (b)) confirmed that only a small loss of acetamidase had occurred during the experiment. The concentration of Mdh-α during ammonia pulse experiments did not change by more than ±1.5% (data not shown).

From the activity and concentration data whole cell $k_{\text{cat}}$ values for acetamidase were calculated. Before the addition of ammonia to the culture the calculated $k_{\text{cat}}$ was 147s$^{-1}$ (i.e. of the order expected for fully switched-on acetamidase) but, 4h after the ammonia pulse it had decreased to 66s$^{-1}$ (i.e. close to the 17-47s$^{-1}$ previously reported for switched-
Table 4.3 Switch-off of acetamidase activity following the addition of ammonia to *M. methylotrophus* culture growing under dual-limitation

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under dual methanol/acetamide limitation (C:N ratio 4.7). Sufficient sterile ammonium sulphate solution to produce an ammonia concentration of 2mM was added to the culture at time zero. Culture acetamidase activity, cellular acetamidase concentration and culture supernatant ammonia concentration were measured as described in Materials & Methods. Prior to the addition of ammonium sulphate the acetamidase activity was 1.78±0.11(7) μmol min⁻¹ (mg cells)⁻¹ and the acetamidase concentration was 4.8±0.3(4) % cell protein.

<table>
<thead>
<tr>
<th>Time after ammonia pulse (h)</th>
<th>[Ammonia] (mM)</th>
<th>Acetamidase activity (%)</th>
<th>[Acetamidase] (%)</th>
<th>Calculated kcat (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.00</td>
<td>100</td>
<td>100</td>
<td>147</td>
</tr>
<tr>
<td>1</td>
<td>1.56</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1.05</td>
<td>58</td>
<td>89</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>0.61</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.26</td>
<td>29</td>
<td>65</td>
<td>66</td>
</tr>
</tbody>
</table>
Figure 4.2 SDS-PAGE and Western blotting of whole cells following the addition of ammonia to *M. methylotrophus* culture growing under dual-limited

*M. methylotrophus* growing in continuous culture (D=0.1h⁻¹) under dual limitation was subjected to a 2mM-ammonia pulse and washed cell samples prepared at time intervals of 0, 2 and 4h. Cell samples were subjected to SDS-PAGE and stained with Kenacid blue R and also Western blotted as described in Materials & Methods. Tracks 1, t=0; 2, t=2h; 3, t=4h; 4, pure acetamidase; 5, Mr standards. (a), SDS-PAGE; (b), Western blot developed with acetamidase antiserum.
off, purified acetamidase; Wyborn, 1994). Therefore, it appeared that at least partial switch-off of acetamidase activity could be brought about by the addition of ammonia to dual-limited culture, but could not be reversed by heating either the culture or washed cells. It was subsequently discovered, however, that at least partial heat-reactivation could be brought about in broken cells prepared 4h after an ammonia pulse. The reason why cells required to be broken before heat reactivation was observed remains obscure. Therefore, switch-off of acetamidase activity could be brought about by the introduction of exogenous ammonia into dual-limited culture in a similar manner to the switch-off of nitrogenase activity in *R. rubrum* in response to excess ammonia (Ludden & Roberts, 1989), albeit rather more slowly (see section 4.10).

The relationship between ammonia and switch-off was further investigated to see if reactivation of acetamidase activity occurred after ammonia had been depleted from the culture following an ammonia pulse. A 2mM-ammonia pulse was carried out into a dual-limited culture and the acetamidase activity followed for an 8h period. The activity decreased by 76% over the first 4h as seen previously (Table 4.4), then decreased slightly more after 5h, at which point the culture ammonia concentration was below detectable levels (indicating that at this point *M. methylotrophus* was utilising a mixed nitrogen source of both acetamide and ammonia; without assimilation the ammonia concentration would have decreased to 50% in 10h at $D=0.1h^{-1}$). Within 1h of ammonia being depleted from the culture the acetamidase activity had increased to 61% of the starting activity, which remained approximately constant for the remainder of the experiment. Washed cells were prepared, subjected to SDS-PAGE and the concentration of acetamidase determined at 2h intervals during the experiment. After 4h the acetamidase concentration had decreased by approximately 46%, again slightly larger than expected by repression and dilution, suggesting that some degradation of acetamidase had occurred. However, an increase in acetamidase concentration was seen once ammonia had been depleted from the culture, suggesting that synthesis of acetamidase resumed once the ammonia concentration in the culture had decreased to zero. The decrease in acetamidase concentration in the presence of ammonia was not sufficient to account for the decrease in acetamidase activity. From acetamidase activity and concentration data, whole cell $k_{cat}$ values were calculated; $k_{cat}$ decreased from 120.7s$^{-1}$ to 54.1s$^{-1}$ during the first 4h of the experiment, suggesting
Table 4.4 Switch-off of acetamidase activity following the addition of ammonia to M. methylotrophus culture growing under dual limitation, followed by reactivation once the culture ammonia concentration had decrease to zero

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under dual methanol/acetamide limitation. Sufficient sterile ammonium sulphate solution to produce an ammonia concentration of 2mM was added to the culture at time zero. Culture acetamidase activity, cellular acetamidase concentration and culture supernatant ammonia concentration were measured as described in Materials & Methods. Prior to the addition of ammonium sulphate the acetamidase activity was 1.8μmol min⁻¹ (mg cells)⁻¹

<table>
<thead>
<tr>
<th>Time after pulse (h)</th>
<th>[Ammonia] (mM)</th>
<th>Acetamidase activity (%)</th>
<th>[Acetamidase] (% cell protein)</th>
<th>Calculated kcat (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.00</td>
<td>100</td>
<td>5.96</td>
<td>120.7</td>
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<tr>
<td>1</td>
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<td>75</td>
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<td>59</td>
<td>4.57</td>
<td>93.1</td>
</tr>
<tr>
<td>3</td>
<td>0.61</td>
<td>43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.26</td>
<td>24</td>
<td>3.23</td>
<td>54.1</td>
</tr>
<tr>
<td>5</td>
<td>&lt;0.01</td>
<td>22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>&lt;0.01</td>
<td>61</td>
<td>4.98</td>
<td>88.6</td>
</tr>
<tr>
<td>7</td>
<td>&lt;0.01</td>
<td>55</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>&lt;0.01</td>
<td>57</td>
<td>5.22</td>
<td>78.4</td>
</tr>
</tbody>
</table>
switch-off of acetamidase activity. Within 1h of the ammonia concentration in the culture having decreasing to below detectable levels the calculated acetamidase $k_{\text{cat}}$ had increase to $88.6s^{-1}$, this value remaining approximately constant for the remainder of the experiment. The decrease in calculated $k_{\text{cat}}$ during conditions of excess ammonia suggested that acetamidase was switched-off in response to excess ammonia as seen previously. However, the increase in acetamidase activity following depletion of ammonia from the culture was accompanied by acetamidase synthesis. This increase in activity was commensurate with the acetamidase present in the cell at the point of ammonia depletion remaining switched-off, and the newly synthesised enzyme being fully active.

4.4.2 Acetamide pulse

The effect upon acetamidase activity of an acetamide pulse was investigated to see whether the combination of excess ammonia and acetate, produced by the action of acetamidase, potentiated more rapid or extensive switch-off than ammonia alone. When enough sterile acetamide solution to give a final concentration of 3mM was pulsed into dual-limited culture (the culture acetamidase activity was sufficient to hydrolyse all of the added acetamide to ammonia and acetate within approximately 3min), a decrease in acetamidase activity of 79% was observed over 4h (data not shown). This was very similar to that seen following an ammonia pulse, indicating that excess acetate had little or no effect on acetamidase switch-off.

4.4.3 Acetate pulse

The effect upon acetamidase activity of acetate was further investigated by carrying out acetate pulses. Enough neutralised, sterile potassium acetate solution to give a final concentration of 3mM or 10mM was pulsed into a dual-limited culture and the acetamidase activity followed for 4h (data not shown). No effect was seen on acetamidase activity, demonstrating that acetate was not a potentiator of acetamidase switch-off.
4.4.4 Methanol pulse

The effect of methanol upon acetamidase activity was investigated by carrying out methanol pulses. Enough sterile methanol to give a final concentration of 39mM (1.25g l⁻¹; 50% of the input methanol concentration) was pulsed into a dual-limited culture and the acetamidase activity followed for 4h (data not shown). No decrease in acetamidase activity was seen, demonstrating that methanol was not a potentiator of acetamidase switch-off.

4.5 The effect on acetamidase activity of removing acetamide from the medium and replacing it with ammonia

It was concluded from nutrient pulse experiments that ammonia could act as a “signal” for acetamidase switch-off in dual-limited cultures. However, it should be noted that during ammonia-pulse experiments the inducer of acetamidase (acetamide) was constantly being added to the culture in the in-flowing culture medium. High levels of switch-off of acetamidase activity were observed in cultures grown at ZENECA Bio Products under pseudo dual limitation, at high cell density, where alternate acetamide- and ammonia-containing media were used to counteract the toxic effect of acetate accumulation (See Chapter 1; Carver & Jones, 1993). Hence, the effect on acetamidase activity of removing acetamide from the in-flowing medium and replacing it with ammonia was investigated. This was carried out by replacing dual acetamide/methanol-limited medium with dual ammonia/methanol-limited medium, keeping the carbon and nitrogen inputs constant. When such a medium exchange was carried out, the acetamidase activity decreased by 74% over 4h (Table 4.5), even though no ammonia was detected in the culture supernatant during the experiment. This decrease in acetamidase activity was very similar to that observed following an ammonia pulse into dual-limited culture. Washed cells were prepared at intervals during the experiment, then subjected to SDS-PAGE and gel analysis. The acetamidase concentration decreased by 18% over 4h (compared to an expected decrease of 24% by repression and dilution, and the 74% decrease in acetamidase activity). Whole cell k_cat values decrease from 147s⁻¹ to 47s⁻¹ during the experiment. Hence, upon changing the growth regime of M. methylotrophus from dual acetamide/methanol...
limitation to dual ammonia/methanol limitation switch-off of acetamidase activity was seen.

The effect upon acetamidase switch-off of changing the medium from dual acetamide/methanol limitation to dual ammonia/methanol limitation was further investigated by carrying out combinations of medium exchange and pulse experiments in tandem. When medium exchanges were carried out along with pulses of ammonia, acetamide and acetate at the time of medium exchange, similar decreases in acetamidase activity and $k_{cat}$ to those observed following a medium-exchange alone or an ammonia pulse alone were observed (data not shown). This demonstrated that excess ammonia, acetate or a combination of both had no effect upon the rate and extent of acetamidase switch-off observed when changing the nitrogen source from acetamide to ammonia.

It was therefore concluded from these \textit{in vivo} experiments that switch-off of acetamidase activity could only be brought about in non-steady state cultures, the switch-off being potentiated by either (i) introducing excess ammonia into a dual-limited culture (either by the direct addition of ammonia or by the addition of acetamide with the concomitant production of ammonia by acetamide hydrolysis) or (ii) changing the nitrogen source from acetamide to ammonia during growth under dual C/N limitation.

\section*{4.6 Purification and properties of acetamidase following an ammonia pulse to \textit{M. methylotrophus} growing under dual methanol/acetamide-limitation}

Acetamidase was purified from dual-limited cells 4h after an ammonia pulse as described in Materials & Methods. This was carried out to establish that acetamidase was truly switched-off following an ammonia pulse and remained in its low activity form during purification. At each step in the purification procedure the acetamidase specific activity ± heat (60°C, 1h) and the acetamidase concentration (% total protein) was measured and $k_{cat}$ calculated. Acetamidase activity remained low during the purification procedure but exhibited varying heat-reactivation (highest in Mono-Q (2) and Superose-6 samples in the presence of exogenous activator fraction) (Table 4.6). The unusual presence of heat-reactivation in washed cells was probably due to them having first been stored frozen overnight and then thawed before assay, the cells becoming partially broken by this
Table 4.5 Switch-off of acetamidase activity by exchanging a continuous culture of *M. methylotrophus* from dual acetamide/methanol limitation to dual ammonia/methanol limitation

*M. methylotrophus* was grown in continuous culture (\(D=0.1 \text{h}^{-1}\)) under dual acetamide/methanol limitation. The medium was changed to dual ammonia/methanol-limited at time zero, keeping the C and N inputs constant. Cellular acetamidase activities, concentration and culture supernatant ammonia concentration were measured as described in Materials & Methods. Before the exchange was carried out the acetamidase activity was 1.41\(\mu\)mol min\(^{-1}\) (mg cells\(^{-1}\)) and the acetamidase concentration was 3.8% of cell protein.

<table>
<thead>
<tr>
<th>Time after exchange (h)</th>
<th>[Ammonia] (mM)</th>
<th>Acetamidase activity [%]</th>
<th>[Acetamidase] (% cell protein)</th>
<th>Calculated (k_{\text{cat}}) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.01</td>
<td>100</td>
<td>100</td>
<td>147</td>
</tr>
<tr>
<td>1</td>
<td>&lt;0.01</td>
<td>95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.01</td>
<td>70</td>
<td>100</td>
<td>103</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.01</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>&lt;0.01</td>
<td>26</td>
<td>82</td>
<td>47</td>
</tr>
</tbody>
</table>
process. Some loss of acetamidase activity was seen during cell breakage, which was not heat reactivatable, suggesting that this reflected denaturation. The $k_{\text{cat}}$, although somewhat variable, generally decreased during purification suggesting that some further switch-off occurred \textit{in vitro}. The pure acetamidase (Fig. 4.3) exhibited a low specific activity (4.70 \mu mol min$^{-1}$ [mg protein]$^{-1}$) which could be heat-reactivated 6-fold in the presence of activator fraction. This suggested that a truly switched-off form of acetamidase could be produced by adding ammonia to a dual-limited culture, which did not become switched-on (in fact becoming rather more switched-off) during purification.

4.7 Switch-off of acetamidase activity \textit{in vitro}

The decrease in $k_{\text{cat}}$, and concomitant increase in heat reactivation, of switched-off acetamidase described above can be explained by further switch-off occurring \textit{in vitro} during purification. It was further discovered that upon attempted purification of switched-on acetamidase from a dual-limited culture, samples of broken cells and high-speed supernatant stored at -20$^\circ$C for a few days demonstrated lower-than-expected activity and increased heat-reactivation. However, broken cell and high-speed supernatant samples measured on the day of preparation did not demonstrate heat-reactivation, and neither did samples of high-speed supernatant which had been frozen and thawed rapidly several times on the day of preparation. Hence, it appeared that acetamidase could undergo switch-off \textit{in vitro} simply by storage at -20$^\circ$C (see also Wyborn, 1994). This was in contrast to a previous report that acetamidase purified from cells grown under acetamide limitation did not show switch-off \textit{in vitro} (Silman, 1990). Hence, the effect of growth regime upon acetamidase switch-off during storage at -20$^\circ$C was investigated further. \textit{M. methylotrophus} was grown under acetamide limitation and dual acetamide/methanol limitation, with and without an ammonia pulse; high-speed supernatants were prepared and assayed ± heat (60$^\circ$C, 1h) at the time of preparation and after various periods of storage at -20$^\circ$C. However, highly variable and inconsistent results were seen between similar samples, making the correlation and interpretation of data impossible. In view of this, no further investigations of the mechanism and/or cause of switch-off \textit{in vitro} were carried out.
Table 4.6 Purification of switched-off acetamidase purified from a dual-limited culture of *M. methylotrophus* following an ammonia pulse

*M. methylotrophus* grown in continuous culture (D=0.1h⁻¹) under dual limitation was subjected to a 2mM ammonia pulse at time zero. The culture was harvested after 4h and the acetamidase purified as described in Materials & Methods. At each stage in the purification procedure acetamidase activity (+ heat; 60°C, 1h) and acetamidase concentration were determined from which $k_{cat}$ was calculated. Heat reactivation of Mono-Q (2) and Superose-6 samples was determined in the presence of exogenous activator fraction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acetamidase activity (µmol min⁻¹[mg protein]⁻¹)</th>
<th>[Acetamidase] (% protein)</th>
<th>Calculated $k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Heat</td>
<td>+Heat</td>
<td></td>
</tr>
<tr>
<td>Washed cells</td>
<td>0.86</td>
<td>1.39</td>
<td>5.3</td>
</tr>
<tr>
<td>Broken cells</td>
<td>0.25</td>
<td>0.61</td>
<td>5.3</td>
</tr>
<tr>
<td>HSS</td>
<td>0.25</td>
<td>0.96</td>
<td>8.4</td>
</tr>
<tr>
<td>Mono-Q (2)</td>
<td>5.32</td>
<td>23.40</td>
<td>46.1</td>
</tr>
<tr>
<td>Superose-6</td>
<td>4.70</td>
<td>29.70</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Acetamidase was purified from *M. methylotrophus* 4h after a dual-limited continuous culture (D=0.1h⁻¹) was pulsed with ammonia. Samples were subjected to SDS-PAGE and then stained for protein with Kenacid blue R, as described in Materials & Methods. Tracks 1, Mr standards; 2, washed cells; 3, broken cells; 4, high-speed supernatant; 5, Mono-Q (2) pool; 6, Superose-6 pure acetamidase; 7, Mr standards.
4.8 Nutrient pulses into non-growing culture samples

It had been conclusively demonstrated that switch-off of acetamidase activity could be initiated either by pulsing ammonia into a dual-limited culture or by changing the nitrogen source from acetamide to ammonia. To investigate further the potential mechanism of this process, experiments were carried out in which additions of 2mM (final concentration) ammonia, acetamide and formamide were made to samples taken from dual-limited cultures (i.e. to non-growing cultures). Little loss in acetamidase activity was observed in such culture samples incubated for 4h without additions (Table 4.7). A slightly larger decrease in activity was seen following the addition of 2mM-ammonia, but this decrease was substantially smaller than that seen following an ammonia pulse into a growing dual-limited culture (13% cf. 71%). A slightly larger decrease in activity was seen following the addition of acetamide, but again this decrease was much smaller than when acetamide was added to growing dual-limited culture (23% cf. -71%). The largest decrease in activity was seen following the addition of formamide. The larger decrease in activity following the addition of formamide could have been due to the switch-off mechanism being energy requiring and formate produced by formamide hydrolysis supplying energy to the cell via formate dehydrogenase. It was noted that the respiration rate of a dual-limited culture increased upon addition of formamide, but not upon addition of acetamide. This potential energy requirement for switch-off was further investigated by carrying out formamide additions ± 20μM-FCCP (data not shown). It was found that upon the addition of FCCP the decrease in activity was not seen; however, it was also noted that the decrease in activity caused by formamide alone was accompanied by a large increase in pH during the first hour of incubation (from pH 7.0 to approximately pH 8.0). Conversely, when formamide+FCCP was added to the reaction mixture the pH did not increase substantially. It was therefore postulated that the decrease in activity was related to the increase in pH and not to the availability of energy to the cell. This hypothesis was tested by pulsing samples taken from a dual-limited culture with acetamide or formamide at several different pH values (pH 7.0, 7.5 and 7.9) and measuring the acetamidase activity and pH after 1h (data not shown). Similar experiments were also carried out where dual-limited culture was incubated at the various pH values in the absence of added amide. The results strongly
Table 4.7 Acetamidase activities of non-growing dual-limited cultures of *M. methylotrophus* following the addition of ammonia, acetamide and formamide

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under dual limitation, samples were removed and maintained under fully aerated conditions at 37°C, pH 7.0. At time zero enough neutralised nitrogen source to give a final concentration of 2mM was added to the reaction mixture. The average acetamidase activity at time zero was 3.19±0.08 (18) µmol min⁻¹ (mg cells)⁻¹.

<table>
<thead>
<tr>
<th>Time after addition (h)</th>
<th>Control</th>
<th>Acetamidase activity (%)</th>
<th>Ammonia</th>
<th>Acetamide</th>
<th>Formamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>95±1.6(7)</td>
<td>90±2.5(2)</td>
<td>89±3.3(6)</td>
<td>84±4.5(3)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>91±2.0(7)</td>
<td>90±2.1(2)</td>
<td>85±4.1(6)</td>
<td>72±6.1(3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>91±2.4(7)</td>
<td>87±2.5(2)</td>
<td>79±5.0(6)</td>
<td>76±9.7(3)</td>
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</tr>
<tr>
<td>4</td>
<td>92±2.6(7)</td>
<td>87±2.1(2)</td>
<td>77±7.4(6)</td>
<td>70±7.8(3)</td>
<td></td>
</tr>
</tbody>
</table>
suggested that the decrease in acetamidase activity was related to the increase in pH and not to the amide added (or the energy status of the cell). Also heat-reactivation could be demonstrated in broken cells prepared after incubation for 4h at pH 7.8 or in the presence of formamide, demonstrating that the decrease in activity in both cases was due to switch-off. However, further investigation of the effect of pH upon acetamidase switch-off in non-growing cultures was inconsistent and highly variable results were seen, making the further elucidation of the mechanism of switch-off impossible.

4.9 The effect upon formamidase activity of nutrient pulses

The effect on formamidase activity was also measured following nutrient pulses into a dual-limited culture, in order to investigate whether control of amidase activity by switch-off was a common feature of both amidases of M. methylotrophus. A similar decrease in formamidase activity and calculated whole cell kcat to that seen for acetamidase was observed after a 2mM-ammonia pulse into dual-limited culture (Table 4.8). This decrease suggested that formamidase was also subject to ammonia-induced switch-off. When dual acetamide/methanol-limited medium was exchanged for dual ammonia/methanol-limited medium a slightly smaller decrease in formamidase activity was seen compared with acetamidase (50% cf. 74% over 4h). When a 2mM-formamide pulse was carried out a decrease in formamidase activity similar to that seen upon ammonia pulse was observed. Formate (2mM) had no effect upon formamidase activity. No heat-reactivation of formamidase was seen in whole cells, broken cells or high-speed supernatant, suggesting that the putative modification to formamidase was different from acetamidase.

Formamidase was purified from cells harvested from a dual-limited culture 4h after an ammonia pulse as described in Materials & Methods. This was carried out to establish whether formamidase, like acetamidase, was truly switched-off and remained switched-off during purification. At each step in the purification procedure the formamidase specific activity and concentration (% total protein) was measured and kcat calculated (Table 4.9; Fig. 4.4). During purification the formamidase specific activity increased substantially more than that expected simply due to protein purification, with a concomitant increase in kcat to a value similar to that calculated for whole cells grown under acetamide limitation.
Table 4.8 Switch-off of formamidase activity following the addition of ammonia to *M. methylotrophus* culture growing under dual limitation

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under dual limitation. Sufficient sterile ammonium sulphate solution to produce an ammonia concentration of 2mM was added to the culture at time zero. Culture formamidase activity, cellular formamidase concentration and culture supernatant ammonia concentration were measured as described in Materials & Methods. Prior to the addition of ammonium sulphate the formamidase activity was 1.07(2) μmol min⁻¹ (mg cells)⁻¹ and the formamidase concentration was 3.3(2) % of cell protein.

<table>
<thead>
<tr>
<th>Time after ammonia pulse (h)</th>
<th>[Ammonia] (mM)</th>
<th>Formamidase activity (%)</th>
<th>[Formamidase] (% cell protein)</th>
<th>Calculated k_{cat} (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.00</td>
<td>100</td>
<td>100</td>
<td>108</td>
</tr>
<tr>
<td>1</td>
<td>1.56</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1.05</td>
<td>56</td>
<td>79</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>0.61</td>
<td>42</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.26</td>
<td>34</td>
<td>77</td>
<td>48</td>
</tr>
</tbody>
</table>
(see section 3.4), and switched-on formamidase purified from these cells. This apparent loss of formamidase switch-off during purification suggested that the putative modification may have been very labile and was lost during the purification procedure. Due to this inability to purify, switched-off formamidase, and also the problems encountered in elucidating the mechanism of switch-off of acetamidase, the phenomenon of formamidase switch-off was not investigated further.
Table 4.9 Purification of switched-off formamidase purified from a dual-limited culture of *M. methylotrophus* following an ammonia pulse

*M. methylotrophus* grown in continuous culture (D=0.1h⁻¹) under dual limitation was subjected to a 2mM ammonia pulse at time zero. The culture was harvested after 4h and the formamidase purified as described in Materials & Methods. At each stage in the purification procedure formamidase activity and concentration were determined from which *k*ₐₙₜ was calculated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity (µmol min⁻¹ [mg protein]⁻¹)</th>
<th>[Formamidase] (% protein)</th>
<th>Calculated <em>k</em>ₐₙₜ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed cells</td>
<td>0.82</td>
<td>2.2</td>
<td>63.5</td>
</tr>
<tr>
<td>Broken cells</td>
<td>0.45</td>
<td>2.2</td>
<td>68.5</td>
</tr>
<tr>
<td>HSS</td>
<td>0.76</td>
<td>2.5</td>
<td>52.5</td>
</tr>
<tr>
<td>Mono-Q(2)</td>
<td>30.32</td>
<td>54.3</td>
<td>94.9</td>
</tr>
<tr>
<td>Superose-6</td>
<td>100.00</td>
<td>95.0</td>
<td>178.0</td>
</tr>
</tbody>
</table>
Formamidase was purified from *M. methylotrophus* 4h after a dual-limited continuous culture (D=0.1h⁻¹) was pulsed with ammonia. Samples were subjected to SDS-PAGE and then stained for protein with Kenacid blue R, as described in Materials & Methods. Tracks 1, Mr standards; 2, washed cells; 3, broken cells; 4, high-speed supernatant; 5, Mono-Q (2) pool; 6, Superose-6 pure formamidase; 7, Mr standards.
4.10 Discussion

During the growth of wild-type *M. methylotrophus* in steady-state continuous culture under dual limitation, no significant switch-off of acetamidase activity was seen. This was in contrast to a previous report that switch-off occurred in dual-limited culture at low cell density (M. A. Carver, personal communication), and at high cell density during growth with an alternating nitrogen source (Carver & Jones, 1993). Silman (1990) also carried out a similar, but less detailed, investigation of acetamidase switch-off in *M. methylotrophus* growing at different C:N ratios, but in this case strain MM8 was used. When this was carried out steady-state switch-off of acetamidase was reported at C:N ratios between 6.6-4.7. This is in stark contrast to that seen during this study when wild-type *M. methylotrophus* was grown under similar conditions. This disparity in the physiological conditions which potentiate switch-off, from study to study, highlights the apparently inexplicable differences seen in acetamidase switch-off. The different behaviour of strain MM8 grown at different C:N ratios, compared to that reported here, may have been due to strain to strain variability, again suggesting inexplicable variability in switch-off between similar experiments.

During growth of *M. methylotrophus* at different C:N ratios, dual C/N-limitation was observed at C:N ratios from 4.7-10.4. This suggested that *M. methylotrophus* could alter its cellular carbon and nitrogen content over a wide range of values. However, in the absence of more detailed analysis (e.g. by on-line effluent gas analysis, total biomass C and N analysis or analysis of, for example, extracellular polymer production) this conclusion remains tentative. It is possible to predict the theoretical boundaries of dual limitation from growth yields under carbon limitation (\(Y_{XC}\): g cells g carbon\(^{-1}\)) and nitrogen limitation (\(Y_{XN}\): g cells g nitrogen\(^{-1}\)), by substituting \(Y_{XN}\) and \(Y_{XC}\) calculated under both growth regimes into the following equation (Egli, 1991):

\[\frac{C_0}{N_0} = \frac{Y_{XN}}{Y_{XC}}\]

where \(\frac{C_0}{N_0}\) is the theoretical boundary between carbon limitation and dual limitation using yield values for carbon-limited growth, and the theoretical boundary between dual
Table 4.10 \( \text{Y}_{X/N} \) and \( \text{Y}_{X/C} \) values of \( M. \ methylotrophus \) grown in continuous culture 
\((D=0.1\text{h}^{-1})\) with acetamide as the nitrogen source

\( M. \ methylotrophus \) was grown in continuous culture \((D=0.1\text{h}^{-1})\) with acetamide as nitrogen source under methanol-limited and methanol-excess conditions, steady-state cell densities were measured and biomass yields calculated (see Table 3.4).

<table>
<thead>
<tr>
<th>Limiting nutrient</th>
<th>( \text{Y}_{X/N} ) ( \text{(g cells g nitrogen}^{-1})</th>
<th>( \text{Y}_{X/C} ) ( \text{(g cells g carbon}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>4.11</td>
<td>1.46</td>
</tr>
<tr>
<td>Acetamide</td>
<td>5.47</td>
<td>0.42</td>
</tr>
</tbody>
</table>
limitation and nitrogen limitation using yield values for nitrogen-limited growth. When $c_0/n_0$ was calculated using yield data for *M. methylotrophus* growing on acetamide as nitrogen source (Table 4.10), the average theoretical boundaries were 2.8 and 13. In the experiment, the boundary between carbon limitation and dual limitation (2.8-4.7) was therefore close to the theoretical value, whereas the boundary between dual limitation and nitrogen limitation (10.4-12.3) was slightly lower than predicted.

It is interesting to note the variation in the concentrations of acetamidase and Mdh-α during growth at different C:N ratios, especially during dual limitation. Thus (Fig. 4.1, Fig. 4.5, Table 4.2), as the C:N ratio increased from 1.8 to 4.7, with a concomitant decrease in culture supernatant ammonia concentration, the concentration of acetamidase increased sharply; once the culture was dual-limited (and subsequently acetamide-limited), with no detectable ammonia in the culture supernatant, the concentration of acetamidase did not change substantially. Conversely, the concentration of Mdh-α during growth under methanol limitation was approximately 17% of cell protein (Table 4.2; Fig. 4.5), and this concentration gradually decreased during dual-limited conditions to reach a concentration of approximately 5% of cell protein during culture under acetamide limitation. Therefore, the concentration of Mdh-α during dual-limitation (even though the culture was methanol-limited) changed significantly with the increasing C:N ratio, whereas the concentration of acetamidase (even though the culture was acetamide-limited) did not. Hence, it can be seen that the concentration of both acetamidase and Mdh are regulated, in-tandem, in relation to the C:N ratio of the culture. However, even under these tightly controlled growth regimes, at different C:N ratios, switch-off of acetamidase activity was not seen.

When ammonia was pulsed into a dual limited culture, in contrast to all of the steady-state growth regimes investigated, a large decrease in acetamidase activity and calculated $k_{cat}$ was observed and heat-reactivation of activity could be demonstrated in broken cells. Similar results were obtained following an acetamide pulse, but not following acetate or methanol pulses, suggesting that ammonia was probably the physiological signal which caused switch-off of acetamidase activity. This down-regulation of acetamidase activity in response to exogenous ammonia was reminiscent of the switch-off of nitrogenase activity in a number of bacterial species in response to ammonia (Zumft & Castillo, 1978; Yoch & Cantu, 1980; Sweet & Burris, 1981; Kush *et al.*, 1985; Hartmann *et al.*, 1986; Reich *et al.*, 1986).
Figure 4.5 Acetamidase and Mdh-α concentrations during growth of *M. methylotrophus* in continuous culture (D=0.1h⁻¹) at different C:N ratios

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) with acetamide as nitrogen source at various C:N ratios. Washed cell samples were subjected to SDS-PAGE, stained for protein with Kenacid blue R and the gel analysed as described in Materials & Methods. Acetamidase concentration (-○-); Mdh-α concentration (-▲-).
was substantially slower than that of nitrogenase (50% decrease in 2.5h cf. approximately 30min respectively; Sweet & Burris, 1981). Hence, a clear comparison can be drawn between acetamidase and nitrogenase in that they are both the first enzyme involved in nitrogen assimilation and they are both switched-off by ammonia. Interestingly, RubisCO activity in *Rhodobacter sphaeroides* has been found to be controlled by switch-off in response to the addition of fixed carbon, in the form of organic acids, to cultures growing photolithoautotrophically under nitrogen-excess conditions (Wang & Tabita, 1992a & b) with reactivation being observed upon depletion of fixed carbon from the culture. The switch-off and subsequent reactivation were reliant not only upon the addition of organic acid, but also on the nitrogen status of the culture. In nitrogen-limited cultures low RubisCO activity was seen, and reactivation of switched-off activity was not seen. Hence, in this organism switch-off of a carbon-assimilating enzyme appears to be co-ordinated with the nitrogen status of the cell. Acetamidase conversely represents a nitrogen-assimilating enzyme which is switched-off in response to the nitrogen status of the cell only, with no switch-off by acetate or methanol.

Switch-off of acetamidase activity could also be potentiated by changing the growth regime from dual acetamide/methanol limitation to dual ammonia/methanol limitation in a similar manner to that employed by ZENECA Bio Products during growth at high cell density. As no excess ammonia (<0.01mM) was detected in the culture supernatant during such a medium exchange, it is likely that the cause of switch-off was either the exchange of nitrogen source from acetamide to ammonia, or that the concentration of excess ammonia required to cause switch-off was very low i.e. <0.01mM. Switch-off of nitrogenase activity has also been found to occur at very low micromolar ammonia concentrations (Zumft & Castillo, 1978; Hartmann *et al.*, 1986; Yoch *et al.*, 1988).

When the loss of acetamidase activity following an ammonia pulse or medium exchange was compared to that seen when *M. methylotrophus* was grown at high cell density with an alternating nitrogen source by ZENECA Bio Products, it was clear that the rate of switch-off was slower at low cell density (Fig. 4.6). The acetamidase activity at low cell density decrease by 50% in approximately 2.5h, compared to approximately 1h at high cell density. The major differences between growth at low cell density and high cell density was that
Figure 4.6 Comparison of the decrease in acetamidase activity seen upon ammonia pulse or nitrogen source exchange at low cell density and that at high cell density with an alternating nitrogen source.

*M. methylotrophus* was grown at low cell density (0.8 g l\(^{-1}\)) and either a 2mM-ammonia pulse carried out as described in Materials & Methods, or the in-flowing medium changed from dual acetamide/methanol-limited to dual ammonia/methanol-limited. *M. methylotrophus* was also grown at high cell density (>10 g l\(^{-1}\)) by ZENECA Bio Products with an alternating nitrogen (acetamide/ammonia) source (Carver & Jones, 1993). At time zero either ammonia was added to the culture, or the nitrogen source was exchanged from acetamide to ammonia, and the acetamidase activity followed. Low cell density, ammonia pulse (—○—); low cell density, nitrogen source exchange (−Δ−); high cell density, ZENECA Bio Products (−□−).
excess acetate was present at high cell density. However, it has been shown at least at low cell density that acetate does not potentiate more rapid switch-off, as demonstrated by similar rates between ammonia and acetamide pulses and also following acetate pulse after exchanging the nitrogen source from acetamide to ammonia. Hence, cell density may play some role in switch-off. Cell density has recently been shown to play a role in several cell signalling events in bacteria. Bioluminescence in the marine bacterium *Vibrio fischeri* and antibiotic biosynthesis in *Erwinia carotovora* have been found to be dependent upon the concentration of small molecules, termed autoinducers (e.g. N-(3-oxohexanoyl) homoserine lactone; HAS) in the culture supernatant (Williams *et al.*, 1992; Bainton *et al.*, 1992). These autoinducers are produced by the bacteria and only at high cell density do they reach a threshold value in the culture supernatant which initiates gene expression. The presence of autoinducers in many genera of bacteria has also been reported (Bainton *et al.*, 1992), however, the role that these play in gene expression and cell physiology has yet to be determined. Hence, this mechanism allows the bacterial cell to sense the abundance of its own species in the environment and only when the population is sufficiently high does appropriate gene expression occur. It could be tentatively hypothesised that such a cell signalling event may operate during the growth of *M. methylotrophus* at high cell density. A chemical signal may build up to a threshold value in the culture supernatant which then produces more rapid switch-off in response to the signals described above. Experiments to further investigate the effect of cell density upon the rate of switch-off were unsuccessful for technical and limited time reasons. Hence, the reason for this difference in the rate of switch-off remains obscure.

The ability to purify switched-off acetamidase from an ammonia-pulsed dual-limited culture demonstrated that this procedure produced genuinely switched-off acetamidase. Furthermore, incubation of purified high-activity acetamidase with ammonia *in vitro* did not cause switch-off (Wyborn, 1994), demonstrating that ammonia switch-off was not a simple allosteric effect of ammonia. However, during purification of acetamidase from an ammonia-pulsed culture further increases in the level of switch-off were seen, suggesting that switch-off could occur *in vitro*. It was subsequently found that this *in vitro* switch-off could also be caused by storage of acetamidase at -20°C. A large amount of work was carried out to try and characterise the effect that growth regime had on *in vitro* switch-off.
However, the results obtained were highly variable, making further investigation of this non-physiological form of switch-off impossible. It is interesting to note, however, that inactivation of pyruvate carboxylase from chicken liver mitochondria and of 17β-hydroxy steroid dehydrogenase by cold has been reported previously (Jarabak et al., 1966; Irias et al., 1969). Reactivation of pyruvate carboxylase could be brought about simply by rewarming the enzyme solution at 23°C. Cold inactivation was caused by dissociation of the tetrameric form of the enzyme. This is in contrast to that seen with acetamidase as upon thawing and warming of enzyme to 37°C low activities were still seen. It was not until the enzyme had been heated at 60°C for 1h that reactivation was observed. Cold inactivation of 17β-hydroxy steroid dehydrogenase has been reported to be due to conformational changes within the enzyme molecule. This hypothesis seems a more adequate explanation for the switch-off of acetamidase activity in vitro by freezing. In the absence of definitive proof as to the modification/mechanism of switch-off, the suggestion that switched-off acetamidase is altered by a “locked” conformational change (Carver & Jones, 1993; Wyborn, 1994) becomes very attractive.

Experiments with samples taken from dual-limited cultures (and hence no longer able to grow) suggested that switch-off of acetamidase activity could also be potentiated by elevated pH. This decrease in activity was also accompanied by heat-reactivation demonstrating that loss of activity was not due to simple denaturation. This mechanism of switch-off, in similarity to that of in vitro switch-off by freezing, was non-physiological and when an ammonia pulse into dual-limited culture was carried out no increase in culture pH was observed. Hence, this mechanism of switch-off by elevated pH cannot account for the decrease in acetamidase activity upon ammonia pulse. The rate and extent of switch-off by incubation of non-growing cells at elevated pH was highly variable between apparently identical experiments. It was therefore concluded that a number of physiological and non-physiological conditions could potentiate switch-off of acetamidase. These observations, together with the highly variable nature of switch-off at any level beyond dual-limited culture, made further investigation of the mechanism of switch-off impossible within the time available.

Formamidase activity in dual-limited culture also appeared to undergo switch-off in a similar manner to that of acetamidase. Large decreases in formamidase activity and $k_{cat}$
were observed upon ammonia and formamide pulse, and also when the nitrogen source was changed from acetamide to ammonia. Formate had no effect upon formamidase activity. No heat-reactivation of switched-off formamidase activity was seen. Moreover, when formamidase was purified following an ammonia pulse, reactivation was observed during the purification procedure. This suggested that the putative modification to formamidase was different to that of acetamidase and labile to purification. Due to this inability to purify switched-off formamidase and the difficulties encountered investigating acetamidase switch-off, formamidase switch-off was not pursued further.

It has previously been suggested that the physiological role of switch-off of acetamidase activity in *M. methylotrophus* is to conserve cellular resources and/or rapidly to prevent the accumulation of high concentrations of potentially toxic acetate when an alternative nitrogen source becomes available to a culture growing on acetamide (Carver & Jones, 1993). This hypothesis appears rational when considering that switch-off was seen when ammonia was pulsed into a dual-limited culture or the limiting nitrogen source was changed from acetamide to ammonia. The rate of switch-off seen following either of these physiological conditions (t1/2 2.5h), although slow in comparison to other switch-off events, was substantially faster than that due to simple repression of enzyme synthesis and subsequent dilution (t1/2 10h). However, as switch-off was also seen in vitro by storing high-activity enzyme at -20°C, or in non-growing cells when incubated at elevated pH, it appears that switch-off of acetamidase activity is not solely a physiological event. This suggested that either the switch-off seen upon freezing enzyme and incubation of cells at high pH is produced by a different mechanism to that in vivo upon ammonia pulse or nitrogen source exchange, or that switch-off in vivo is brought about by spurious physical events within the cell potentiated by certain physiological conditions. It appears unlikely that a chance, physical event in vitro, such as low temperature or elevated pH, could mimic a biochemical mechanism for switch-off of acetamidase activity which has been evolved over millions of years in response to physiological conditions. To discern whether the mechanism of switch-off is common to all of the conditions found to potentiate it, both in vivo and in vitro, would require a substantial effort using bio-physical, biochemical and possibly further molecular biology techniques (See Wyborn, 1994) which were beyond the scope of this study. However, the physiological signals which potentiate switch-off in vivo
have been elucidated and added further to our knowledge about this event. Unfortunately, further attempts to elucidate the mechanism of switch-off proved unfruitful and very frustrating, due to an innate variability between apparently identical experiments at levels beyond growing culture, making this problem impossible to unravel further. Therefore, to date, the exact biochemical mechanism of acetamidase switch-off in *M. methylotrophus* remains an interesting, but unsolved problem.
CHAPTER 5

EVOLUTION OF *M. METHYLOTROPHUS* IN FORMAMIDE-LIMITED CONTINUOUS CULTURE
CHAPTER 5

EVOLUTION OF M. METHYLOTROPHUS IN FORMAMIDE-LIMITED CONTINUOUS CULTURE

5.1 Introduction

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5.6 Discussion
5.1 Introduction

The cultivation of microorganisms in nutrient-limited continuous culture not only allows the study of the physiological regulation of enzymes under clearly-defined conditions, as previously discussed in Chapter 3, but also applies a strong selective pressure. This selective pressure is highest upon so called "substrate-capturing" enzymes (Harder et al., 1977), i.e. those which have a direct effect upon the rate of utilisation of the growth-limiting nutrient such as uptake systems or enzymes of the catabolic pathway which demonstrate high flux-control coefficients (see Dykhuizen et al., 1987). Selective pressure during continuous culture is highest at low dilution rates, where the standing concentration of the limiting nutrient (S) is concomitantly low. Spontaneous mutations which endow a mutant with a selective advantage compared with the wild-type will eventually allow it to out-grow the parent strain and take over the culture. The selection of mutants in continuous culture was realised as early as 1950 (Novick & Szilard, 1950b), and has been widely studied and utilised to isolate microbial strains with enhanced performance characteristics (for reviews see Harder et al., 1977; Dykhuizen & Hartl, 1983). Selection in continuous culture is often referred to as "directed evolution," but must not be confused with "directed mutation" as suggested by Cairns et al. (1988; see also Hall, 1991), or the recently described "molecular directed evolution" as reviewed by Osuna et al. (1994).

During steady-state growth in chemostat culture the growth rate of a microorganism is dependent upon the maximum specific growth rate (μmax), the saturation constant of the organism for the growth-limiting nutrient (numerically equivalent to the growth-limiting substrate concentration which gives a growth rate of 0.5 μmax; Ks) and the concentration of growth-limiting substrate in the culture (S) by the classical Monod equation (see also section 3.1):

\[
\mu = \frac{\mu_{\text{max}} S}{K_s + S}
\]  

(1)
Hence, any mutant arising within a continuous culture which has a higher $\mu_{\text{max}}$ or a lower $K_s$ would have a higher specific growth rate than the parental strain and would eventually take over the culture. For example, if a culture was growing at a dilution rate (and therefore a specific growth rate) of 0.05h$^{-1}$, and a spontaneous mutant arose within the culture with a $\mu_{\text{max}}$ under the same conditions of $>0.05h^{-1}$, this strain would eventually outgrow the parent and take over the culture. Mathematical analyses of competition of mutants in continuous culture have been carried out by Powell (1958) and Dykhuizen & Hartl (1981). Selective advantage can be conferred on the mutant by a number of mutations to “nutrient-capturing” enzymes, which are generally of two types; (i), $V_{\text{max}}$ mutations, which increase the rate of uptake or metabolism of the limiting nutrient either by the over-production of wild-type enzyme or by the production of an altered enzyme with an increased $k_{\text{cat}}$ (i.e. giving a higher $\mu_{\text{max}}$); (ii), $K_m$ mutations, which increase the affinity of the organism for the growth-limiting nutrient (i.e. giving a lower $K_s$). It has been concluded that, in general, $\mu_{\text{max}}$ mutants are usually selected at high dilution rates and $K_s$ mutants are usually selected at low dilution rates (Wiebe et al., 1994a).

The selection process observed within an evolving continuous culture can be of two types reflecting the metabolic activities measured during the selection period. The first has been termed “periodic selection” (also referred to as “nonspecific selection”; Atwood et al., 1951; Dykhuizen & Hartl, 1983) which is characterised by a pattern of periodic increases and decreases in a given activity during chemostat culture. By definition, the given activity which demonstrates periodic/nonspecific selection is not the one which confers the selective advantage, but one which “hitchhikes” in a mutant which does have a selective advantage (see Dykhuizen & Hartl, 1983). Conversely, “specific selection” is where the activity followed during selection is that which conveys the selective advantage. During specific selection the activity measured does not follow an unpredictable, periodic increase and decrease in activity, as seen with periodic selection, but usually exhibits a sudden step-wise increase in activity.

A very wide range of examples of evolution in chemostat culture have been reported in the literature. However, only a few will be considered here, and will concentrate mainly on lactose metabolism. Directed evolution of $\beta$-galactosidase in *E. coli* during growth under lactose limitation has been studied in detail (Horiuchi et al., 1962; Dykhuizen & Hartl,
lactose limitation has been studied in detail (Horiuchi et al., 1962; Dykhuizen & Hartl, 1983). When *E. coli* was grown under lactose limitation \((D=0.115\text{hr}^{-1})\) for 10-20 generations a sharp increase in the β-galactosidase activity was observed. This increase in activity was found to be due to the isolation of strains which were constitutive for the synthesis of β-galactosidase. The selective advantage conferred on the constitutive mutant over the inducible wild-type was that β-galactosidase was produced at maximum concentration (though not hyper-produced) even during growth at lactose concentrations which were normally insufficient to induce the enzyme fully in the wild-type organism. Thus, at the low lactose concentrations which prevailed during lactose-limited growth at low dilution rates the constitutive mutant had a higher growth rate than the wild-type and therefore eventually replaced it in the culture. After several hundred further generations of growth under lactose limitation, strains exhibiting even higher β-galactosidase activities were isolated and were designated as "hyper strains". These hyper strains were shown to over-produce wild-type β-galactosidase up to approximately 25% of cell protein (cf. approximately 6% of cell protein in wild-type). However, β-galactosidase is not the first enzyme in the metabolism of lactose, since the latter is transported into the cell via a proton symport system specific to β-galactosides (West, 1970). Due to the method of selection (prolonged growth under lactose limitation at low dilution rate) it may have been expected that strains would have been isolated with an improved β-galactoside permease, either by the over-expression of wild-type permease or by the production of a more efficient permease (higher \(k_{\text{cat}}\) and/or lower \(K_{\text{M}}\)). However, the hyper-producing strains were found to have lactose uptake rates similar to those of the wild-type, indicating that no advantageous mutation in the lactose transport system had occurred. This suggested that lactose uptake was probably not a major rate-limiting step in lactose metabolism under the conditions employed.

*Agrobacterium radiobacter* can also be grown upon lactose as a carbon source. In this organism lactose is transported into the cell via a lactose-binding protein (LBP)-dependent uptake system, and subsequently hydrolysed using a highly active β-galactosidase. When *A. radiobacter* was grown in continuous culture at low dilution rate \((0.045\text{hr}^{-1})\) under lactose limitation for approximately 40 generations, a sudden increase in the rate of lactose uptake was observed (Williams et al., 1990) due to the culture being taken over by a
spontaneous mutant strain which was later purified and referred to as AR50. The latter was constitutive for the expression of both the transport system and $\beta$-galactosidase, and both LBP and $\beta$-galactosidase were over-expressed compared with the wild-type. Hence, in both *A. radiobacter* and *E. coli* the $\beta$-galactosidase had become constitutive and over-expressed. But, in contrast to *E. coli*, the lactose uptake system in *A. radiobacter* had also become over-expressed. Therefore, in strain AR50 the selective advantage over the wild-type was conferred not only by over-expression of $\beta$-galactosidase (as in hyper strains of *E. coli*), but also by the over-expression of the transport system (such that LBP comprised up to 30% of cell protein cf. 8% in the wild-type). The over-expression of other binding protein-dependent sugar transport systems has also been shown previously in *A. radiobacter* during prolonged growth under glucose limitation (Cornish et al., 1988), demonstrating the prime importance of nutrient uptake to growth at low substrate concentrations.

The isolation of mutant strains with improved uptake systems by directed evolution in continuous culture has been reported in *E. coli* (Collins et al., 1976) and *Ps. aeruginosa* (Williams et al., 1994). The amino acid alanine is transported into *E. coli* via a proton symport system similar to that for lactose. When *E. coli* was grown in continuous culture ($D=0.1h^{-1}$) for approximately 200 generations with alanine as the limiting nutrient, the culture demonstrated more rapid alanine uptake than the wild-type. This was found to be due to the presence of several novel strains demonstrating altered stoichiometries of proton-coupled alanine uptake, the consequence of which was to increase the $V_{\text{max}}$ of the uptake system.

When *Ps. aeruginosa* was grown in continuous culture ($D=0.05h^{-1}$) under glycerol limitation for prolonged periods (up to 260 generations), increases in the activity of the energy-independent glycerol uptake system, glycerol kinase and glycerol-3-phosphate dehydrogenase were observed. These increases were found to be accompanied by a shift to constitutivity and to over-expression of the kinase and dehydrogenase as well as an outer membrane protein. The latter was also over-expressed following prolonged growth under glucose limitation, and was subsequently identified as a previously-characterised glucose porin, OprB. It was concluded that *Ps. aeruginosa* transported both glucose and glycerol via OprB, which was over-expressed during prolonged culture under either glucose or glycerol limitation in order to facilitate the rapid passage of low concentrations of these
substrates through the outer membrane. The increase in glycerol kinase and glycerol-3-phosphate dehydrogenase activity was probably a response to the requirement that, once transported through the inner membrane, free glycerol must be rapidly removed in order to maintain a glycerol concentration gradient across the membrane and thus maintain transport by facilitated diffusion. *P. aeruginosa* therefore over-expressed several proteins in response to prolonged growth under glucose or glycerol limitation, similar to the over-expression of the lactose transport system and β-galactosidase in response to the prolonged growth of *A. radiobacter* under lactose limitation.

Directed evolution of the filamentous fungus *Fusarium graminearum* A3/5 which is important commercially as a source of myco-protein (Quorn) has recently been reported during growth of the organism in glucose-limited continuous culture at low dilution rate (D=0.05h\(^{-1}\); Wiebe *et al.*, 1994a). Under these conditions six peaks of periodic selection were observed in advantageous mutants carrying cyclohexamide-resistance over 69 generations, representing five adaptive changes in the microbial population. The mutant strain isolated from the last adaptive peak (referred to as strain A28-S) was found to have a significantly lower \(K_m\) for glucose uptake (12 cf. 29\(\mu\)M) than the parent strain A3/5, representing a \(K_s\) mutation. It is interesting to note that no “colonial” mutants (selectively advantageous mutants which demonstrate highly branched mycelia) were isolated during growth at low dilution rate. Conversely, colonial mutants were selected during culture of *F. graminearum* A3/5 under glucose limitation at higher dilution rates (0.18 or 0.19h\(^{-1}\); Wiebe *et al.*, 1994b), suggesting that any advantages associated with colonial mutations are not selected at low dilution rates. Selection of colonial mutants in prolonged glucose-limited continuous cultures represent a problem during commercial production of Quorn myco-protein, as these mutants cause alterations to both the filtration characteristics of culture broth and the texture of the final product. Hence, a series of glucose-limited chemostat cultures (D=0.18h\(^{-1}\)) were used to isolate novel *F. graminearum* strains with delayed colonial mutant formation (Wiebe *et al.*, 1994b). However, the physiological/biochemical basis for the selection of these strains was not described.

Another important aspect of microbial selection and evolution is the isolation of mutant strains which acquire the ability to utilise “new” substrates *i.e.* ones which the organism was previously unable to metabolise. Many examples of such “metabolic acquisitions”
examples will be considered here. One of the most widely studied of such systems is the evolved β-galactosidase (EBG) of E. coli. When lacZ deletion mutants of E. coli K12 were incubated for several weeks on solid medium containing lactose as the sole source of carbon and energy, colonies developed which could utilise lactose (Hall, 1984). These isolates were found to contain a lactase which was physically, biochemically and immunologically different to LacZ. The locus responsible for the lactase was mapped to an area of the E. coli chromosome near to tolC, which is on the opposite side of the chromosome to the lac operon. This enzyme became known as EBG. In the unevolved lacZ deletion E. coli strain the EBG protein (EBG0) was found to be induced by lactose, and was a β-galactosidase (showing activity to o-nitrophenyl galactopyranoside; ONPG) but not a lactase. Only after sufficient selective pressure in the presence of lactose did EBG acquire the ability to hydrolyse lactose at a significant rate, due to a mutation in the ebgA gene causing a change in the genotype from ebgA0 to ebgA+. The ebgA+ mutants isolated fell into two groups; type I which synthesised EBG constitutively and type II which only synthesised EBG when induced by lactose. The regulation of EBG enzyme synthesis was subject to negative control by a repressor that was the product of the ebgR gene, located very close to ebgA. Type I mutants had mutations in both ebgA (enabling growth on lactose) and ebgR (causing constitutive expression of EBG), whereas type II mutants had a mutation only in ebgA (enabling growth on lactose). It was subsequently discovered that some constitutive isolates only grew slowly on lactose, but had obtained the ability to grow on lactulose, suggesting that upon suitable selections the substrate utilised by the EBG enzyme could be varied. Subsequent selections carried out with an ebgA0, ebgR+ strain on lactose, lactulose, galactosyl-arabinoside and lactobionate lead to the isolation of mutants which could utilise these substrates. Purification and kinetic analysis of the EBG enzymes from these strains demonstrated clearly that selection for the utilisation of a particular substrate always increased the catalytic specificity of the enzyme for that substrate (increased Vmax, decreased Km).

Another example of metabolic acquisition is afforded by Klebsiella aerogenes mutants which can utilise xylitol as a carbon and energy source. Enterobacteria oxidise ribitol, a more naturally common isomer of xylitol, to D-ribulose using an inducible NAD+-linked ribitol dehydrogenase (Rdh), then phosphorylate D-ribulose to D-ribulose 5-phosphate by
ribitol dehydrogenase (Rdh), then phosphorylate D-ribulose to D-ribulose 5-phosphate by D-ribulokinase which is then fed into the pentose-phosphate pathway (Rigby et al., 1974; Mortlock, 1982; Hartley, 1984). Similarly, arabitol is oxidised to xylulose by Rdh which is then phosphorylated to xylulose 5-phosphate by xylulokinase and fed into the pentose-phosphate pathway. Xylitol and arabitol will not support growth of wild-type K. aerogenes, but mutants that are constitutive for Rdh will grow slowly on these substrates. Rdh has a low affinity for xylitol and arabitol (Kₘ approximately 1M cf. 1mM for ribitol) and only when it is produced constitutively is growth on these substrates possible. Hartley and his co-workers have studied the evolution of Rdh in continuous culture for the utilisation of xylitol (Rigby et al., 1974; Hartley, 1984). When an Rdh constitutive mutant was grown in xylitol-limited continuous culture new mutant strains took over the culture after 50-200 generations. These mutant strains were all found to over-produce the wild-type Rdh, allowing the mutants to grow faster on xylose than the original strain and hence take over the culture. Further selections rendered mutants which contained higher concentrations of Rdh, but did not produce any with improved characteristics towards xylitol (i.e. lower Kₘ and/or higher Vₘₐₓ). It was only following chemical mutagenesis and further rounds of selection in the chemostat that mutants producing Rdh with enhanced kinetic characteristics were isolated. The evolution of Ps. aeruginosa acetamidase to allow the utilisation of new amides as carbon and nitrogen sources for growth has also been reported (see Chapter 1; Mortlock, 1982; Clarke, 1984; Clarke & Drew, 1988).

The isolation of acetamidase hyperactive strains of M. methylotrophus in acetamide- and acrylamide-limited continuous culture (D=0.025h⁻¹) has been reported previously (see Chapter 1; Silman et al., 1989; 1991; Silman 1990). This chapter describes the directed evolution of M. methylotrophus in formamide-limited continuous culture.
5.2 Prolonged growth of \textit{M. methylotrophus} in formamide-limited continuous culture

The growth of wild-type \textit{M. methylotrophus} in acetamide- and acrylamide-limited continuous culture at low dilution rate has previously been shown to facilitate the isolation of acetamidase hyperactive mutants (Silman \textit{et al.}, 1989; 1991; Silman 1990). In view of the successful application of chemostat selection to acetamidase, it was considered appropriate to see if this approach could also be used to produce mutants with altered formamidase and/or alterations in formate metabolism. Wild-type \textit{M. methylotrophus} was grown in formamide-limited continuous culture (D=0.1h\(^{-1}\)) for 101 days, equivalent to approximately 350 generations. A dilution rate of 0.1h\(^{-1}\) was used as at lower dilution rates, with concomitantly higher selection pressure, steady-state formamide limitation was not obtainable (see section 3.8).

5.2.1 Enzyme activities during directed evolution

Washed cells were prepared at intervals and assayed for formamidase, acetamidase and urease activity and formate oxidation rate. Formamidase activity almost doubled during the course of the experiment (Fig. 5.1), but this occurred gradually rather than by a sudden, step-wise change of the type more commonly seen as mutant strains take over a culture (Horiuchi \textit{et al.}, 1962; Collins \textit{et al.}, 1976; Harder \textit{et al.}, 1977; Tsang & Grootwassink, 1988; Cheng \textit{et al.}, 1989; C.W. Jones, personal communication). In contrast, acetamidase activity remained very low during the course of the experiment, and the slight increase observed was probably a reflection of the 5\% activity which formamidase exhibits towards acetamide. Urease activity initially increased in-line with the formamidase activity, but reached a maximum after approximately 114 generations and then declined slightly.

The formate oxidation rate observed also demonstrated a substantial increase (Fig. 5.1) suggesting that the increased ability to oxidise formate during formamide limitation conferred a selective advantage. However, unlike formamidase activity the increase in formate oxidation rate occurred principally by a sharp step-wise increase after approximately 160 generations.
Figure 5.1 Directed evolution of *M. methylotrophus* under formamide limitation

*M. methylotrophus* was grown in continuous culture (D=0.1hr\(^{-1}\)) under formamide limitation for approximately 350 generations. Culture samples were periodically removed from the chemostat, and washed cells prepared and assayed for formamidase, acetamidase and urease activity and formate oxidation rate as described in Materials & Methods. Formamidase activity (○); acetamidase activity (■); urease activity (△); formate oxidation rate (♦).
5.2.2 SDS-PAGE and Western blotting of cell samples collected during directed evolution

To investigate further the apparent selection of a formamidase hyperactive mutant during prolonged growth under formamide limitation, washed cells prepared at intervals during the experiment were subjected to SDS-PAGE followed by gel analysis and Western blotting (Figure 5.2). No significant increase in formamidase concentration was observed during selection (Figure 5.3), which averaged 4.0±0.1(8)% of cell protein; this conclusion was substantiated by the Western blot (Fig. 5.2(c)). Only very low concentrations of acetamidase were seen, which were only just detectable by Western blotting (Fig. 5.2(b)), as observed previously during growth under formamide limitation (see section 3.4). However, a striking and continuous increase in the concentration of a protein(s) with an approximate Mr of 40,000 was observed during the experiment, the concentration of which tripled to approximately 18.7% of cell protein during the course of the experiment (Fig. 5.3). This protein had previously been observed following growth under formamide and urea limitation and shown to be composed of two unknown proteins with different N-terminal amino acid sequences (see section 3.4). The observation that one or both of the proteins became over-expressed during prolonged growth under formamide limitation suggested that they conferred a selective advantage under these conditions. To find out whether one or both of the proteins had become over-expressed, a sample of washed cells collected after 350 generations was subjected to SDS-PAGE, the Mr 40,000 protein band excised and the first 6 residues of the N-terminal amino acid sequence(s) determined. The composition and relative quantity of the sequences indicated that the two proteins (GATISF-) and (ADYPTA-) were present in approximately the same ratio as in the wild-type (Table 5.1), demonstrating that both proteins had become over-expressed.

It was also noted that an unknown protein of Mr approximately 32,000 exhibited a step-wise increase in concentration during the experiment (Figs. 5.2; 5.3), from a very low concentration to approximately 3.0% of cell protein after 10-60 generations, but only marginally increased to 4.2% of cell protein thereafter. However, the identity and function of this protein was not pursued further due to time limitations.
M. methylotrophus was grown in continuous culture (D=0.1h$^{-1}$) under formamide limitation for approximately 350 generations. Periodically, culture samples were removed from the chemostat, washed cells prepared and subjected to SDS-PAGE and Western blotting as described in Materials & Methods. Tracks 1, $M_r$ standards; 2, 17 generations; 3, 66 generations; 4, 139 generations; 5, 197 generations; 6, 211 generations; 7, 260 generations; 8, 284 generations; 9, 308 generations; 10, $M_r$ standards. (a), SDS-PAGE; (b), Western blot developed with acetamidase antiserum; (c), Western blot developed with formamidase antiserum.
Figure 5.3 Evolution of *M. methylotrophus* in formamide-limited continuous culture

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under formamide limitation for approximately 350 generations. Culture samples were periodically removed from the chemostat, and washed cells prepared and subjected to SDS-PAGE and gel analysis as described in Materials & Methods. Formamidase (-o-); Mdh-α (-.o-); Mr 40,000 protein (-o-); Mr 32,000 protein (-Δ-).
Table 5.1 N-terminal amino acid sequences of Mr 40,000 proteins following directed evolution of *M. methylotrophus* under formamide limitation

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under formamide limitation for 350 generations, then washed cells were prepared and subjected to SDS-PAGE and the over-expressed Mr 40,000 protein(s) excised and subjected to N-terminal amino acid sequencing as described in Materials & Methods.

<table>
<thead>
<tr>
<th>N-terminal amino acid sequence of Mr 40,000 proteins</th>
<th>Amount of PTH amino acid (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>18.45</td>
</tr>
<tr>
<td>A</td>
<td>18.45</td>
</tr>
<tr>
<td>T</td>
<td>13.84</td>
</tr>
<tr>
<td>I</td>
<td>18.75</td>
</tr>
<tr>
<td>S</td>
<td>7.98</td>
</tr>
<tr>
<td>F</td>
<td>17.69</td>
</tr>
<tr>
<td>A</td>
<td>22.40</td>
</tr>
<tr>
<td>D</td>
<td>32.42</td>
</tr>
<tr>
<td>Y</td>
<td>20.30</td>
</tr>
<tr>
<td>P</td>
<td>18.49</td>
</tr>
<tr>
<td>T</td>
<td>12.62</td>
</tr>
<tr>
<td>A</td>
<td>16.34</td>
</tr>
</tbody>
</table>
In contrast, the concentration of Mdh-α during the experiment decreased slightly during the early part of the selection process, but returned to approximately the starting concentration later (Fig. 5.3), giving an average concentration of 7.2±0.3(8)% of cell protein.

5.3 Isolation of *M. methylotrophus* strain MM25

In order to investigate further the changes which had occurred during directed evolution under formamide limitation, it was necessary to isolate the *M. methylotrophus* strain exhibiting the evolved phenotype. A sample of culture was taken after approximately 350 generations (culture A) and streaked on SEII-agar containing ammonium as the nitrogen source. One of the resultant single colonies was picked at random and grown in continuous culture under formamide limitation. This mutant exhibited a phenotype very similar, but not identical to culture A, suggesting that some heterogeneity existed within culture A. The mutant was subsequently termed strain MM25, in convention with the nomenclature system previously used by Silman et al. (1989).

5.4 Comparison of strain MM25 with wild-type *M. methylotrophus* and culture A following growth in continuous culture under formamide limitation

5.4.1 Amidohydrolase activities and formate oxidation rates

Strain MM25 was grown in formamide-limited continuous culture (D=0.1h⁻¹), washed cells prepared and acetamidase, formamidase and urease activities determined together with formate oxidation rate. The formamidase activity of MM25 was substantially higher than that of both wild-type (from previous experiments) and culture A, from which it was derived (Table 5.3). This demonstrated that some level of heterogeneity still existed in culture A even after approximately 350 generations under formamide limitation. The acetamidase activity of MM25 under formamide limitation was higher than that in both wild-type and culture A grown under the same conditions, but this probably reflected the ability of formamidase to hydrolyse acetamide slowly (see also section 3.8).
<table>
<thead>
<tr>
<th></th>
<th>2.94f0.25(3)</th>
<th>0.25f0.25(3)</th>
<th>6.3f0.16(3)</th>
<th>4.35</th>
<th>2.4f0.13(3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>2.92</td>
<td>2.02</td>
<td>2.02</td>
<td>2.15</td>
<td>2.4f0.13(7)</td>
</tr>
<tr>
<td></td>
<td>0.07f0.03(7)</td>
<td>2.6f0.13(7)</td>
<td>2.18f0.2(7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table S.2** Comparison of mitochondrial and oxidation rates of wild-type *M. meliloti* and *M. phaseoli* under formamide-illumination.
Urease activity conversely, was only slightly higher in culture A and MM25 compared with the wild-type, whereas the formate oxidation rate of MM25 was substantially higher than that of wild-type, but slightly lower than in culture A (Table 5.3). These results were again compatible with heterogeneity within culture A from which MM25 was derived.

5.4.2 SDS-PAGE analysis

Whole cells of wild-type *M. methylotrophus*, culture A and MM25 were subjected to SDS-PAGE and gel analysis (Fig. 5.4). The formamidase concentration in culture A and MM25 were similar to that seen in wild-type (4.6 and 4.9 cf. 4.5% of cell protein, respectively). It therefore appeared that during selection no significant increase in formamidase concentration occurred, indicating that MM25 was not a hyper-expressing mutant in contrast to the isolation of MM6 by directed evolution under acetamide limitation (Silman *et al.*, 1989; Silman 1990). The formamidase activities and concentrations yielded calculated whole cell k\(_{\text{cat}}\) values of 195, 330 and 431s\(^{-1}\) for wild-type, culture A and MM25 respectively. Hence, it appeared that the increase in formamidase activity during directed evolution under formamide limitation was due to the selection of one or more mutant strains (represented by MM25) with increased k\(_{\text{cat}}\) values.

Formamidase was subsequently purified to electrophoretic homogeneity from MM25 in parallel with the U/F-BP (see Chapter 6), and partially characterised (Table 5.4). The purified enzyme exhibited a substantially higher specific activity and k\(_{\text{cat}}\) than previously reported for wild-type formamidase (Wyborn *et al.*, 1994; Wyborn, 1994; see also section 4.9). The MM25 formamidase also demonstrated a slightly lower K\(_{\text{m}}\) for formamide than that of the wild-type. However, this small difference in K\(_{\text{m}}\) may not have been significant, as K\(_{\text{m}}\) values of 1.7-2.3mM have previously been observed for wild-type formamidase (N.R. Wyborn, personal communication). It was therefore concluded that MM25 was expressing a mutant formamidase with an enhanced k\(_{\text{cat}}\).

The concentration of formate dehydrogenase in whole cells could not be measured as this protein is present at undetectably low levels (S. Callan, J. A. Greenwood & C. W. Jones, unpublished), therefore the mechanism by which the formate oxidation rate increased in culture A and MM25 remains obscure.
Figure 5.4 SDS-PAGE of wild-type *M. methylotrophus*, culture A and MM25 washed cells following growth under formamide limitation.

Wild-type *M. methylotrophus*, culture A and MM25 were grown in continuous culture (D=0.1h⁻¹) under formamide limitation, washed cells prepared, subjected to SDS-PAGE and stained for protein with Kenacid blue as described in Materials & Methods. Tracks 1, Mr standards; 2, wild-type *M. methylotrophus*; 3, culture A; 4, MM25; 5, Mr standards.
Table 5.4 Kinetic properties of purified wild-type and MM25 formamidase

The specific activity, $k_{cat}$ and $K_m$ of purified MM25 formamidase was determined with formamide as substrate at 37°C as described in Materials & Methods. The values quoted for purified wild-type formamidase are taken from (a), Wyborn et al., 1994; (b), this study (see Table 4.9).

<table>
<thead>
<tr>
<th>Formamidase</th>
<th>Specific activity</th>
<th>$k_{cat}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmol min$^{-1}$ [mg protein]$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td>(mM)</td>
</tr>
<tr>
<td>Wild-type (a)</td>
<td>37.4</td>
<td>64</td>
<td>2.1</td>
</tr>
<tr>
<td>Wild-type (b)</td>
<td>100.0</td>
<td>178</td>
<td>-</td>
</tr>
<tr>
<td>MM25</td>
<td>148.5±34.3(3)</td>
<td>322±74(3)</td>
<td>1.62±0.14(3)</td>
</tr>
</tbody>
</table>
The Mr 40,000 proteins which became over-expressed during selection was found to constitute approximately 7.5% of cell protein in wild-type, which increased to 13.1% in culture A and 12.3% in MM25. The Mr 32,000 protein band which became rapidly over-expressed during selection under formamide limitation was undetectable in the wild-type but constituted approximately 4.0% of cell protein in culture A and MM25. Due to time limitations the identity and function of this protein were not investigated further.

5.5 Subcellular location of Mr 40,000 proteins

5.5.1 Crude membrane and high-speed supernatant fractions

To further characterise the Mr 40,000 proteins, crude membranes and high-speed supernatant were prepared from a formamide-limited culture of MM25 and subjected to SDS-PAGE. Protein bands of similar intensity with Mr of approximately 40,000 were seen in both fractions (Fig 5.5). N-terminal amino acid sequencing of the excised bands containing the membrane-associated and soluble proteins of Mr approximately 40,000 showed that the proteins had the sequences GAT1- and ADYP- respectively. It was therefore concluded that one protein (GAT1-) was membrane bound and the other (ADYP-) was soluble. It was also noted that the Mr 32,000 protein was also membrane bound, whereas, formamidase was located entirely in the supernatant fraction.

5.5.2 Attempted preparation of inner and outer membranes

As one of the Mr 40,000 proteins (GAT1-) was membrane bound, attempts were made to separate crude membranes into inner and outer membrane fractions in order to determine the location of this protein more accurately. Several attempts at separation were made using sucrose density-gradient centrifugation as described in Materials & Methods. However, satisfactory separation of NADH oxidase activity (a marker of inner membranes) and KDO (a marker of outer membranes) was never achieved on any of the sucrose gradients employed (Table 5.5.a-d). It was therefore not possible, by this methodology, to
determine whether the over-expressed protein was located in the inner or outer membrane of *M. methylotrophus*.

5.5.3 Attempted preparation of periplasmic and cytoplasmic fractions

Due to the Gram-negative nature of *M. methylotrophus*, soluble proteins can either be periplasmic or cytoplasmic. In an attempt to identify the soluble Mr 40,000 protein (ADYP-) as either cytoplasmic or periplasmic, several shock fluid (periplasm) preparations were carried out as described in Materials & Methods. However, formamidase and glucose-6-phosphate dehydrogenase activity (both cytoplasmic markers) and/or NADH oxidase activity were observed in all the preparations, suggesting that the shock fluids were contaminated by cytoplasm and/or inner membranes due to spheroplast breakage (Table 5.6.a-c). It was therefore not possible, by this methodology, to determine whether the soluble Mr 40,000 protein (ADYP-) was periplasmic or cytoplasmic.
Figure 5.5 SDS-PAGE of crude membranes and high-speed supernatant of MM25 grown in continuous culture under formamide limitation

*M. methylotrophus* strain MM25 was grown in continuous culture (D=0.1h⁻¹) under formamide limitation, crude membranes and high-speed supernatant were prepared and then subjected to SDS-PAGE and stained for protein with Kenacid blue as described in Materials & Methods. Tracks 1, Mr standards; 2, crude membranes; 3, high-speed supernatant; 4, Mr standards.
Table 5.5 Attempted separation of *M. methylotrophus* crude membranes into inner and outer membranes

Crude membranes prepared from *M. methylotrophus* grown in continuous culture (D=0.1h⁻¹) under formamide limitation were separated upon sucrose density gradients by a modification of the method of Hancock & Carey (1979) as described in Materials & Methods. Four attempted separations were carried out; Table 5.4.a, gradient of 20, 60 and 70% (w/v) sucrose; Table 5.4.b, gradient of 20, 52, 58, 64 and 70% (w/v) sucrose; Table 5.4.c, gradient of 20, 55, 58, 60 and 70% (w/v) sucrose; Table 5.4.d, essentially identical to 5.4.c except broken cells were treated with lysozyme before crude membrane preparation (Hancock & Nikaido, 1978). Membrane fractions collected from the interfaces between different sucrose concentrations after centrifugation of gradients to equilibrium were assayed for NADH oxidase activity and KDO concentration as described in Materials & Methods, the amount of each marker in the fractions is expressed as a percentage of the total.

Table 5.5.a

<table>
<thead>
<tr>
<th>Fraction</th>
<th>NADH oxidase activity (%)</th>
<th>KDO concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>47</td>
</tr>
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</table>
### Table 5.5.b

<table>
<thead>
<tr>
<th>Fraction</th>
<th>NADH oxidase activity (%)</th>
<th>KDO concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>29</td>
</tr>
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<td>4</td>
<td>9</td>
<td>12</td>
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</table>

### Table 5.5.c

<table>
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<tr>
<th>Fraction</th>
<th>NADH oxidase activity (%)</th>
<th>KDO concentration (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>53</td>
<td>28</td>
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<tr>
<td>2</td>
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<td>42</td>
</tr>
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<td>3</td>
<td>19</td>
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### Table 5.5.d

<table>
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<tr>
<th>Fraction</th>
<th>NADH oxidase activity (%)</th>
<th>KDO concentration (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>69</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>29</td>
</tr>
</tbody>
</table>
Shock fluids (periplasm) were prepared from \textit{M. methylotrophus} washed cells following growth in continuous culture (D=0.1h\textsuperscript{-1}) under formamide limitation by three different protocols as described in Materials & Methods. Table 5.5.a, method of Silman, (1990); Table 5.5.b, method of Eftekhar & Schiller (1994); Table 5.5.c, method of Cheng \textit{et al.} (1970). Following shock fluid preparation broken cells and membranes were prepared and all three sub-cellular fractions assayed for formamidase, NADH oxidase and glucose-6-phosphate dehydrogenase activity as described in Materials & Methods, the amount of each marker in the fractions is expressed as a percentage of the total.

**Table 5.6 Attempted preparation of M. methylotrophus shock fluid (periplasm)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Formamidase activity (%)</th>
<th>NADH oxidase activity (%)</th>
<th>Glucose-6-phosphate dehydrogenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shock-fluid</td>
<td>36</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td>Membranes</td>
<td>0</td>
<td>49</td>
<td>17</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>64</td>
<td>27</td>
<td>56</td>
</tr>
</tbody>
</table>
### Table 5.6.b

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Formamidase activity (%)</th>
<th>NADH oxidase activity (%)</th>
<th>Glucose-6-phosphate dehydrogenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shock-fluid</td>
<td>0</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>Membranes</td>
<td>0</td>
<td>29</td>
<td>2</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>100</td>
<td>46</td>
<td>97</td>
</tr>
</tbody>
</table>

### Table 5.6.c

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Formamidase activity (%)</th>
<th>NADH oxidase activity (%)</th>
<th>Glucose-6-phosphate dehydrogenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shock-fluid</td>
<td>9</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Membranes</td>
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<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>91</td>
<td>0</td>
<td>70</td>
</tr>
</tbody>
</table>
5.6 Discussion

During directed evolution of *M. methylotrophus* in continuous culture (D=0.1h⁻¹) under formamide limitation the formamidase activity increased by approximately two-fold. This increase was gradual rather than a sudden, step-wise increase which is generally associated with the selection of hyper-expressing strains (Horiuchi *et al.*, 1962; Collins *et al.*, 1976; Harder *et al.*, 1977; Tsang & Grootwassink, 1988; Cheng *et al.*, 1989). The formate oxidation rate of the culture also increased approximately three-fold, but in contrast to formamidase activity, this increase was step-wise. The concentration of two Mr of 40,000 proteins also increased by approximately four-fold; this change in concentration, in similarity to formamidase activity, was gradual rather than step-wise. Another protein with an Mr of approximately 32,000 also increased in concentration by >three-fold, in a step-wise manner.

Strain MM25 was found to express a number of mutant characteristics following growth under formamide limitation in continuous culture (D=0.1h⁻¹). The formamidase activity of MM25 during growth under formamide limitation was substantially higher than that of wild-type *M. methylotrophus*, but the enzyme had not become over-expressed. Purification of MM25 formamidase showed that this high activity was due to the production of a formamidase with a substantially enhanced *k*<sub>cat</sub>. Hence, MM25 was similar to strain MM8 in that the respective amidase exhibited an enhanced *k*<sub>cat</sub>, but in the case of MM25 hyper-production of the formamidase had not occurred (Silman *et al.*, 1989; 1991; Silman, 1990). MM25 also over-expressed two Mr 40,000 proteins which were induced during growth under formamide and urea limitation (see section 3.4), suggesting that these proteins were important for formamide metabolism or transport. The formate oxidation rate of MM25 was substantially higher than that of wild-type, possibly reflecting the increased need to remove potentially-toxic concentrations of formate produced due to the higher formamidase activity exhibited by this strain. A further advantage of the increased formate oxidation rate would be to generate increased amounts of NADH (and hence ATP by oxidative phosphorylation).

That MM25 expressed a formamidase with higher activity than that observed in culture A, but similar Mr 40,000 proteins concentration and formate oxidation rate, suggested that
culture A was not homogeneous. However, MM25 probably represented the major strain within culture A and exhibited the dominant phenotype viz. over-expression of Mr 40,000 proteins, increased formamidase $k_{cat}$ and increased formate oxidation rate.

The identity of the Mr 40,000 proteins was further investigated by combining the biochemical evidence described above with molecular biology data (N. R. Wyborn and S. G. Williams, unpublished). A 3.2 kbp $PstI$ restriction fragment from *M. methylotrophus* chromosomal DNA has recently been shown to contain the formamidase structural gene (*fmdA*) (see Chapter 1; section 3.2; Wyborn *et al.*, 1994), together with a second gene (*fmdB*) which encodes a regulatory protein and appears to constitute a presumed formamidase operon with *fmdA*; incomplete open-reading frames (orfC and orfD) were also present. Due to the truncation of orfC and orfD a larger (8.4kbp) $Sph$ 1-fragment of *M. methylotrophus* DNA containing the entire *fmdC, fmdA, fmdB* and *fmdD* gene sequences has subsequently been cloned and sequenced (S. G. Williams & C. W. Jones, unpublished). Recloning and sequencing of this larger DNA fragment containing complete orfC and orfD showed that both encoded hydrophilic proteins (Wyborn, 1994; N. R. Wyborn, S. G. Williams & C. W. Jones, unpublished). The derived amino acid sequences of the orfC and orfD products were therefore scrutinised to see if either contained the N-terminal amino acid sequences of the two Mr 40,000 proteins. The sequence *GATISF-* was present within the translated orfC (subsequently called *fmdC*), starting at amino acid residue 27. This suggested that FmdC had a peptide signal sequence; a conclusion which was commensurate with its membrane-bound location. The sequence *ADYPTA-* was present within the translated orfD (subsequently called *fmdD*), starting at amino acid 31. This also suggested that FmdD possessed a signal sequence, which given the soluble nature of the mature protein, indicated that it was located in the periplasm.

The *fmdC* gene was found to have a putative -12, -24 NtrA-type promoter. The amino acid sequence preceding the first amino acid of the mature FmdC protein was characteristic of a signal peptide (Duffaud *et al.*, 1985; Gierasch, 1989; Goodwin & Anthony, 1995). The sequence contained 26 amino acids, with two positively charged residues (two adjacent lysine residues at -21 and -22 close to the N-terminal end of the signal sequence) (Fig. 5.6). The central region of the sequence was highly hydrophobic with glycine residues at -9 and -7, and small side-chain residues (alanine) were present at -3 and -1. Therefore, the first 26
amino acids of FmdC represented an archetypal signal sequence. Once the signal peptide region had been removed the mature FmdC protein was predicted to be hydrophilic, contain 364 amino acids and have an $M_r$ of 39,204, which was similar to the approximate $M_r$ of 40,000 as determined by SDS-PAGE. From the membrane location, hydrophilic nature and pattern of expression (see section 3.4) of FmdC it was tentatively concluded that it acts as a porin (Nikaido, 1993; 1994) to facilitate the passage of amides and urea through the outer membrane. Further evidence for the outer membrane location of the FmdC protein was not possible as satisfactory separation of inner and outer membranes on sucrose density gradients was not achieved. However, this hypothesis was given further credence as simple structural analyses showed that FmdC contained extensive $\beta$-pleated sheet secondary structure (a characteristic of porins; Saier, 1994) and substantial structural homology with the *E. coli* porin OmpF (S. G. Williams & C. W. Jones, unpublished).

The *fmdD* gene was also found to have a putative -12, -24 NtrA-type promoter. The derived amino acid sequence preceding the first amino acid of the mature FmdD was also found to demonstrate archetypal signal peptide characteristics. The signal peptide contained 30 amino acids, with three positively charged residues (two adjacent arginine residues at -24 and -25 and a lysine at -20, all close to the N-terminal end of the sequence) (Fig. 5.6). The central region of the sequence was highly hydrophobic with a glycine residue at the -9 position, and with alanine residues at the -3 and -1 positions. Therefore, the first 30 amino acids of the FmdD product encoded a typical peptide signal sequence. Once the signal peptide region had been removed the mature FmdD protein was predicted to be hydrophilic, 382 amino acid in length and have an $M_r$ of 41,870, which was similar to the approximate $M_r$ of 40,000 as determined by SDS-PAGE. The amino acid sequence of the mature FmdD showed a strong homology (29% strict identity; Fig. 5.7) to the primary sequence of AmiC from *Ps. aeruginosa* (Wilson *et al.*, 1993). This protein is a member of a structurally-related family of periplasmic binding proteins (Tam & Saier, 1993). However, AmiC has become cytoplasmic and involved in the regulation of amidase expression (Wilson *et al.*, 1993; Chapter 1). The FmdD protein sequence also demonstrated significant homology to the leucine-isoleucine-valine-binding proteins from *E. coli* (Adams *et al.*, 1990), *S. typhimurium* (Ohnishi *et al.*, 1990) and *Ps. aeruginosa* (Hoshino & Kose, 1989) (12%, 17% and 23% strict identity respectively; Fig. 5.7) and therefore is also
The nucleotide and derived amino acid sequence of *fmdC* and *fmdD* from plasmid pSW1 insert DNA (S. G. Williams & C. W. Jones, unpublished). Positively charged amino acid residues in the N-terminal portion of the signal peptide are represented in parentheses. Hydrophobic amino acids within the signal peptide hydrophobic region are underlined. Glycine residues within the hydrophobic domain are marked with an asterisk (*). The C-terminal amino acid of the signal peptide is designated as -1, with the N-terminal amino acid of the mature protein being designated as +1. The extent of N-terminal amino acid sequencing of proteins is marked with an arrow (^).
GCCACGTACTCCATGAAGATTCGCTGACTGAACACATCTGAGCAACACTTGCATGGCACTGTGGTGGGTGAAGAG
WPRTSMKIARKHIEQHLHGTVVGEE
TACATCGCTTTGGACGACACTCACTCGTGGTCCGATCAACAAATCAAACTGAAAATACTGACGTTGACTCT
YIALDDTQFGSVDNKIKLKDVI
ACGCAGCGGTGGTAGGCAGTAACGCAGCTAGTATGGGTATCGGTGGTGAAAACCTGGCAGGTTTC
YTMLTISVTEDEVLGIGGGENLAGP
TACTCAGCCATGAAATATTCCCAATCCCCAGCAATCTCTGAGAACCAGAAATTTTGTAGAAGGCTTTCAAAAAAC
YSAMKFQFSQDNPEKAKPFVEAFKKK
GCTGGGTAAAGAAGCTCTGAGCTGACGTGACCAGCAGCTGGTGCCCTGTGTTGACAAAACCTGGCAGGTTTC
KWGDVKDAVIGBDVTQAAYLGPWLYKAA
ACGCAGCGGTGGTAGGCAGTAACGCAGCTAGTATGGGTATCGGTGGTGAAAACCTGGCAGGTTTC
VERSFGDFDVDKVQAALPGYTFKDP
CCAGAAGGTCCCTTGAGCTCTAGTAACCACACCTGACACCCAGAGCTGTGTATCGATCATTACCGGCTGAATGAA
EGPVTEAAANHHLTTKRLIQGQWGK
ATGCACAGCTAAAGTGTGTTACACCTCTGACTACATAACACCATTACCCAGACTGACATTGATCAATGGGTGAA
DGQAARVYSTDYIKPDPPPGBKGYQ
Figure 5.7 Amino acid alignments of bacterial proteins showing high homology to *M. methylotrophus* FmdD (U/F-BP)

*M. methylotrophus* mature FmdD (U/F-BP) amino acid sequence was subjected to a BLAST database search and peptide sequences showing high levels of homology were aligned using a GAP alignment algorithm. Amino acids demonstrating strict identity between FmdD and other peptide sequences are represented in bold type. EcLivI, *E. coli* leucine-isoleucine-valine-binding protein; StLivC, *Salmonella typhimurium* leucine-isoleucine-valine-binding protein; PaLiv, *Ps. aeruginosa* leucine-isoleucine-valine-binding protein; MmFmdD, *M. methylotrophus* urea/formamide-binding protein; PaAmiC, *Ps. aeruginosa* AmiC.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcLivJ</td>
<td>NAKGOGDKRNQGIAKVTGSGPVAQTVGSQ FMDQAVAQAI</td>
</tr>
<tr>
<td>StLivC</td>
<td>NAKGOGDKRNQGIAKVTGSGPVAQTVGSQ FMDQAVAQAI</td>
</tr>
<tr>
<td>PaLiv</td>
<td>NAKGOGDKRNQGIAKVTGSGPVAQTVGSQ FMDQAVAQAI</td>
</tr>
<tr>
<td>MmFmdD</td>
<td>NAKGOGDKRNQGIAKVTGSGPVAQTVGSQ FMDQAVAQAI</td>
</tr>
<tr>
<td>PaAmiC</td>
<td>NAKGOGDKRNQGIAKVTGSGPVAQTVGSQ FMDQAVAQAI</td>
</tr>
</tbody>
</table>
probably a member of this structurally-related family of proteins. Due to its structural similarity to AmiC and various amino acid binding-proteins, putative periplasmic location and physiological regulation, it was hypothesised that FmdD may be a periplasmic urea/formamide-binding protein, hence explaining the over-expression of this protein by prolonged growth under formamide-limitation. The FmdD protein has subsequently been demonstrated to be periplasmic in recombinant *E. coli* carrying the *fmdD* gene (S. G. Williams, personal communication).

A further orf (*fmdE*) has recently been identified downstream of *fmdD* which is transcribed from the same promoter and encodes a putative transmembrane protein (S. G. Williams & C. W. Jones, unpublished). It is likely, therefore, that *fmdD* and *fmdE* encode part of an “ABC-type” transporter (Ames, 1986; Ames *et al.*, 1990; Higgins *et al.*, 1990; Tam & Saier, 1993; Chapter 1) for formamide and urea, similar to that used by other species of bacteria for the transport of sugars and amino acids (Ames, 1986; Greenwood *et al.*, 1990; Williams *et al.*, 1990; Adams *et al.*, 1990; Ohnishi *et al.*, 1990; Hoshino & Kose, 1989). Similarly, the presence of FmdC during growth under acetamide, formamide and urea limitation implied that it may act as an amide and urea specific porin (for reviews see Nikaido, 1993; 1994) to facilitate the passage of amides and/or urea across the outer membrane.

It was therefore concluded that MM25 over-expressed an outer-membrane porin and a binding protein-dependent transport system for formamide and urea, as well as synthesising a formamidase with an increased $k_{cat}$ and demonstrating an increased capacity for formate oxidation. This scenario was similar to that seen when *Ps. aeruginosa* was subjected to directed evolution under glycerol-limitation; not only was the uptake system over-expressed, but so also were enzymes involved in the subsequent metabolism of the transported glycerol (Williams *et al.*, 1994). Both the over-expression of wild-type enzyme and the expression of an altered enzyme with an increased $k_{cat}$ leads to the more rapid utilisation of the substrate. In *M. methylotrophus* the next “more fit” phenotype, following the selection of a more efficient uptake system, would be to utilise formamide more rapidly, as was seen in MM25. Hence, the presence of the formamidase $k_{cat}$ mutation within MM25 may not have been a happenstential periodic selection event and within time MM25 may have taken over the culture completely.
It has previously been noted that hyper-producing strains can only be selected in continuous culture when the growth-rate of the organism at low substrate concentration is limited by the activity of the enzyme responsible for the initial utilisation of the substrate (Harder et al., 1977). Under these conditions it is frequently the rate of substrate transport into the cell which limits the growth rate, and therefore mutants exhibiting a more efficient transport system are selected. This must have been the case for formamidase as over-expression of a putative uptake system was observed. However, it must be noted that a mutant which over-expressed the putative amide porin was apparently not isolated during directed evolution of *M. methylotrophus* under acetamide limitation (Silman et al., 1989; Silman, 1990). This suggested that the rate of diffusion of acetamide through the outer membrane was not a major growth-rate limiting step, whereas conversely the rate of transport of formamide was. The reason for this appears obscure as both amides are small uncharged molecules, as also is urea, which would be expected to diffuse freely into the cell. The significance of this will be discussed in more detail in the next chapter.
CHAPTER 6

PURIFICATION OF FmdD AND ITS IDENTIFICATION AS A UREA/FORMAMIDE-BINDING PROTEIN
CHAPTER 6

PURIFICATION OF FmdD AND ITS IDENTIFICATION AS A UREA/FORMAMIDE-BINDING PROTEIN

6.1 Introduction

6.2 Purification of FmdD from MM25

6.3 N-terminal amino acid sequence of purified FmdD

6.4 Determination of subunit and native Mr

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6.7 Discussion
PURIFICATION OF FmdD AND ITS IDENTIFICATION AS A UREA/FORMAMIDE-BINDING PROTEIN

6.1 Introduction

It is a common assumption that small, uncharged molecules enter the bacterial cell by simple diffusion by virtue of exhibiting high diffusion coefficients. However, it has recently become apparent that transport systems operate for many such molecules during growth at low substrate concentrations (e.g. in chemostat culture at low dilution rate). The uptake of glycerol by *Ps. aeruginosa* and *E. coli* occurs via a high-affinity facilitated-diffusion system (Lin, 1976; Williams *et al.*, 1994), and urea is actively-transported into a number of bacterial species including *Ps. aeruginosa* and the marine bacterium *Deleya venusta* HG1 (Jahns, 1992a & b; see also Jahns *et al.*, 1988). Furthermore, urea uptake by the cyanobacterium *Anabaena cycadeae* is independent of urease activity (therefore discounting uptake due to a urease-dependent urea concentration gradient across the cell membrane) (Singh, 1991). However, none of these urea uptake systems has been characterised.

Many bacterial binding protein-dependent solute transport systems have been well characterised (for recent reviews see Higgins *et al.*, 1990; Tam & Saier, 1993; Saier, 1994; Chapter 1). The most commonly purified and characterised component of these systems is the binding protein, due to its soluble nature and relatively high concentration. All solute-binding-proteins so far characterised are monomeric proteins of Mr 25,000-56,000 (Ames, 1986; Ames *et al.*, 1990), which show a bilobate structure with the cleft between the two lobes containing the binding site (Ames, 1986; Ames *et al.*, 1990; Nikaido & Ames, 1992). They demonstrate high affinity for their substrate (*K_D* ≤1μM) and undergo a conformational change upon substrate binding. Binding-proteins have recently been grouped into 8 clusters by way of amino acid sequence homology (Tam & Saier, 1993; Chapter 1).

*A. radiobacter* has been shown to transport glucose and lactose using specific binding protein-dependent uptake systems (Cornish *et al.*, 1988; Greenwood *et al.*, 1990). Two glucose-binding proteins (GBP1 and GBP2) were purified to near homogeneity from shock
fluid using anion exchange FPLC, and shown to be monomeric proteins (Mr 36,500 and 33,500 respectively) using SDS-PAGE and gel filtration FPLC. Both proteins were found by equilibrium dialysis to bind glucose with high affinity (GBP1 $K_D=0.23\mu M$; GBP2 $K_D=0.07\mu M$) using a single binding site. A lactose-binding protein (LBP) was purified from shock fluid in a similar manner to GBP1 and GBP2, and also shown to be a monomeric protein (Mr 41,000) with a high affinity for lactose ($K_D=0.14\mu M$) and a single binding site (Greenwood et al., 1990). This binding protein-dependent system is of particular interest as lactose is transported into other bacterial species by way of the H$^+$ symport and phosphotransferase systems. The N-terminal amino acid sequences of all three binding proteins showed significant homology with sugar binding proteins from other species of bacteria.

The amino acids lysine, arginine and also ornithine are transported in to S. typhimurium by way of a binding protein-dependent uptake system. The lysine-, arginine-, ornithine-binding protein (LAO) has been purified to homogeneity from shock fluid by way of acid precipitation, carboxymethyl cellulose chromatography and HPLC ion-exchange chromatography (Nikaido & Ames, 1992). The binding-protein has an $M_r$ of approximately 30,000, and binds L-arginine, L-lysine and L-ornithine with $K_D$ values of 14, 15 and 29nM respectively, probably at a single binding site.

The branched-chain amino acids leucine, isoleucine and valine can be transported into Ps. aeruginosa, S. typhimurium and E. coli by a binding protein-dependent uptake system. The binding-protein from Ps. aeruginosa (LIV-BP; BraC) has been purified to homogeneity from shock fluid by ammonium sulphate precipitation, DEAE-cellulose and DEAE-Sephadex column chromatography (Hoshino & Kageyama, 1980). The binding-protein has an $M_r$ of 40,000-43,000 as determined by SDS-PAGE and gel filtration, and a predicted $M_r$ of 39,767 as determined from the braC gene sequence (Hoshino & Kageyama, 1980). Leucine, isoleucine and valine are bound with $K_D$ values of 0.4, 0.3 and 0.5μM respectively. The branched-chain amino acid binding protein from S. typhimurium (LIVT-BP; LivB) has also been purified to homogeneity from shock fluid by DEAE-cellulose and DEAE-Sephadex chromatography, and isoelectric focusing (Ohnishi & Kiritani, 1983). LIVT-BP has an $M_r$ of 34,000-38,000 as determined by SDS-PAGE and gel filtration, and a predicted $M_r$ of 36,627 as determined from the livB gene sequence.
Leucine, isoleucine and valine are bound with $K_D$ values of 0.43, 0.15 and 0.89$\mu$M respectively. However, a binding stoichiometry of $\leq 1$ was observed. The LIV-BP (LivJ) from *E. coli* has also been purified from shock fluid by ammonium sulphate precipitation and chromatography on DEAE-cellulose and DEAE-Sephadex columns (Amanuma & Anraku, 1974). This LIV-BP has an $M_r$ of 36,770 as determined by amino acid sequencing of the protein (Ovchinnikov et al., 1977). Leucine and isoleucine are bound with $K_D$ values of 0.12 and 0.19$\mu$M respectively, at a single binding site. However, a $K_D$ value for valine was not reported (Amanuma et al., 1976). It is interesting to note that AmiC from *Ps. aeruginosa*, which has been demonstrated to be an amide binding-protein (Wilson et al., 1993), has been grouped with aliphatic amino acid-binding proteins (Tam & Saier, 1993). AmiC has recently been purified and characterised and found to have a sub-unit $M_r$ of 42,834 and exist as a dimer (Wilson & Drew, 1991). Acetamide, propionamide, lactamide and butyramide all bind to AmiC, with $K_D$ values of 3.7 and 3.1$\mu$M for acetamide and propionamide respectively, with the other two amides binding approximately 100-fold less tightly (Wilson et al., 1993). Interestingly, the binding stoichiometry of acetamide:AmiC was found to be 1:2. However, AmiC is a cytoplasmic protein and involved in amidase gene expression, not amide transport, which may explain its unusual binding stoichiometry and dimeric composition.

Directed evolution of *M. methylotrophus* under formamide limitation has led to the isolation of a mutant MM25 which over-expressed two proteins, FmdC and FmdD (Chapter 5). Based on their physiological regulation, derived amino acid sequences and sub-cellular locations, it was tentatively concluded that FmdC is an outer membrane porin for amides and urea, and that FmdD is a periplasmic urea/formamide-binding protein (section 5.7). This chapter describes the purification of FmdD and its identification as a urea/formamide-binding protein.
6.2 Purification of FmdD from MM25

*M. methylotrophus* strain MM25 was grown in formamide-limited continuous culture (D=0.1 hr⁻¹). Washed cells were disrupted, then centrifuged to produce a high-speed supernatant as described in Materials & Methods. An initial experiment was carried out to investigate the ammonium sulphate concentration at which FmdD was precipitated from the high-speed supernatant. An aliquot of high-speed supernatant (5ml) was taken and fractionated at 0-35, 35-50, 50-65 and 65-80% ammonium sulphate saturation. At each ammonium sulphate concentration the precipitate was removed by centrifugation and redissolved in 20mM-Bis/Tris buffer pH 6.8 as described in Materials & Methods. The ammonium sulphate fractions were subsequently analysed by SDS-PAGE and the majority of FmdD was found to precipitate at ammonium sulphate concentrations of 65-80% (Fig. 6.1). Most of the formamidase also precipitated within this fraction, such that FmdD and formamidase were the major proteins present.

Purification of FmdD for later characterisation was therefore initiated by preparing a high-speed supernatant, making it 65% saturated with ammonium sulphate and then taking the supernatant for further purification. The ammonium sulphate fraction was dialysed and further purified by Mono-Q anion-exchange chromatography FPLC as described in Materials & Methods. FmdD eluted from the Mono-Q column in two fractions as a sharp, well-separated protein peak at a salt concentration of approximately 115mM-KCl (Fig. 6.2). SDS-PAGE and gel analysis of the bulked fractions showed that FmdD was ≥95% pure at the end of this procedure with a yield of 12.6% (Fig. 6.3; Table 6.1). Interestingly, formamidase eluted from the Mono-Q column at a salt concentration of approximately 385mM-KCl, and was thus co-purified by this simplified procedure to a high level of purity (≥95%; see section 5.4.2).

6.3 N-terminal amino acid sequence of purified FmdD

Purified FmdD was subjected to SDS-PAGE, then excised from the gel and subjected to N-terminal amino acid sequencing. This was carried out to check the identity of the purified protein as no easy assay was available for the protein during purification besides
Figure 6.1 Ammonium sulphate precipitation of a high-speed supernatant fraction from *M. methylotrophus* strain MM25

*M. methylotrophus* strain MM25 was grown in continuous culture under formamide limitation (D=0.1h⁻¹). Washed cells were disrupted, then centrifuged to produce a high-speed supernatant which was fractionated at various concentrations of ammonium sulphate as described in the text. Ammonium sulphate fractions were subjected to SDS-PAGE and stained for protein with Kenacid blue R as described in Materials & Methods. Tracks 1, Mr standards; 2, high-speed supernatant; 3, 0-35% ammonium sulphate fraction; 4, 35-50% ammonium sulphate fraction; 5, 50-65% ammonium sulphate fraction; 6, 65-80% ammonium sulphate fraction; 7, >80% ammonium sulphate fraction; 8, Mr standards.
Figure 6.2 Purification of FmdD and formamidase from *M. methylotrophus* strain MM25 by Mono-Q anion-exchange chromatography following ammonium sulphate precipitation

*M. methylotrophus* strain MM25 was grown in formamide-limited continuous culture (D=0.1h⁻¹). Cells were harvested, washed, disrupted and then centrifuged to produce a high-speed supernatant which was then fractionated by ammonium sulphate precipitation as described in Materials & Methods. The dialysed ammonium sulphate fractionated supernatant (>65%) was loaded on to a Mono-Q anion exchange column and bound proteins eluted with a linear 0-1M-KCl salt gradient (Materials & Methods). A₂₈₀nm (-), KCl gradient (-).
A280nm

Fraction number

KCl concentration (M)
Table 6.1: Partition of FMD from M. melitensis strain M125 following growth in continuous culture under formamide-limitation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Built Monolayer Subpellet</th>
<th>HSS</th>
<th>Broken Cells</th>
<th>Washed Cells</th>
</tr>
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<tr>
<td>13.2</td>
<td>72</td>
<td>8.9</td>
<td>32.4</td>
<td>44.6</td>
</tr>
<tr>
<td>9.6</td>
<td>4.3</td>
<td>3.9</td>
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</tr>
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<td>0.1</td>
<td>1.5</td>
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</tr>
<tr>
<td>0.001</td>
<td>0.1</td>
<td>1.0</td>
<td>4.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Cells was calculated assuming the concentration of FMD and FND within the MFL 40,000 protein band to be 55% and 45%, respectively.

Samples were subjected to SDS-PAGE and gel analysis as described in Materials and Methods. The concentration of FMD in washed cells and broken cells was calculated to produce a high-speed supernatant from which FND was separated using ammonium sulfate precipitation and anion-exchange FPLC.

*M. melitensis* strain M125 was grown in formamide-limited continuous culture (D = 0.15 h−1). Cells were harvested, washed, disrupted and then
Figure 6.3 SDS-PAGE of samples during the purification of FmdD from *M. methylotrophus* strain MM25 grown in continuous culture under formamide-limitation

*M. methylotrophus* strain MM25 was grown in formamide-limited continuous culture (D=0.1h⁻¹). Cells were harvested, washed, disrupted and then centrifuged to produce a high-speed supernatant from which FmdD was purified using ammonium sulphate precipitation and anion-exchange FPLC as described in Materials & Methods. Tracks 1, Mr standards; 2, washed cells; 3, broken cells; 4, high-speed supernatant; 5, 65% ammonium sulphate supernatant; 6, bulked Mono-Q fractions; 7, Mr standards.
subjective identification due to Mr upon SDS-PAGE. Only a single sequence (ADYP-) was
found, and was identical to that of FmdD following the removal of the signal sequence,
thus confirming that the purified protein was also the soluble protein which was induced
under formamide and urea limitation and over-expressed (together with FmdC) in MM25.

6.4 Determination of subunit and native Mr

Purified FmdD was subjected to SDS-PAGE, stained for protein with Kenacid blue R and
analysed to determine the subunit Mr as described in Materials & Methods (Fig. 6.3). The
Mr of 41,000 was very similar both to the Mr of approximately 40,000 determined
previously in whole cells and to the predicted Mr of 41,870. The native Mr was determined
by gel-filtration chromatography; a small amount (approximately 28μg) of protein was
chromatographed with Mr standards on a Superose-12 HR 10/30 gel-filtration column as
described in Materials & Methods and yielded a native Mr of 45,000. The native Mr of the
FmdD has recently been determined using polyacrylamide gel electrophoresis in the
presence of only a very low concentration of SDS (under conditions where both
formamidase and acetamidase are not dissociated), and similar values were obtained as for
gel-filtration and conventional SDS-PAGE (J. A. Greenwood, personal communication). It
was concluded, therefore, that FmdD is a monomer.

6.5 Identification of FmdD as a urea/formamide binding protein (U/F-BP) using
equilibrium dialysis

6.5.1 Determination of the binding constant of U/F-BP for urea

As no radiolabelled formamide was available, binding was investigated using [14C]urea.
Urea bound well to FmdD, but not to boiled FmdD or BSA. The binding constant for urea
(KD; μM) was determined by equilibrium dialysis over a 20-fold range of [14C]urea
concentrations (13.3-261.7μM) as described in Materials & Methods. Scatchard analysis
yielded an average KD of 7.15μM and a stoichiometry of approximately 0.75mol urea
mol protein$^{-1}$ (Fig. 6.4) from two independent determinations. It was concluded, therefore, that FmdD bound urea with high affinity and probably at a single binding site.

6.5.2 Substrate-binding specificity

The ligand-binding specificity of FmdD was investigated by carrying out competitive equilibrium dialysis using $[^{14}C]$urea and various unlabelled amides, organic acids and ammonia at concentrations of 0.8µM and 32.5µM cf. 1.3µM $[^{14}C]$urea, as described in Materials & Methods. Binding of $[^{14}C]$urea to FmdD was decreased essentially as expected by the presence of unlabelled urea; viz. to 65% cf. 60% at 0.8µM and to 6% cf. 4% at 32.5µM (Table 6.2). The presence of unlabelled formamide and acetamide decreased binding to 71% and 77% respectively at 0.8µM and to 6% and 29% respectively at 32.5µM; the effect of unlabelled formamide was therefore similar to that of urea, but greater than that of acetamide, suggesting that the affinities of FmdD for formamide and urea were similar, but much greater than for acetamide. The effect of unlabelled propionamide, butyramide, formate, acetate and ammonia on $[^{14}C]$urea binding was also investigated. Propionamide and butyramide had a small effect upon binding at concentrations of 32.5µM, the effect of butyramide being slightly larger than that of propionamide, but none of the other solutes had any significant effect upon binding (Table 6.2). The affinity of FmdD for various solutes was therefore in the order urea>formamide>acetamide>butyramide>propionamide>formate/acetate/ammonia. It was concluded that FmdD is a urea/formamide binding protein (U/F-BP).

6.6 Physiological regulation of U/F-BP determined by SDS-PAGE and Western blotting

Antiserum specific to U/F-BP were raised by J. A. Greenwood in a similar manner to that already described for acetamidase and formamidase (Chapter 3; Materials & Methods), and subsequently used by the author. The U/F-BP antiserum demonstrated cross-reactivity with purified U/F-BP but not with formamidase, acetamidase or other $M$. methylotrophus proteins. Washed cells of $M$. methylotrophus prepared from continuous cultures ($D=0.1$h$^{-1}$)
The binding of $[^{14}\text{C}]$urea to purified FmdD was measured by equilibrium dialysis as described in Materials & Methods. The concentration of FmdD was 0.83μM and the $[^{14}\text{C}]$urea concentration ranged from 13.3-261.7μM. The amount of urea bound (nmol [nmol protein]$^{-1}$) divided by the free urea concentration (μM) was plotted against the amount of urea bound (nmol nmol protein$^{-1}$).
Table 6.2 Specificity of purified FmdD for urea, amides, organic acids and ammonia

Competitive equilibrium dialysis was carried out with purified FmdD (0.5nmol) in the presence of $[^{14}\text{C}]$urea (0.8nmol; 1.3µM) and various unlabelled solutes (0.48nmol; 0.8µM or 20nmol; 32.5µM) as described in Materials & Methods. The amount of binding is expressed as a percentage of that in the absence of the unlabelled solute.

<table>
<thead>
<tr>
<th>Unlabelled solute</th>
<th>0.8µM Binding (%)</th>
<th>32.5µM Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>65±6 (5)</td>
<td>6±2 (5)</td>
</tr>
<tr>
<td>Formamide</td>
<td>71±5 (4)</td>
<td>6±3 (4)</td>
</tr>
<tr>
<td>Acetamide</td>
<td>77±8 (3)</td>
<td>29±18 (3)</td>
</tr>
<tr>
<td>Propionamide</td>
<td>108 (2)</td>
<td>91 (2)</td>
</tr>
<tr>
<td>Butyramide</td>
<td>97 (2)</td>
<td>85 (2)</td>
</tr>
<tr>
<td>Formate</td>
<td>94 (2)</td>
<td>104 (2)</td>
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<tr>
<td>Acetate</td>
<td>112 (2)</td>
<td>121 (2)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>83 (2)</td>
<td>100 (2)</td>
</tr>
</tbody>
</table>
grown under various nutrient limitations were subjected to SDS-PAGE, then either stained for protein or subjected to Western blotting using U/F-BP antiserum (Fig. 6.5 a, b). No U/F-BP was detected following growth with ammonia as the nitrogen source, and only very low concentrations following growth under methanol limitation with formamide, acetamide and urea as the nitrogen source (i.e. when ammonia was present in excess). A rather higher concentration of U/F-BP was seen following growth under acetamide limitation, an observation which contrasted with the results of N-terminal amino acid sequencing of the Mr 40,000 protein band from SDS-polyacrylamide gels of cells grown under acetamide limitation where only the sequences of the putative outer membrane porin FmdC (GATI-) and acetamidase (MIHG-) were detected (section 3.4). It would appear, therefore, that N-terminal amino acid sequencing was not sensitive enough to detect the low concentration of U/F-BP in the presence of higher concentrations of FmdC and acetamidase. This observation again highlights the high sensitivity of Western blotting relative to other procedures for the detection of proteins.

High concentrations of U/F-BP were detected following growth under formamide and urea limitation in agreement with the results of the earlier N-terminal amino acid sequencing of the Mr 40,000 protein band in these cells. It was concluded, therefore, that U/F-BP was strongly induced by formamide and urea, weakly induced by acetamide and repressed by ammonia.
Figure 6.5 SDS-PAGE and Western blotting of *M. methylotrophus* whole cells grown in continuous culture (D=0.1h⁻¹) under various nutrient limitations

*M. methylotrophus* was grown in continuous culture (D=0.1h⁻¹) under various nutrient limitations, washed cells prepared and subjected to SDS-PAGE and Western blotting as described in Materials & Methods. Tracks: 1, Mr standards; 2, ammonia limitation/methanol excess; 3, ammonia excess/methanol limitation; 4, acetamide limitation/methanol excess; 5, acetamide excess/methanol limitation; 6, formamide limitation/methanol excess; 7, formamide excess/methanol limitation; 8, urea limitation/methanol excess; 9, urea excess/methanol limitation; 10, Mr standards. (a), SDS-PAGE; (b), Western blot developed with U/F-BP antiserum.

(a) $10^{-3} \times Mr$

(b)

- Mdh-α
- Formamidase
- Acetamidase/
- U/F-BP/FmdC

- Mdh-β
6.7 Discussion

The urea/formamide-binding protein (U/F-BP; FmdD) from *M. methylotrophus* strain MM25 was purified and shown to be a monomer with an $M_r$ of approximately 41,000. U/F-BP bound urea with a $K_D$ of 7.15$\mu$M and with a stoichiometry of 0.75 mol urea mol protein$^{-1}$, indicating the presence of a single binding site. Binding was also observed with formamide and acetamide in the order urea/formamide > acetamide. U/F-BP exhibited only a low affinity for longer chain amides (propionamide and butyramide), and no significant affinity for organic acids (formate and acetate) and ammonia. U/F-BP was identified from its N-terminal amino acid sequence as the mature form of FmdD. It was therefore concluded that FmdD is a periplasmic urea/formamide-binding protein which probably comprises part of a binding protein-dependent uptake system for urea and formamide, a function which is fully commensurate with the observed high expression of FmdD during growth under formamide and urea limitation.

The binding constant of U/F-BP for urea of 7.15$\mu$M was quite high compared with that of other solute-binding proteins. In general, the affinity of sugar-binding proteins for their ligands is in the range 0.1-1$\mu$M, and around 0.1$\mu$M for amino acid-binding proteins (Ames *et al.*, 1990). Hence, U/F-BP binds urea considerably less tightly than sugar- and amino acid-binding proteins bind their respective ligands. However, the dicarboxylate-binding protein from *Rhodobacter capsulatus* has recently been reported to bind L-malate with a $K_D$ of 8.4$\mu$M, demonstrating the existence of at least one binding protein with a higher $K_D$ (Shaw *et al.*, 1991). Furthermore, AmiC from *Ps. aeruginosa* exhibits $K_D$ values for acetamide and propionamide of 3.7 and 3.1$\mu$M, respectively, with other amides binding much less tightly (Wilson *et al.*, 1993).

All periplasmic solute binding-proteins so far characterised are monomers with $M_r$ values in the range 25,000-56,000 (Ames, 1986; Ames *et al.*, 1990), and *M. methylotrophus* U/F-BP thus fits well within this generalisation. Furthermore, all of the proteins bind their ligands at a single binding site, thought to be a cleft between the two lobal domains (Ames, 1986; Ames *et al.*, 1990; Nikaido & Ames, 1992), and again the stoichiometry of 0.75 mol urea mol protein$^{-1}$ for U/F-BP with urea would suggest this protein behaves similarly. Observed stoichiometries of binding other than the expected value of 1.0
have previously been seen in the histidine- and leucine-binding proteins of *S. typhimurium* (0.7mol histidine mol protein$^{-1}$; Lever, 1972 and 0.45mol leucine mol protein$^{-1}$; Ohnishi & Kiritani, 1983), the galactose-binding protein from *E. coli* (2.0mol galactose mol protein$^{-1}$; Boos et al., 1972) and the arabinose- and histidine-binding proteins from *E. coli* (0.7-0.8mol arabinose mol protein$^{-1}$ and 0.4-0.6mol histidine mol protein$^{-1}$ respectively; Miller et al., 1983). The disparity between observed and expected stoichiometries of binding have been explained by inaccurate protein concentration determinations (Shaw et al., 1991), tightly-bound ligands which remain bound to the binding protein during purification (see Ames, 1986), and adsorption of binding protein to dialysis membranes or compartment walls of equilibrium dialysis apparatus (Miller et al., 1983).

SDS-PAGE and Western blotting of washed cells prepared from continuous cultures grown under various nutrient limitations demonstrated that U/F-BP was induced by acetamide, formamide and urea (urea/formamide>acetamide), and repressed by ammonia. This pattern was very similar to that of formamidase (Chapter 3) and was consistent with U/F-BP being involved principally in the uptake of urea and formamide.

It is possible at this stage, using these various data, to speculate about the transport of acetamide, formamide and urea into *M. methylotrophus*. The observation that this organism possesses a periplasmic urea/formamide-binding protein (presumably as part of a binding protein-dependent, ABC-type transport system) which is maximally induced during formamide- or urea-limited growth, and over-expressed as a result of prolonged growth under formamide limitation, is indicative that such systems are required for small, uncharged molecules during growth under substrate-limiting conditions. Many environments (such as the oceans, ground waters and microenvironments in sewage; from which *M. methylotrophus* was originally isolated) can be envisaged where urea would be present in low concentrations and where the possession of a high-affinity uptake system for urea, would put an organism such as *M. methylotrophus* at an evolutionary advantage. The same is true of the uptake of formamide, which is produced in nature from the biodegradation of cyanide (Knowles & Bunch, 1986; Fallon, 1992). Both the U/F-BP (FmdD) and the putative outer-membrane protein (FmdC) became over-expressed during prolonged growth under formamide limitation. Hence, with both a general amide/urea porin and a periplasmic binding protein-dependent uptake system the transport of
formamide and urea would presumably be an advantage during growth at any imposed growth rate \( \leq \mu_{\text{max}} \) under urea or formamide limitation. Unfortunately, due to the very high urease activities of formamide-limited washed cells, it was not possible to measure \(^{14}\text{C}\)urea uptake rates by conventional filtration methods (for example see Cornish et al., 1988). This could perhaps be carried out if urease-negative mutants of \textit{M. methylotrophus} could be obtained, or by cloning and expressing the \textit{M. methylotrophus} uptake system in a urease-negative strain of \textit{E. coli} (unfortunately there is some evidence that pSW1 does not encode the whole of the uptake system; S. G. Williams & C. W. Jones, unpublished).

Only low concentrations of U/F-BP were induced during growth under acetamide limitation in comparison to those seen under formamide or urea limitation. This, together with the relatively-low affinity of U/F-BP for acetamide and the failure to detect active transport of acetamide into \textit{M. methylotrophus} seen previously (section 3.7), suggested that U/F-BP was not involved in acetamide uptake.

Another protein with an \( M_r \) of approximately 32,000 (section 5.2) also became overexpressed following directed evolution under formamide limitation, in addition to U/F-BP (FmdD) and FmdC. It is unlikely, however, that this membrane-bound protein was a component of the urea/formamide-binding protein transport system, since the inner-membrane components of such systems are found at very low concentrations (approximately one-thirtieth of the concentration of the binding protein; Ames, 1986) and would therefore not be visible upon SDS-PAGE. Also FmdE (a putative inner membrane protein encoded by the \textit{fmdE} gene immediately downstream of \textit{fmdD}; see section 5.7) has a predicted \( M_r \) of 11,377 which is considerably smaller than the \( M_r \) of the unknown protein. The identity and function of this protein therefore remains obscure.
CHAPTER 7

IMMUNOLOGICAL COMPARISON OF BACTERIAL AMIDASES
CHAPTER 7

IMMUNOLOGICAL COMPARISON OF BACTERIAL AMIDASES

7.1 Introduction

7.2 SDS-PAGE and Western blot analysis of bacterial amidases

7.3 Determination of *My. smegmatis* "acetamidase" substrate profile

7.4 Discussion
IMMUNOLOGICAL COMPARISON OF BACTERIAL AMIDASES

7.1 Introduction

Microorganisms possessing specific amidases have been found in numerous genera; both Gram-positive and Gram-negative bacteria as well as fungi (see Chapter 1; Introduction, Chapter 3). With the advent of molecular biology techniques more information is becoming available, from gene sequencing, and hence from derived amino acid sequences, about the interrelatedness of amidases from these various organisms. The enantiomer-selective amidase from the Gram-positive bacterium *Brevibacterium* sp. R312 has been purified, the structural gene cloned and the primary sequence determined (Mayaux et al., 1990). Upon comparison of the derived amino acid sequence of this amidase with other protein sequences present in the data bases, highly-significant homologies were seen with the sequences of other amidases such as the indolacetamide hydrolase from *Ps. syringae* and *A. tumefaciens* (both Gram-negative bacteria) and the acetamidase from *A. nidulans* (a fungus). The highest degree of homology between the different amidases, which was conserved from species to species, was observed in the N-terminal half of the protein. This strongly suggested that this region of the protein was involved in forming the amidase active site (Mayaux et al., 1990). A newly isolated strain of *Rhodococcus* possessing an enantiomer-selective amidase was also shown by gene sequencing and comparison of the derived amino acid sequence, to demonstrate significant homology to the corresponding enzymes from *Brevibacterium, Ps. syringae, A. tumefaciens* and *A. nidulans* (Mayaux et al., 1991). The observation that these amidases showed no significant homology to the acetamidase of *Ps. aeruginosa* indicated that at least two different classes of amidase exist in microorganisms.

*Brevibacterium* sp. R312, like some other microorganisms, has been shown to contain more than one amidase. In addition to the enantiomer-selective amidase, this organism contains specific enzymes for the hydrolysis of a wide number of aliphatic amides (wide-spectrum amidase), formamide and α-amino amides (Maestracci et al., 1984; 1988; Mayaux et al., 1990). The structural gene encoding the wide-spectrum amidase has been cloned and sequenced (Soubrier et al., 1992); the derived amino acid sequence was more
than 80% identical with the acetamidase of *Ps. aeruginosa*, but no significant homology to the enantiomer-selective amidase or related enzymes. The N-terminal amino acid sequences of the enzymes from *Ps. aeruginosa* and *Brevibacterium* sp. R312 are identical over the first 19 amino acids and demonstrate only two substitutions in this region when compared to the sequence of *M. methylotrophus* acetamidase (Silman *et al.*, 1991; see section 4.1). It was suggested, upon the similarity between *Ps. aeruginosa* and *Brevibacterium* sp. R312, that the aliphatic amidases may indeed represent a separate and distinct “family” from the enantiomer-selective amidases. Moreover, the high level of homology between the *Brevibacterium* and *Ps. aeruginosa* enzymes was suggested to be indicative of horizontal gene transfer between Gram-positive and Gram-negative species during evolution (Soubrier *et al.*, 1992).

The gene encoding the formamidase of *M. methylotrophus* has been cloned, sequenced and shown to be highly homologous (57.3% strict identity) to the “acetamidase” of *My. smegmatis* (Wyborn *et al.*, 1994; Wyborn, 1994). These two enzymes also showed no significant homology to the acetamidase of *Ps. aeruginosa* or the enantiomer-selective amidase of *Brevibacterium* sp. R312 (Mahenthiralingam *et al.*, 1993; Wyborn, 1994). These observations suggested that these enzymes may represent a third distinct group of amidases.

This chapter describes the immunological comparison of amidases from various genera of bacteria. The results are discussed in relation to the classification of amidases into three distinct groups based upon biochemical, molecular and immunological data.
7.2 SDS-PAGE and Western blot analysis of bacterial amidases

Samples of pure or partially-pure acetamidase and formamidase from *M. methylotrophus*, acetamidase from *Ps. aeruginosa* acetamidase (donated by Dr P. Brown, Kings College, London), "acetamidase" from *My. smegmatis* (donated by Dr P. Draper, National Institute for Medical Research, London) and enantiomer-selective amidase from *Rh. rhodochrous* I1 (donated by Dr M. Kobayashi, Kyoto University, Japan) were subjected by SDS-PAGE and Western blotting (Materials & Methods). Blots were developed using antisera raised against *M. methylotrophus* acetamidase and formamidase (see Chapter 3), and *My. smegmatis* "acetamidase" (donated by Dr P. Draper). Antiserum to *M. methylotrophus* acetamidase strongly cross-reacted with the acetamidases from *M. methylotrophus* and *Ps. aeruginosa*, but not with any of the other amidases (Fig. 7.1). Antiserum to *M. methylotrophus* formamidase strongly cross-reacted with the formamidase from *M. methylotrophus* and the "acetamidase" from *My. smegmatis*, but not with any of the other amidases. Similar results were also obtained using antiserum raised against the "acetamidase" from *My. smegmatis*. No cross-reactivity was seen between any of the antisera and the enantiomer-selective amidase from *Rh. rhodochrous*. These results indicated that the acetamidases from *M. methylotrophus* and *Ps. aeruginosa* are immunologically similar, as are the formamidase from *M. methylotrophus* and the "acetamidase" from *My. smegmatis*, whereas the enantiomer-selective amidase from *Rh. rhodochrous* is immunologically different from all the other enzymes.

7.3 Determination of *My. smegmatis* "acetamidase" substrate profile

As the *My. smegmatis* "acetamidase" demonstrated cross-reactivity with antiserum raised against the formamidase from *M. methylotrophus*, and the *M. methylotrophus* formamidase cross-reacted with antiserum raised against the "acetamidase" from *My. smegmatis*, the substrate profile of the *My. smegmatis* enzyme was further investigated using partially pure (ammonium sulphate precipitated) protein. The enzyme exhibited high activity with formamide as substrate, but only marginal activity with longer-chain amides (acetamide, acrylamide, propionamide and butyramide), and no activity with urea (Table 7.1). These
Figure 7.1 SDS-PAGE and Western blotting of various bacterial amidases with antiserum raised against acetamidase and formamidase from M. methylotrophus and "acetamidase" from My. smegmatis.

Samples of various bacterial amidases were subjected to SDS-PAGE and Western blotting as described in Materials & Methods. Tracks: 1, Mr standards; 2, M. methylotrophus acetamidase; 3, M. methylotrophus formamidase; 4, Ps. aeruginosa acetamidase; 5, Rh. rhodochrous broad spectrum amidase; 6, My. smegmatis "acetamidase"; 7, Mr standards. (a), SDS-PAGE; (b), Western blot developed with acetamidase antiserum (M. methylotrophus); (c), Western blot developed with formamidase antiserum (M. methylotrophus); (d), Western blot developed with "acetamidase" antiserum (My. smegmatis).
results were similar to those seen previously with purified formamidase from *M. methylotrophus* (Wyborn, 1994).
Table 7.1 Comparison of the substrate profile of *M. smegmatis* "acetamidase" and *M. methylotrophus* formamidase

The substrate profile of *M. smegmatis* "acetamidase" (donated by Dr P. Draper) was determined at 37°C with a range of amides and urea all at final concentrations of 50mM (Materials & Methods). Figures in brackets for *M. smegmatis* "acetamidase" are taken from Draper (1967). The data for *M. methylotrophus* formamidase are taken from Wyborn (1994). The activity with each substrate is expressed as a percentage of that with formamide.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amidohydrolase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>My. smegmatis</em> &quot;acetamidase&quot;</td>
</tr>
<tr>
<td>Urea</td>
<td>0</td>
</tr>
<tr>
<td>Formamide</td>
<td>100 (100)</td>
</tr>
<tr>
<td>Acetamide</td>
<td>0.41 (1.0)</td>
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<tr>
<td>Acrylamide</td>
<td>0.75 (1.6)</td>
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<tr>
<td>Propionamide</td>
<td>0.49 (1.3)</td>
</tr>
<tr>
<td>Butyramide</td>
<td>1.29 (2.6)</td>
</tr>
</tbody>
</table>
7.4 Discussion

Antiserum raised against *M. methylotrophus* formamidase demonstrated good cross-reactivity with “acetamidase” from *M. smegmatis*, and antisera raised against the *M. smegmatis* “acetamidase” demonstrated good cross-reactivity with formamidase from *M. methylotrophus*, suggesting that these two enzymes are highly similar. Furthermore, the derived amino acid sequence of the *M. smegmatis* “acetamidase” is 57.3% identical to that of the *M. methylotrophus* formamidase (Wyborn, 1994), but exhibits no notable homology to any other published amidase sequences (Mahenthiralingam et al., 1993). This striking sequence homology together with the immunological similarity to the formamidase from *M. methylotrophus*, and the substrate profile determined during this study and previously (Daraper, 1967), suggests that *M. smegmatis* “acetamidase” is in fact a formamidase. The occurrence of enzymes which are specific for formamide hydrolysis are rare in the literature (see Chapter 1) compared with other amidases, which may help explain the incorrect classification of the *M. smegmatis* enzyme as an acetamidase rather than a formamidase.

The *M. methylotrophus* acetamidase has previously been shown to be highly similar to that from *Ps. aeruginosa* (Silman, 1990; Silman et al., 1991), in respect to subunit Mr and N-terminal amino acid sequence (only two residues within the first 19 are different). Antiserum raised against the acetamidase of *M. methylotrophus* cross-reacted with the *Ps. aeruginosa* amidase, demonstrating that these two enzymes are also immunologically similar. However, the intensity of the band seen upon Western blotting of the *Ps. aeruginosa* enzyme with antisera to the *M. methylotrophus* acetamidase was not as strong as that against the enzyme from *M. methylotrophus* (Fig. 7.1), suggesting that the enzymes contained a number of non-identical epitopes. Although the acetamidase gene from *M. methylotrophus* has not been cloned, sequenced and the amino acid sequence predicted, total amino acid content has previously been determined (Silman et al., 1991). The *M. methylotrophus* acetamidase was found to contain substantially more glycine, serine and histidine, but less methionine and arginine, than the *Ps. aeruginosa* amidase. Hence, there appear to be significant differences between these two enzymes which could explain the
slightly lower avidity of the antiserum raised against the *M. methylotrophus* enzyme for the amidase from *Ps. aeruginosa*.

Immunological comparison of amidases from *Pseudomonas* species has previously shown that significant differences can exist within this single genus (Clarke, 1972). Antiserum raised against *Ps. aeruginosa* acetamidase cross-reacted strongly in immunodiffusion experiments with cell extracts of *Ps. putida* and *Ps. acidovorans*, but only a weak reaction was seen with cell extracts of *Ps. cepacia*. The weak reaction between *Ps. aeruginosa* amidase antiserum and cell extracts of *Ps. cepacia* suggested that there were significant antigenic differences between the amidase from this species and the other three. The observation that such immunological differences can exist between amidases from the same genus, while the acetamidase from *M. methylotrophus* shows fairly good immunological similarity to the *Ps. aeruginosa* enzyme, suggests either that all of the acetamidases of the type found in *M. methylotrophus* and *Ps. aeruginosa* had a common ancestor which has changed to varying extents from species to species during evolution, or that horizontal gene transfer may have occurred between *Ps. aeruginosa* and *M. methylotrophus*.

The enantiomer-selective amidase from *Rh. rhodochrous J1* exhibited no cross-reactivity to antisera raised against the formamidases of *M. methylotrophus* or *My. smegmatis*, or to antiserum raised against the acetamidase of *M. methylotrophus*, suggesting that this amidase was very different to all of the others.

Using a combination of immunological, biochemical and molecular characteristics it is possible to classify microbial amidases into three groups (Table 7.2). Group I contains the formamidases from *M. methylotrophus* and *My. smegmatis*. No other formamidases have been characterised, although it has been suggested from physiological studies that formamidases may be present in *A. eutrophus* (Friedrich & Mitrenga, 1981), *A. nidulans* (Hynes, 1970) and *Brevibacterium* (Maestracci et al., 1984; 1988).

Group II contains the acetamidases similar to that from *Ps. aeruginosa*. Substrate profile, Western blotting, N-terminal amino acid sequence and amino acid composition data suggest that the acetamidase from *M. methylotrophus* is similar to that from *Ps. aeruginosa*. In addition, the derived amino acid sequence of the wide-spectrum amidase from *Brevibacterium* sp. R312 is ≥81% identical to that of the acetamidase of *Ps.*
<table>
<thead>
<tr>
<th>Group</th>
<th>Organism</th>
<th>Enzyme N°</th>
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</table>

Table 7.2: Classification of microbial amides
| 19 | 60.160 | *Aquimarina* sp. N-7.74 | N.74 | Acetaminidase | 54.671 |
| 18 | 48.315 | *Aquimarina* | N.315 | Indoleacetamidase | 64.153 |
| 17 | 49.788 | *Aquimarina* | N.788 | Indoleacetamidase | 61.176 |
| 16 | 30.266 | *Burkholderia* | N.266 | Indoleacetamidase | 98.069 |
| 15 | 49.788 | *P. aviseptatum* | N.788 | Indoleacetamidase | 100.82 |
| 14 | 54.178 | *P. chrysogenum* | B23 | Enzyme-Selective: G2 | 89.012 |

<table>
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<tr>
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</tr>
<tr>
<td><em>Aquimarina</em></td>
<td>N.788</td>
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<td><em>Burkholderia</em></td>
<td>N.266</td>
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<tr>
<td><em>P. aviseptatum</em></td>
<td>N.788</td>
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<tr>
<td><em>P. chrysogenum</em></td>
<td>B23</td>
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Table 7.2 References

1, Wyborn (1994); 2, Mahenthiralingam et al. (1993); 3, Silman (1990); 4, Silman et al. (1991); 5, Clarke & Drew (1988); 6, Ambler et al. (1987); 7, Thiery et al. (1986a); 8, Maestracci et al. (1984); 9, Soubrier et al. (1992); 10, Kobayashi et al. (1993); 11, Mayaux et al. (1990); 12, Mayaux et al. (1991); 13, Nishiyama et al. (1991); 14, Ciskanik et al. (1995); 15, Keel et al. (1984); 16, Yamada et al. (1985); 17, Sekine et al. (1989); 18, Corrick et al. (1987); 19, Hashimoto et al. (1991).
aeruginosa (Soubrier et al., 1992), and the N-terminal amino acid sequence over the first 19 residues is completely identical (and therefore is only slightly different from that of the M. methylotrophus acetamidase). Hence, these enzymes are all structurally very similar, although the oligomeric structure of the Ps. aeruginosa amidase is $\alpha_6$ cf. $\alpha_4$ for the other two enzymes. Other members of this group of amidases are probably wide-spread within microorganisms, but to date little amino acid sequence homology data is available. However, the Arthrobacter sp. J1 amidase (Asano et al., 1982) is probably a member of this group since the enzyme is specific to acetamide, acrylamide and propionamide, has a subunit $M_r$ of 39,000 and an oligomeric structure of $\alpha_8$ (no amino acid sequence data is currently available).

Group III contains a growing number of enzymes which are similar to the enantiomer-selective amidase from Rh. rhodochrous J1. This enzyme exhibited no cross-reactivity to acetamidase or formamidase antiserum, demonstrating its immunological disparity from Group I and Group II amidases. The amino acid sequences of Group III amidases show no homology to amidases from either Group I or Group II, but show significant sequence homologies to each other in spite of the diversity of their substrate profiles. The enzymes from Rh. rhodochrous J1, Brevibacterium sp. R312, Rhodococcus strain and Ps. chlororaphis B23 all show enantiomer-specificity to aromatic amides, whereas the enzymes from A. tumefaciens, Ps. savastanoi and Bradyrhizobium japonicum are all specific for the hydrolysis of indolacetamide, and the enzymes from A. nidulans and Rhodococcus sp. N-774 are short-chain aliphatic amidases. This biochemical sub-division of structurally-related enzymes may be due to the enzymes all having a common progenitor which has become specific to different amide substrates during evolution. A putative amidase gene exhibiting significant similarity to the enzymes from A. nidulans and Ps. savastanoi has also recently been identified in Saccharomyces cerevisiae (Chang & Abelson, 1990). It is interesting to note that of the two eukaryote amidases so far reported both belong to Group III, even though the A. nidulans enzyme has a similar substrate profile to that of the Group II amidases. There have been many other reports of amidases (beyond the scope of this thesis), mostly those specific to aryl-amides, which may eventually upon cloning, sequencing and amino acid sequence derivation be shown to belong to Group III or to novel groups.

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The classification of amidases based upon amino acid sequence similarities has previously been suggested by Hashimoto et al. (1991), Mayaux et al. (1991) and Soubrier et al. (1992). The former two authors noted that the amidases from \textit{Rhodococcus} sp. N-774 and \textit{Rhodococcus} strain showed no significant homology to the acetamidase of \textit{Ps. aeruginosa}, whilst showing significant homology to other amidases in Group III. Soubrier et al. (1992) noted the high level of homology between the wide-spectrum amidase of \textit{Brevibacterium} sp. R312 and the acetamidase of \textit{Ps. aeruginosa}, which did not exhibit any significant homology to the enantiomer-selective amidases of Group III. All of the above authors therefore concluded that microbial amidases belonged to at least two groups. The Group I amidases were not identified until now due to the lack of published data on formamidases and the misnaming of the "acetamidase" from \textit{My. smegmatis}. Mayaux et al. (1991) pondered as to whether different amidases expressed by the same microorganism can be of the same type or belong to different groups. The data presented here clearly show that amidases from different groups can be expressed in the same organism e.g. \textit{M. methylotrophus} and \textit{Brevibacterium} sp. R312.

Each group contains both Gram-positive and Gram-negative organisms, e.g. \textit{M. methylotrophus} and \textit{My. smegmatis} in Group I, \textit{Brevibacterium} sp. R312 and \textit{Ps. aeruginosa} in Group II and \textit{Rh. rhodochorous} J1 and \textit{Ps. savastanoi} in Group III. This has previously been suggested to be due to horizontal gene transfer during evolution between Gram-positive and Gram-negative bacteria, as opposed to the very slow change in sequence of a progenitor enzyme evolved before the divergence of Gram-positive and purple bacteria (Soubrier et al., 1992). This suggestion remains possible but seems unlikely when considering the biochemical diversity between structurally similar amidases in Group III. For this hypothesis to be true several horizontal gene transfer events would have had to occur followed by sufficient time for the evolution of enzymes specific to diverse amides. Also, eukaryote amidases show homology to the Group III amidases (but not to the other two groups), suggesting that the Group III amidases may well represent descendants of an ancient enzyme present before the divergence of prokaryotes and eukaryotes, with Group I and Group II amidases arising after divergence.
CHAPTER 8

SUMMARY AND DISCUSSION
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8.1 Physiological regulation of acetamidase, formamidase and urease

8.2 Switch-off of acetamidase activity

8.3 Directed evolution of *M. methylophilus* under formamide limitation

8.4 Immunological comparison of bacterial amidases

8.5 Closing remarks
SUMMARY AND DISCUSSION

8.1 Physiological regulation of acetamidase, formamidase and urease

Extensive investigation of the physiological regulation of acetamidase, formamidase and urease in continuous culture confirmed that acetamidase is maximally induced during growth under acetamide limitation. However, it is not induced by formamide or urea. Formamidase is also induced during growth under acetamide limitation, but demonstrated much higher activities following growth under formamide and urea limitation (the highest activity being seen when urea was the nitrogen source). Urease activity is not significantly induced by growth under acetamide limitation, but high activities were seen during growth under formamide- and urea-limited conditions. All three enzymes are repressed by ammonia. This repression by ammonia is a reflection of the role of these enzymes in M. methyloptrophus, which is to supply nitrogen (not carbon) for growth. Repression is therefore seen when ammonia, the most readily utilised nitrogen source, becomes available and prevents the further production of the above enzymes. This pattern of regulation is different to that observed in other species of amidase-possessing microorganisms which utilise amides as a source of both carbon and nitrogen for growth (see Chapter 3).

It is interesting to note the apparent physiological disparity of regulation between the formamidase of M. methyloptrophus (induced by amides and urea, and repressed by ammonia) and the “acetamidase” of My. smegmatis (induced by acetamide and acetate). However, it must be stated that the latter enzyme has not been physiologically investigated as thoroughly as the former. A large quantity of DNA both upstream and downstream of the M. methyloptrophus formamidase structural gene (fmdA) has now been cloned and sequenced. It would be interesting to compare the corresponding regions of the My. smegmatis genome to investigate differences/similarities which may explain the apparently different patterns of expression. The induction of M. methyloptrophus formamidase by urea appears to be the first such report of gratuitous induction of formamidase. It may also be interesting to investigate further the physiological/ecological function of formamidase with respect to the ecological niche from which M. methyloptrophus was isolated (viz. activated sludge from a Teesside sewage treatment plant). Cyanide can be biodegraded by way of
hydration to formamide, by the enzyme cyanide hydratase, and then the action of a formamidase and formate dehydrogenase (see Knowles & Bunch, 1986). As *M. methylotrophus* possesses both of the latter enzymes it would be interesting to test for the presence of the former and therefore an operational pathway for the degradation of this compound. In fact it can be hypothesised that cyanide (both man-made and biologically-formed) may have been a common compound in the environment from which *M. methylotrophus* was isolated.

Little is currently known about the urease of *M. methylotrophus* besides the physiological regulation and brief biochemical properties reported here. No other urease has been reported in the literature to be induced by formamide. Hence this enzyme represents an interesting possibility for the further study of nitrogen assimilating enzymes in *M. methylotrophus*.

### 8.2 Switch-off of acetamidase activity

Switch-off of acetamidase activity was not seen during any steady-state growth regime including dual acetamide/methanol limitation and under more closely-controlled conditions at different C:N ratios (Chapter 4). However, switch-off can be brought about in dual-limited cultures either by introducing excess ammonia (by way of an ammonia or acetamide pulse) or by changing the nitrogen source from acetamide to ammonia. It is this latter observation which is most pertinent to the original growth regime which was found to potentiate switch-off by ZENECA Bio Products (*i.e.* high cell density culture with an alternating nitrogen source; See Chapter 1; Carver & Jones, 1993). Previous, extensive physico-chemical studies have been unable to identify any form of common post-translational modification to the switched-on or switched-off forms of acetamidase (Silman, 1990; Wyborn, 1994). The latter study concluded that switched-off acetamidase had probably undergone a conformational change which may have been related to the dissociation of an allosteric effector. It can be concluded from the physiological studies that switch-off is produced, by an as yet unknown mechanism, when an alternative nitrogen source (ammonia) becomes available to an *M. methylotrophus* culture growing under dual methanol/acetamide limitation. However, the actual signal which potentiates switch-off is
unlikely to be excess ammonia, as no ammonia was detected in culture supernatant samples during exchange experiments in which the nitrogen source was changed from limiting acetamide to limiting ammonia. It is difficult to envisage a system which allows the cell to detect the removal of limiting acetamide from the medium and its replacement by limiting ammonia, when it is considered that acetate was found to have no effect upon switch-off. Unfortunately, further elucidation of the biochemistry of this event was hampered by the failure to obtain consistent results with non-growing cultures. These attempts were further frustrated by the observations that switch-off could be caused in such samples simply by increasing the pH or by storing cell extracts at -20°C. These obviously non-physiological phenomena made any further attempts to unravel the mechanism of acetamidase switch-off, beyond the whole-cell level, both difficult and unattractive. Hence, the biochemical mechanism of acetamidase switch-off remains obscure. As several approaches on both the physiological (reported here), molecular and physico-chemical levels (see Wyborn, 1994) have been unfruitful, it is difficult to recommend further possible routes to the elucidation of the mechanism of switch-off. Any further efforts would have to be conducted on many different multi-disciplinary levels, probably involving those mentioned above together with kinetic and structural studies.

Formamidase activity also appeared to undergo switch-off in response to similar physiological signals as acetamidase. However, heat-reactivation of formamidase activity was never observed and activity was regained following purification of formamidase from ammonia pulsed culture. This suggested that the putative modification to formamidase was different to that of acetamidase. Due to the problems encountered in the attempted elucidation of acetamidase switch-off, formamidase switch-off was not further investigated. Switch-off of urease activity was not seen.

8.3 Directed evolution of M. methylotrophus under formamide limitation

A mutant M. methylotrophus strain (MM25) was isolated following prolonged growth (approximately 350 generations) under formamide limitation. This mutant exhibited approximately twice the formamidase activity of the wild-type, together with a higher formate oxidation rate and a striking increase in the concentrations of U/F-BP (FmdD) and
a membrane protein (FmdC) which may be an amide/urea outer membrane porin. The rate at which the formamidase activity increased during selection was substantially slower than that which was observed when acetamidase was evolved under acetamide limitation (Silman et al., 1989; 1991; Silman, 1990). This latter selection was characterised by a five-fold increase in acetamidase activity within approximately 10 generations in acetamide-limited continuous culture, which is in stark contrast to the approximate two-fold increase seen in formamidase activity following 350 generations under formamide-limited growth. It should be noted, however, that the acetamidase mutants were selected at a much lower dilution rate than the formamidase mutant (0.025h⁻¹ cf. 0.1h⁻¹), which may explain the more rapid selection of the former due to stronger selection pressure at low dilution rate. Another factor which may have influenced these selection rates is that during growth under formamide limitation a binding protein-dependent uptake system is used for the uptake of the limiting nutrient, whereas there is no evidence for an analogous system under acetamide limitation. Hence, under formamide limitation the selection pressure was strongest upon the uptake system which thus resulted in the selection of a mutant strain which over-expressed that system rather than formamidase.

The formamidase from the mutant was purified and partially characterised. The increase in formamidase activity seen in MM25 was found to be due to the production, at wild-type concentrations, of an enzyme with an increased $k_{cat}$. However, the mutant formamidase was not fully characterised and it would be of value to investigate this enzyme further. The $fmdA$ gene from MM25 has recently been cloned and sequenced (J. A. Greenwood, S. G. Williams & C. W. Jones, unpublished), and found to contain five base changes which produce four amino acid substitutions in the formamidase protein (two aspartate residues to glutamates and two valine residues to leucines). The wild-type formamidase has been crystallised and preliminary x-ray analysis carried out (O'Hara et al., 1994). It would therefore be interesting to crystallise the formamidase from MM25 and to look for possible changes in the three dimensional structure of this enzyme which confer the increased $k_{cat}$. This structure/function approach could be extended by the isolation of further mutants with altered formamidases by further rounds of selection in continuous culture or by site-directed mutagenesis of $fmdA$.  
The physiological regulation of FmdC and FmdD was investigated by protein sequencing of the M, 40,000 proteins from cells grown under various nutrient limitations (see Chapter 3), and by Western blotting with FmdD (U/F-BP) specific antiserum (see Chapter 6). FmdC was induced by acetamide, formamide and urea, and repressed by ammonia. FmdD was only weakly induced by acetamide, but induced to higher concentrations by formamide and urea, and repressed by ammonia. Hence, FmdC and FmdD were regulated concurrently with their functions as an amide outer membrane porin and a urea/formamide-binding protein respectively.

The U/F-BP was purified from MM25 and characterised (see Chapter 6). The observed $K_d$ of the U/F-BP from *M. methylotrophus* strain MM25 for its substrate was however high in comparison with various amino acid- or sugar-binding proteins (7.15µM cf. 0.1-1µM). This value could possibly be re-determined following denaturation and renaturation to remove any ligand which may remain tightly bound during purification. The observed binding stoichiometry could also be re-determined with this renatured protein to investigate whether the value of <1 was due to the presence of bound ligand. The U/F-BP could be further investigated, structurally, by carrying out crystallisation trials and if attempts were successful by the solving of its three dimensional structure. This could be carried out both in the presence and absence of substrate to allow the elucidation of any conformational changes which occur upon liganding. These structural studies could then be used as a basis for further investigations into the structure/function relationship of this binding protein. The U/F-BP has also been shown to be a member of a family of structurally related binding proteins (including amino acid-binding proteins from various bacteria and AmiC from *Ps. aeruginosa*; see also Tam & Saier, 1993). It would be interesting to investigate further the interrelatedness of these proteins by an immunological comparison using the antiserum raised against the U/F-BP from *M. methylotrophus*.

The putative amide/urea outer membrane porin (FmdC) was not conclusively located to the outer membrane or further characterised. Hence, this component which also appears to be associated with acetamide passage into the cell, requires further investigation. The reason that a binding protein-dependent uptake system is used by *M. methylotrophus* for the uptake of urea and formamide but not acetamide (a physically similar molecule) remains obscure.
The increase in formate oxidation rate in MM25 was not investigated further and its characterisation could constitute an interesting exploration of this aspect of *M. methylotrophus* carbon metabolism. This study could be carrying out by parallel purification and characterisation of the formate dehydrogenases from wild-type and MM25. Another characteristic of MM25 which warrants further investigation is the identification and functional analysis of the Mr 32,000 protein which was present in an undetectably low concentration in wild-type but became rapidly overexpressed during selection under formamide limitation. Hence, much work remains to be carried out in order to elucidate all of the major changes which occurred in *M. methylotrophus* strain MM25 during directed evolution under formamide limitation.

8.4 Immunological comparison of bacterial amidases

It has been shown by using antiserum raised against the acetamidase and formamidase of *M. methylotrophus*, and the “acetamidase” from *My. smegmatis*, that the acetamidases from *M. methylotrophus* and *Ps. aeruginosa* are similar as are the formamidase from *M. methylotrophus* and the “acetamidase” from *My. smegmatis*. The amidase from *Rh. rhodochrous J1* was different from all the other amidases tested. Based on this, and molecular biological evidence, microbial amidases have been classified into three groups (see Chapter 7). Unfortunately only one representative of the group III amidases was obtained (that from *Rh. rhodochrous J1*). However, the enzymes which have been allocated to this group, mainly due to molecular evidence, are numerous and diverse. It is therefore possible that this group is heterogeneous and only by the further immunological analysis of group III amidases from various sources would it be possible to discern further possible subgroupings.

The immunological evidence together with the substrate profile and comparative amino acid sequence data (see Wyborn, 1994) has conclusively shown that the “acetamidase” of *My. smegmatis* is in fact a mis-named formamidase. Little data has been reported in the literature concerning specific formamidases, however, physiological data has suggested their possible presence in *A. eutrophus*, *A. nidulans* and *Brevibacterium sp. R312*. Hence, further work with these organisms could help fully characterise the similarities or
differences between these enzymes, as well as pointing to possible evolutionary relationships.

The wide-spread presence of amidases in diverse organisms from both prokaryotes and eukaryotes, suggest that they are highly significant enzymes, which to date have largely been ignored in a comparative sense. Therefore a large amount of additional data remains to be gathered about these enzymes, which may lead to a better understanding of the evolutionary relationships of a number of such enzymes and the organisms which possess them.

8.5 Closing remarks

When this study was started some three and a half years ago the physiological regulation of acetamidase activity in *M. methylotrophus* had been investigated in acetamide-limited continuous culture, the acetamidase mutant enzymes purified and characterised, and a preliminary investigation of acetamidase switch-off carried out. Since this time a much more-detailed characterisation of the different forms of acetamidase has been carried out in addition to the more through investigation of the physiological regulation of acetamidase, formamidase and urease activity in *M. methylotrophus*. With respect to acetamidase switch-off the determination of the physiological regimes which potentiate it were of major significance. Although these physiological and biochemical studies have added to the breadth and depth of knowledge about acetamidase switch-off, the underlying mechanism involved is still obscure, even following several man-years of investigation. At many points during this time the problems involved in working with this system have been highly frustrating and at times it appeared to represent an unsolvable riddle, often seeming more akin to "bioalchemy" than biochemistry. However, for these reasons alone the conundrum of acetamidase switch-off remains an academically (if no longer an industrially) stimulating problem. It remains to be seen as to whether this problem is further investigated and finally solved.

Recent emphasis in amidase research in *M. methylotrophus* has been on formamidase. This area of research has led to many new paths of investigation including the crystallisation of the enzyme for ongoing structural analysis, the cloning of the structural
gene and associated regions of DNA (foundA BC D) and the isolation of a mutant formamidase. The most interesting aspects of the latter part of the work described here was the discovery that two of the over-expressed proteins in MM25 were the products of the foundD and foundC genes located upstream and downstream respectively, of the formamidase structural gene and encoding a porin and a binding protein associated with amide/urea uptake. This allowed the very rapid integration and correlation of physiological and molecular data. With further characterisation of formamidase expression at the molecular level this enzyme system could well become the most extensively understood amidase, as attempts to crystallise the acetamidase from Ps. aeruginosa have so far failed.
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