KINETIC AND MECHANISTIC STUDIES
OF HETEROTETRAMERIC
SARCOSINE OXIDASE
FROM ARTHROBACTER SP. 1-IN

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

Richard Harris BSc (Hons, Bristol)
Department of Biochemistry
University of Leicester

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PREFACE

The research described in this thesis was carried out between October 1997 and September 2000. The work reported was conducted by myself, unless stated otherwise in the text. No part of this thesis has been submitted for a degree or diploma or other qualification at any other university.

January, 2001
Leicester University
Leicester

Richard Harris
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Also I would like to thank Professor Steve Chapman for allowing me access to his lab to conduct the redox potentiometry experiments. Thanks as well to The Edinburgh group for being so welcoming and accommodating, particular thanks must go to Dr Andrew Munro and Dr. Mike Noble for their assistance in data collection and later in the analysis of the results.

Finally, the most thanks go to Jenni for supporting me during my studies and write-up and for her constant love and encouragement.
ABSTRACT

Heterotetrameric sarcosine oxidase (TSOX; EC. 1.5.3.1) from Arthrobacter sp. 1-IN is a complex flavoprotein that catalyses the oxidative demethylation of sarcosine to produce glycine, formaldehyde and hydrogen peroxide. TSOX is composed of four subunits with approximate molecular weights of 106, 43, 24 and 15 kDa the genes for which have been cloned and the recombinant protein expressed at low levels. (Meškys et al., 1996)

The Arthrobacter sp. 1-IN genomic DNA containing the sox operon was sequenced and, as well as the four TSOX genes, contains genes for a serine hydroxymethyltransferase and a formyl-tetrahydrofolate deformylase. Native and recombinant TSOX were expressed, purified and characterised. Cofactor analysis showed that TSOX contains 1 mol each of non-covalently bound FAD and NAD⁺ and also 1 mol of covalent flavin.

The reductive half-reaction of TSOX was studied using stopped-flow spectroscopy. pH dependence of flavin reduction by sarcosine indicated no kinetically influential ionisations in the enzyme-substrate complex. Two ionisations with pKa values of 7.4± 0.1 and 10.4 ± 0.2 were identified from a kₚ/kᵦ versus pH plot. Kinetic isotope effect studies of the rate of C-H bond breakage in sarcosine indicated a ground-state quantum tunnelling mechanism for H-transfer assisted by the thermal fluctuations of the protein molecule.

Anaerobic reduction experiments indicated that the two flavins of TSOX were reducible and that two electrons were required per flavin for complete reduction. The redox potentials for the two flavins were determined and indicated that one flavin had an unusually high redox potential. The inter-flavin electron transfer rate was measured using a pH jump technique between pH 7 and 9. The pH jump experiments and redox potential measurements indicated that the intramolecular electron transfer in TSOX was an endergonic process.
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<td>A&lt;sub&gt;450&lt;/sub&gt;</td>
<td>Absorbance at 450 nm</td>
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<tr>
<td>ABM</td>
<td>ADP binding Motif</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumen</td>
</tr>
<tr>
<td>DMGDH</td>
<td>Dimethylglycine dehydrogenase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSOX</td>
<td>Dimeric sarcosine oxidase</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ε&lt;sub&gt;450&lt;/sub&gt;</td>
<td>Extinction coefficient at 450 nm</td>
</tr>
<tr>
<td>EDTA</td>
<td>Diaminoethanetetra-acetic acid</td>
</tr>
<tr>
<td>E&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Midpoint potential</td>
</tr>
<tr>
<td>ETF</td>
<td>Electron transferring protein</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>KIE</td>
<td>Kinetic isotope effect</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MADH</td>
<td>Methylamine dehydrogenase</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine Oxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSOX</td>
<td>Monomeric sarcosine oxidase</td>
</tr>
<tr>
<td>MTA</td>
<td>Methylthioacetic acid</td>
</tr>
<tr>
<td>MTOX</td>
<td>N-Methyltriptophan oxidase</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>OD₆₀₀</td>
<td>Optical density at 600 nm</td>
</tr>
<tr>
<td>OPA</td>
<td>One-phor-all buffer</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCA</td>
<td>Pyrrole-2-carboxylic acid</td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode array</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PIPOX</td>
<td>L-Pipocolate oxidase</td>
</tr>
<tr>
<td>PNACL</td>
<td>Protein and nucleic acid laboratory</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>SOX</td>
<td>Sarcosine oxidase</td>
</tr>
<tr>
<td>SDH</td>
<td>Sarcosine Dehydrogenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetic acid EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>( N, N', N'-\text{tetramethylethylenediamine} )</td>
</tr>
<tr>
<td>TMADH</td>
<td>Trimethylamine dehydrogenase</td>
</tr>
<tr>
<td>Tris</td>
<td>Trizma base (tris [hydroxymethyl] amino methane)</td>
</tr>
<tr>
<td>TSOX</td>
<td>Heterotetrameric sarcosine oxidase (prefix ( \beta ) or ( \alpha ) to indicate subunit)</td>
</tr>
<tr>
<td>TST</td>
<td>Transition state theory</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGST</td>
<td>Vibrationally enhanced ground state tunnelling</td>
</tr>
<tr>
<td>YT</td>
<td>Yeast extract Tryptone</td>
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Chapter 1

Introduction
1.1 Flavoproteins and Redox Systems

1.1.1 Flavoenzymes

Flavoproteins are a large group of enzymes that use flavin as prosthetic groups (Singer, 1978). They can be found in all organisms and catalyse a broad range of biochemical and redox reactions. Flavins are derivatives of riboflavin, which is comprised of a tricyclic isoalloxazine ring connected to a ribityl side chain at the N10 position (Figure 1.1). There are two main forms of flavin; flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN is riboflavin phosphorylated at the ribityl –5'OH where as FAD has an ADP moiety at this position (Figure 1.1). The active portion of flavin is the heterocyclic isoalloxazine ring which is capable of undergoing both one-electron reduction to form a semiquinone, and two-electron reduction to produce the dihydroquinol form. This allows flavoproteins to act as adaptors between one and two electron oxido-reductants, and to operate over a wide range of oxidation-reduction potentials. Flavoenzymes can cycle between (i) fully oxidised and fully reduced, (ii) fully oxidised and semiquinone or (iii) semiquinone and fully reduced redox states. The reactivity of the isoalloxazine ring is modulated by interaction with the protein environment allowing the flavin to perform certain reactions in some enzymes and not in others. The two-electron reduction potential of flavins makes them stronger oxidants than other cofactors such as NAD⁺. For example flavins can facilitate the oxidation of C-C bonds whereas NAD⁺ is used for more facile oxidations.

Unlike other enzymes that bind cofactors transiently, the majority of flavoenzymes bind flavin tightly and non-covalently, with binding constants in the nM range, and thus the flavin does not dissociate during catalysis (Palfey & Massey, 1998). The only exception is bacterial luciferase, which binds reduced FMN and then uses it in the oxidation of aldehydes before releasing oxidised FMN (Baldwin & Zeigler, 1992). There are, however, a minority of cases (approximately 25 known examples) where the flavin is covalently bound to the protein at the 8α methyl or C6 positions of the isoalloxazine ring (Decker & Brandsch, 1991). The covalent attachment is via a histidine, cysteine or tyrosine residue, the mechanisms of autocatalytic covalent attachment are reviewed in Mewies et al. (1997).
Figure 1.1. The structure of riboflavin showing the standard numbering of the atoms of the isoalloxazine ring system and ribityl tail. Riboflavin is the basis for the flavin cofactors FAD and FMN, which have an adenine moiety or a phosphate group attached to the 5' end of the ribityl chain respectively.
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The role of covalent flavins is not fully understood. Enzymes exist that catalyse the same reaction but differ in whether they bind flavin covalently or non-covalently (Fraaije et al., 1999). Covalent attachment is not a strategy for achieving active site saturation, as flavin can bind non-covalently with high affinity (Mewies et al., 1996). In some cases, the covalent attachment has been shown to modulate the redox potential of the flavin. Studies of vanillyl-alcohol oxidase have shown that covalent attachment is required for efficient catalysis, but removing the link does not totally abolish catalytic activity (Fraaije et al., 1999). Mutation of the cysteine residue involved in covalent linkage in trimethylamine dehydrogenase (TMADH) suggests that the covalent link prevents chemical inactivation of the flavin by water (Mewies et al., 1995, Mewies et al., 1996). However, covalent binding or tight non-covalent binding of flavins is an important property of flavoproteins, since in this form, the cofactor cannot be released in an altered redox state, to be reduced or oxidised by another system. Consequently, the flavin must be returned to its original redox state before the enzyme can undergo another catalytic cycle.

The mechanisms by which one- and two-electron reduction of flavin occurs in different flavoenzymes has been hotly contested in the literature. Single electron transfer (SET) reactions between flavins and other redox centres such as hemes, iron sulphur centres, or other flavins, occur by direct long-range electron transfer. Electron transfers can occur through space or along a defined transfer pathway (protein bridge). No particular orientation between the isoalloxazine ring and its electron transfer partner is known to be optimal for efficient electron transfer (Cuane et al., 1999).

Flavoenzymes catalyse a range of chemical reactions including radical, carbanion and direct hydride transfers, and are able to dehydrogenate many different compounds such as dithiols, alcohols, α-hydroxy acids, α-amino acids and amines. As a result of the dehydrogenation reaction, the flavin becomes reduced, and in order to function catalytically the oxidised form of the enzyme has to be regenerated by electron transfer to a redox acceptor. The acceptor is often molecular oxygen or another redox protein, or in the case of disulphide oxidoreductases a disulphide substrate molecule. Almost all flavoenzymes catalyse reactions involving redox transformations of both organic and inorganic substrates. In a few cases, the role of the flavin is to form a redox intermediate, which allows a non-redox reaction to take
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place, followed by the reversal of the intermediate formation, with no net transfer of electrons. Examples of reactions in this category include chorismate synthase (Henstrand et al., 1996), oxynitrilase (Jorns, 1979) and DNA photolyase (Jorns et al., 1987). A few enzymes have been identified with flavin bound to them, but the flavin appears to have no role in catalysis. It is postulated the flavin may either be a remnant of evolution, or may serve a structural or regulatory role (Edmondson & Ghisla, 1999; Palfey & Massey, 1998).

1.2.1 Properties and Methods of Study

Both free FMN and FAD are an intense yellow colour due to an absorbance band at ~450 nm; the characteristic flavin spectrum also has absorbance maxima at 370 and 260 nm. Excitation of the 450 nm band, either directly or by energy transfer from the short wavelength bands gives rise to a green fluorescence maximum at ~540 nm (Siegel, 1978). This fluorescence is approximately ten times more intense for FMN than FAD, since stacking of the adenine ring over the flavin isoalloxazine in FAD causes fluorescence quenching. Binding of flavin co-factors to proteins affects the intensity of the absorbance maxima, and often causes resolution of the 450 nm band, producing shoulders and possibly shifts in the wavelength of the maximum absorbance.

The binding of ligands to many flavoproteins is accompanied by a change in their UV spectra and this has been used to calculate ligand binding constants. Formation of intermediates can also be detected by characteristic changes in absorbance (Figure 1.2). The reduction of flavin results in absorbance bleaching at ~450 nm. This spectral bleaching can be used in spectropotentiometric studies to calculate the flavin reduction potentials. Also, the bleached spectrum of two-electron reduced flavin is distinct from the spectra of the two one-electron reduced semiquinone forms of flavin, which can be either red/anionic (mono-protonated; \( \lambda_{\text{max}} \approx 370 \) and 480 nm) or blue/neutral (protonated; \( \lambda_{\text{max}} \approx 360 \) and 580 nm). Thus, the number of electrons taken up and the ionisation state of the flavin can be deduced from the spectral form of the flavin. The redox potential of two-electron reduced free flavin is -210 mV (pH 7) (Massey, 1991), but in flavoproteins this value can vary by up to 600 mV, owing to the protein environment surrounding the cofactor. Flavoprotein redox potentials range in value from -495 mV for the semiquinone/hydroquinone redox couple of the Azotobacter vinelandii flavodoxin
(Barman & Tollin, 1972) to +190 mV for the oxidised/semiquinone redox couple of *Methylophilus methylotrophus* electron transferring flavoprotein (ETF) (Byron et al., 1989).

**Figure 1.2.** (A) Different oxidation states of the flavin isoalloxazine ring. (B) Absorbance properties of flavin oxidation states. Spectra are from the enzyme 2-Methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (MHPC) taken from Palfey & Massey (1998).
Distinct spectral properties can also be observed in flavoprotein catalysed reactions that report on intermediates in the catalytic cycle. For example, some flavoproteins form charge-transfer complexes that have strong long-wavelength absorption bands. During the stacking of a nicotinamide ring over the isoalloxazine ring of flavin, the $\pi$ electron clouds interact giving rise to charge-transfer character (Shieh et al., 1981; Lee et al., 1998; Gutierrez et al., 2000). These spectral intermediates are in addition to the different spectral forms that are seen during reduction/oxidation of the flavin. Charge-transfer intermediates and flavin reduction intermediates are readily identified using stopped-flow spectroscopy methods.

Flavoprotein oxidation/reduction reactions involve the transfer of two electrons to flavin, followed by reoxidation of the flavin often mediated by molecular oxygen. Consequently, catalysis by flavoenzymes always involves a reductive half-reaction, where the enzyme-bound flavin is reduced, and an oxidative half-reaction, where the reduced flavin is reoxidised. As the spectrum of oxidised flavin is distinct from that of reduced flavin, transitions between the two states can be monitored easily using stopped-flow techniques. Both the reductive and oxidative half-reactions are accessible by stopped-flow methods making this an attractive method for studying flavoprotein mechanism. Clearly, this is a more attractive approach in terms of defining mechanistic information than the more traditional approaches of steady-state analysis. A stopped-flow kinetic characterisation often does not require the detailed analysis and assumptions of steady-state kinetics, and frequently results in the direct determination of most, if not all of the rate constants. The disadvantages of stopped-flow techniques are that they require large quantities of enzyme and often anaerobic conditions.

Several flavoenzyme systems have already been studied in detail, and hence, it is relatively straightforward to identify flavoenzyme intermediates based on the available literature. Other spectroscopic methods such as EPR and ENDOR spectroscopy can be used for studying the semiquinone form of flavins. NMR spectroscopy has been used to study various aspects of flavoenzymology, e.g. the structure of trapped intermediates. X-ray crystallography combined with sequence analysis has lead to well-resolved structures for a number of flavoenzymes, and this has greatly aided mechanistic studies of flavoenzymes. This structural and sequence information has been used to identify broad sequence and structural similarities.
within the flavoenzyme family (Vallon, 2000). FAD is usually bound to a β-α-β unit (i.e. Rossmann fold) with the sequence motif Gly-X-Gly-X-X-Gly, typical of nucleotide binding proteins (Wierenga et al., 1986). FMN is often bound to βα-αβ barrel structure similar to those of triose phosphate isomerase (Phillips et al., 1978). These structures also provide information about the protein effects on flavin reactivity, either by direct interactions with the isoalloxazine ring, or by indicating the bending or twisting of the ring in different oxidation states (Ludwig et al., 1997). Crystallography has been able to demonstrate how an enzyme undergoes various conformational changes in the presence of substrate [e.g. in p-hydroxybenzoate hydroxylase (Palfey et al., 1999) and thioredoxin reductase (Williams, 1995)]; it also shows that charge-transfer complexes between the flavin isoalloxazine ring and planar ligands are the result of stacking interactions of π-orbitals (e.g. old yellow enzyme; Fox & Karplus, 1994).

1.1.3 Flavoprotein Families and Roles

There are currently several hundred members of the flavoprotein family that have been identified and characterised (Massey, 2000). The protein data bank (PDB; Berstien et al., 1977) contains sequence information for the majority of these (Fraaije & Mattevi, 2000). The flavoprotein family consists of enzymes which catalyse a diverse range of redox reactions, using a variety of different mechanisms and structures. The family ranges from very simple enzymes like flavodoxins, which contain a single flavin and have a molecular weight of ~14 kDa (Leenders et al., 1993); to complex enzymes like the P450s which contain two flavins and haem, and have a molecular weight of ~120 kDa (Narhi & Fulco, 1987). Flavoproteins are involved in a wide range of biological processes, including aerobic metabolism where they catalyse the 2 electron dehydrogenation of many substrates and 1 electron transfers to numerous metals through their free radical states. They often form part of multi-redox centre enzymes such as xanthine oxidase/dehydrogenase (Hille & Massey, 1981), cytochrome P450 systems (Porter & Kasper, 1986; Kurzban et al., 1990) and nitric oxide synthases (Griffiths, 1998). Flavoproteins have been shown to be involved in mammalian embryo development (Ramana-Murty & Adiga, 1982), signal transduction of apoptosis [e.g. apoptosis-inducing factor (Daugas et al., 2000)], soil detoxification (Dagley, 1987), photosynthesis [e.g. spinach ferredoxin reductase...
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(Bruns & Karplus, 1995), repair of DNA damage [e.g. DNA photolyase (Jorns et al., 1987)] and plant phototropism (Briggs et al., 1999).

The diversity of structures and reactions catalysed by flavoproteins has complicated classification of the family into smaller subgroups. Many different schemes have been put forward and adapted over the years, particularly when new members of the family have been identified. A widely accepted scheme has been that of Massey and Hemmerich (1980) in which they propose five distinct groups. These groups are the transhydrogenases, dehydrogenases-oxidases, dehydrogenases-oxygenases, dehydrogenases-electron transferases and electron transferases. However, the more recent classification of Palfey and Massey (1998) attempts to overcome problems with previous classifications by describing the classes of the simple flavoproteins only. The simple classifications can then be applied to the more complex flavoproteins dependent on their properties. This taxonomy divides the simple flavoprotein family into four groups: oxidases, disulphide oxidoreductases, monooxygenases, and a group consisting of reductases, dehydrogenases and electron transferases (Palfey & Massey 1998; Figure 1.3). Sarcosine oxidase, whose study is the basis of this thesis, is a complex flavoprotein containing two flavin cofactors. The overall reaction can be viewed as a dehydrogenation with the final electron acceptor being molecular oxygen. However, the roles of the two flavins can be classified as follows: the first flavin (FAD) acts as a dehydrogenase during oxidation of sarcosine, and the second flavin (8α-N3-FMN) acts as an oxidase, passing its electrons to molecular oxygen. The next section summarises the state of knowledge of the various sarcosine oxidases identified to date.
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Figure 1.3. The reactions catalysed by simple flavoproteins (adapted from Palfey & Massey, 1998).

\[
\begin{align*}
XH + O_2 & \rightarrow X + H_2O_2 \\
R-S-R + \text{NAD(P)H} + H^+ & \rightarrow 2 R + \text{NAD(P)}^+ \\
\text{Substrate} + O_2 + \text{NAD(P)H} + H^+ & \rightarrow \text{Substrate-OH} + H_2O + \text{NAD(P)H} + H^+ \\
AH_2 + B & \rightarrow A + BH_2
\end{align*}
\]

Oxidases
Disulphide oxidoreductases
Monoxygenases
Reductases, dehydrogenases and electron transferases
1.2 Sarcosine Oxidase

1.2.1 General Properties

Sarcosine oxidase (SOX; EC 1.5.3.1) has been isolated from various bacterial sources, where it is produced as an inducible enzyme when the microorganisms are grown on sarcosine as the source of carbon and energy. SOXs have been isolated from more than a dozen different bacterial species and several mammalian sources (Table 1.1). Of these, the genes encoding SOX have been cloned from four bacterial sources (Chlumsky et al., 1995; Suzuki et al., 1992; Suzuki et al., 1994; Koyama et al., 1991) and one mammalian source (Rebuer et al., 1997). The bacterial SOX enzymes can be divided into three subclasses: the monomeric (MSOX), the heterodimeric (DSOX) and the heterotetrameric (TSOX) enzymes; of these the MSOX and TSOX enzymes have been the most thoroughly investigated. All bacterial sarcosine oxidases catalyse the oxidative demethylation of sarcosine to yield glycine, hydrogen peroxide and formaldehyde.

\[
\begin{align*}
\text{CH}_3\text{NHCH}_2\text{COOH} + \text{O}_2 + \text{H}_2\text{O} & \rightarrow \\
\text{HCHO} + \text{NH}_2\text{CH}_2\text{COOH} + \text{H}_2\text{O}_2 & \quad \text{Scheme 1.1}
\end{align*}
\]

TSOX from Corynebacterium P-1 contains four different subunits of approximate molecular masses 100, 45, 21, and 11 kDa (Kvalnes-Krick et al., 1986). The enzyme contains non-covalently bound FAD, NAD$^+$ (Willie et al., 1995) and covalently bound FMN attached to the β subunit (His-173) in the form of an 8α(N$^3$-histidyl) FMN (Willie et al., 1996). The MSOX enzymes are similar in size to the β subunit (45 kDa) of TSOX and contain covalently bound FAD. The MSOX enzymes exhibit modest sequence identity (~25%) with the β subunit of Corynebacterial TSOX (Chlumsky et al., 1995). TSOX also contains two sites for binding of tetrahydrofolate (H$_4$folate). The presence of H$_4$folate does not effect the rate of sarcosine oxidation, but in its presence methylenetetrahydrofolate is the product of the reaction instead of formaldehyde (Kvalnes-Krick & Jorns, 1987). Methylentetrahydrofolate is most likely to be the physiological product, as it is the substrate for serine hydroxymethyltransferase (SHMT); an enzyme which is part of the Corynebacterial
sox operon (Chlumsky et al., 1995). It has also been shown that the MSOX enzymes are unable to use H4 folate as a substrate (Wagner & Jorns, 1997).

Table 1.1. Sarcosine oxidising enzymes isolated from various sources, $K_m$ values for sarcosine. Adapted from Suzuki (1994).

<table>
<thead>
<tr>
<th>Source</th>
<th>Mr (Da)</th>
<th>Prosthetic group</th>
<th>$K_m$ (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcosine dehydrogenase</td>
<td>99,000</td>
<td>Covalent FAD</td>
<td>0.5</td>
<td>Sato et al., 1979</td>
</tr>
<tr>
<td>Rat liver mitochondria</td>
<td></td>
<td></td>
<td></td>
<td>Cook &amp; Wagner, 1986</td>
</tr>
<tr>
<td>Human liver mitochondria</td>
<td>100,000</td>
<td>Covalent flavin</td>
<td>N.D.</td>
<td>Eschenbrenner et al., 1999</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>170,000</td>
<td>Flavin</td>
<td>29</td>
<td>Oka et al., 1979</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. WRF</td>
<td></td>
<td>Covalent FAD</td>
<td>N.D.</td>
<td>Pinto &amp; Frisel, 1975</td>
</tr>
<tr>
<td>Sarcosine Oxidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cylindrocarpon didum</em> M-1</td>
<td>45,000</td>
<td>Covalent FAD</td>
<td>1.8</td>
<td>Mori et al., 1980</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. B-0618</td>
<td>42,000</td>
<td>Covalent flavin</td>
<td>12.2</td>
<td>Matsuda et al., 1987</td>
</tr>
<tr>
<td><em>Bacillus</em> sp. NS-129</td>
<td>42,955</td>
<td>Covalent FAD</td>
<td>12.1</td>
<td>Koyama et al., 1991</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. KB210-8SSY</td>
<td>44,000</td>
<td>Flavin</td>
<td>0.91</td>
<td>Inouye et al., 1987</td>
</tr>
<tr>
<td><em>Alcaligenes denitrificans</em></td>
<td>155,000</td>
<td>Flavin</td>
<td>4.2</td>
<td>Kim et al., 1987</td>
</tr>
<tr>
<td>Rabbit liver</td>
<td>44,000</td>
<td>Covalent flavin</td>
<td>6.7</td>
<td>Reuber et al., 1997</td>
</tr>
<tr>
<td><em>Corynebacterium</em> P-1</td>
<td>174,000</td>
<td>Covalent FMN</td>
<td>3.4</td>
<td>Suzuki, 1981</td>
</tr>
<tr>
<td><em>Corynebacterium</em> U-96</td>
<td>174,000</td>
<td>Covalent FMN</td>
<td>2.5</td>
<td>Jorns, 1985</td>
</tr>
<tr>
<td><em>Arthroacter ureafaciens</em></td>
<td>185,000</td>
<td>Noncovalent flavin</td>
<td>6.4</td>
<td>Ogushi et al., 1988</td>
</tr>
<tr>
<td><em>Arthroacter</em> sp. TE1826</td>
<td>43,250</td>
<td>FAD</td>
<td>10</td>
<td>Nishiya &amp; Imanaka 1993</td>
</tr>
<tr>
<td><em>Arthroacter</em> sp. I-1N</td>
<td>184,000</td>
<td>Noncovalent flavin</td>
<td>15.5</td>
<td>Meškys et al., 1996</td>
</tr>
</tbody>
</table>
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During sarcosine oxidation by TSOX, electrons are transferred from sarcosine to the non-covalently bound FAD. Electrons are then subsequently transferred in two one-electron steps from the reduced FAD to the covalently bound FMN, where oxygen is reduced to hydrogen peroxide. The scheme for electron flow is thus:

\[
\text{Sarcosine } \rightarrow \text{ non-covalent FAD } \rightarrow \text{ covalent FMN } \rightarrow \text{ Oxygen} \quad \text{Scheme 1.2}
\]

The role of the tightly bound NAD\(^+\) in the structure or function of TSOX is not known. It has been shown that it is not reduced by sarcosine, and it is not in redox equilibrium with the flavins (Willie et al., 1995). Whether it catalyses a separate redox reaction is unknown, but observed homology of the sequence surrounding an ADP binding motif (attributed to NAD\(^+\) binding) in TSOX with other enzymes (e.g. octopine and nopaline oxidases; Zanker et al., 1994) may indicate primordial substrate binding sites (Chlumsky et al., 1995). Other flavoenzymes have recently been shown to contain adenine nucleotides. Electron transferring flavoprotein (ETF) contains one mol of AMP in addition to FAD (Roberts et al., 1996; Duplessis et al., 1994), and has been shown to affect the rate of reconstitution of the enzyme with FAD (Sato et al., 1993). Trimethylamine dehydrogenase (TMADH) binds one mol of ADP; this latter cofactor plays no role in the redox reaction catalysed by the enzyme (Lim et al., 1988).

The presence of both covalent and non-covalent flavin in TSOX distinguishes the Corynebacterial enzyme from all other currently known flavoproteins, including the other sarcosine oxidising enzymes MSOX and sarcosine dehydrogenase (SDH). There are a few known flavoenzymes that contain two non-equivalent flavins: NADPH cytochrome P450 reductase (Oprian & Coon, 1982), nitric oxide synthase (Hevel et al., 1991), NADPH sulphite reductase (Siegel et al., 1972), methionine synthase reductase (Leclerc et al., 1998) and the protein NR1 (Paine et al., 2000). Each of these enzymes contains one mol of non-covalently bound FAD and FMN per active site. In these enzymes, FAD accepts electrons from NADPH which are subsequently transferred to FMN. FMN acts as the exit port for electron transfer to an acceptor molecule.
SOX belongs to a larger family of enzymes including bacterial N-methyltryptophan oxidase (MTOX; Koyama et al., 1996), and the mammalian enzymes, pipicolate oxidase (PIPOX; Dodt et al., 2000), dimethylglycine dehydrogenase (DMGDH; Wittner & Wagner, 1981) and sarcosine dehydrogenase (SDH; Wittner & Wagner, 1981). All these monomeric enzymes catalyse similar oxidative demethylation reactions of secondary or tertiary amino acids, and bind FAD covalently. Sequence information is available for these flavoproteins and sequence analysis has shown important similarities between DMGDH, and both MSOX and TSOX (Chlumsky et al., 1995). DMGDH is approximately twice the size of MSOX and the β subunit of TSOX (43 kDa), it uses H₂folate as a substrate and contains a covalently bound 8α(N³-histidyl) FAD. The β subunit of Corynebacterial TSOX exhibits ~25% identity with various MSOX enzymes and the N-terminal half of rat DMGDH. The C-terminal half of the α subunit from Corynebacterial TSOX exhibits 30% identity with the C-terminal half of rat DMGDH and various T-proteins, which form part of the multi-enzyme glycine cleavage system (Okamura-Ikeda et al., 1993). Corynebacterial TSOX, rat DMGDH and various T-proteins all catalyse the formation of 5,10-methylenetetrahydrofolate using different one-carbon donors. The observed sequence similarity suggests that the C-terminal portions of the α TSOX subunit and DMGDH might contain a folate-binding domain, whereas the N-terminal half of DMGDH and the β subunit of TSOX are involved in the binding of flavin and the amino acid substrate (Figure 1.4).
Figure 1.4. Alignment of the β and α subunits of Corynebacterial TSOX with MSOX, DMGDH and T-proteins. The figure is based on sequence alignments reported by Chlumsky et al. (1995). The blue region catalyses methylene tetrahydrofolate formation and may contain a folate binding site. The yellow region may contain the substrate-binding site.
1.2.2 Reaction Mechanism of Corynebacterial TSOX

1.2.2.1 Steady-state Analysis

Hayashi et al. (1983) have proposed an overall reaction mechanism for TSOX by measuring the oxygen uptake at various concentrations of oxygen and sarcosine. The enzymatic reaction follows a Ping-Pong bi-bi mechanism (Scheme 1.3):

$$
\begin{align*}
E_0 + S & \leftrightarrow E_0S \rightarrow E_rP \rightarrow E_r + P \\
P + H_2O & \rightarrow \text{Glycine} + \text{HCHO} \text{ (spontaneously)} \\
E_r + H_2O & \rightarrow E_rO_2 \rightarrow E_0H_2O_2 \rightarrow E_0 + H_2O_2
\end{align*}
$$

Scheme 1.3

In Scheme 1.3, $E_0$ and $E_r$ represent the oxidised and reduced forms of the enzyme, respectively, $S$ is the substrate sarcosine and $P$ the postulated iminium intermediate $\text{CH}_2=(\text{NH}^+)-\text{CH}_2\text{COO}^-$ which is then hydrolysed non-enzymatically by water.

1.2.2.2 Reductive and Oxidative Half-reactions of TSOX

As discussed previously (Section 1.2.1) Corynebacterial TSOX contains a covalently bound FMN and a non-covalently bound FAD present in different subunits. The non-covalent FAD can be removed by acid-ammonium sulphate precipitation (Jorns, 1985). The observation that the semi-apoenzyme is not reduced by dithiothreitol or by sarcosine lead to the conclusion that the two flavins have different roles (Hayashi, 1984; Jorns, 1985). The non-covalent FAD accepts electrons directly from dithiothreitol (or the substrate sarcosine), while the covalent FMN accepts electrons from the reduced non-covalent FAD or from an artificial reductant such as dithionite (Hayashi et al., 1980; Jorns, 1985).

Sulphite reacts with TSOX to form a reversible covalent complex with 50% of the enzyme flavin, causing the enzyme to lose its activity (Jorns, 1985). This treatment, however, does not prevent the reduction of the sulphite-unreactive flavin by sarcosine, but interferes with the reoxidation of the reduced enzyme by oxygen. The sulphite-reactive flavin was identified as the covalent form, because the semi-apoenzyme lacking non-covalent flavin still reacted with sulphite. Jorns (1985) proposed from these observations that the non-covalent FAD reacts with sarcosine,
and the covalent FMN with molecular oxygen (i.e. electrons flow from sarcosine to oxygen through the two flavins).

Kawamura-Konishi & Suzuki (1987) were able to measure the rate of anaerobic reduction of the bound flavins using stopped-flow methods by monitoring the absorbance change at 455 nm. Reduction of TSOX by sarcosine was biphasic in nature, with limiting rate constants of 31 and 6.7 s⁻¹; for the fast and slow phases, the rate of oxidation by oxygen was 100 s⁻¹. To explain the overall rate of reaction (18 s⁻¹) in the steady-state the following mechanism (Scheme 1.4) was proposed:

(Reduction)
E₈ + S ⇌ E₈S → E₆ + P → E⁰ + P
E⁰ + S ⇌ E⁰S → E⁰ + P

(Oxidation)
E⁰ + O₂ ⇌ E⁰O₂ → E₆ + H₂O₂ ⇌ E⁰ + H₂O₂
E⁰ + O₂ → E₈ + H₂O₂

Scheme 1.4

In Scheme 1.4, E represents TSOX, the superscript and subscript represent the non-covalent and covalent flavins, respectively, and the term “r” and “o” represent the reduced and oxidised states of the flavins. The half-reduced form of the enzyme E⁰₉ is distributed between two pathways: (i) a pathway in which E⁰₉ reacts with sarcosine to fully reduce the enzyme to the level of 4 electrons, which then reacts with oxygen to regenerate the oxidised form of the enzyme and (ii) the half-reduced enzyme which reacts with molecular oxygen to regenerate the fully oxidised form of the enzyme.

The overall steady-state rate was explained by assuming that 70% of the enzyme becomes reduced to the four-electron level, and 30% of the enzyme is reduced only to the 2-electron level. Ali et al. (1991) observed the semiquinone form of flavin during TSOX reduction, and proposed that the step representing formation of the semiquinone form limits the rate of the overall reaction. Both Suzuki and Ali and co-workers proposed an elaborate steady-state scheme for the mechanism of electron transfer based on their observations (Figure 1.5).
Figure 1.5. Summary of the reaction mechanisms of TSOX according to reports of Kawamura-Konisha and Suzuki (1987) (Scheme I) and Ali et al. (1991) (Scheme II). The enzyme forms are the same as those in Scheme 1.4. SQ represents the semiquinone form of flavin.
The first phase of anaerobic reduction of the Corynebacterial TSOX with sarcosine converts the oxidised enzyme to an equilibrium mixture of the two-electron-reduced forms (EH₂) at a rate (2700 min⁻¹) similar to the maximum rate of aerobic turnover in the steady state (2600 min⁻¹; Ali et al., 1991). The second phase of the anaerobic reaction converts EH₂ to the four-electron reduced enzyme, EH₄, and occurs with a rate of 350 min⁻¹, which is 7-fold slower than steady-state aerobic turnover. The rate of reaction of the EH₂ form with oxygen (4480 min⁻¹) is 2-fold faster than aerobic turnover and 13-fold faster than the conversion of EH₂ to EH₄, suggesting that the oxidised and EH₂ forms of TSOX cycle during turnover with sarcosine.

The formation of a flavin biradical (FADH⁺FMN⁻⁻) has been observed in the EH₂ form and its rate of formation is close to the rate of flavin reduction of oxidised enzyme by sarcosine (Ali et al., 1991). The rate of internal electron transfer between the flavins is fast (45000 min⁻¹) as determined by pH-jump methods (Ali et al., 1991).

1.2.2.3 Substrate Association and Biradical Formation in TSOX

TSOX enzymes appear to catalyse the oxidation of a limited range of substrates, turning over only sarcosine (348 min⁻¹; Ogushi et al., 1988) and a few N-methylated amino acids such as N-methyl-L-alanine (204 min⁻¹; Suzuki, 1981) and N-ethylglycine (132 min⁻¹; Suzuki, 1981). TSOX does not react with the N-methylated amino acids, dimethylglycine, creatine, creatinine or choline (Suzuki, 1981). TSOX forms complexes with various heterocyclic carboxylic acids such as D-proline ($K_d = 22$ mM), 2-furoic acid ($K_d = 0.34$ mM), 2-pyrrololecarboxylic acid ($K_d = 0.38$ mM), and 2-thiophencarboxylic acid ($K_d = 3.3$ mM) (Zeller et al., 1989). These compounds are competitive inhibitors and provide evidence for a single sarcosine binding site near the non-covalent flavin. Anaerobic reduction with L-proline and 2-thiophencarboxylic acid produces the characteristic biradical reaction intermediate which is seen with sarcosine.

1.2.2.4 Cloning and Sequencing of Corynebacterial TSOX Genes

The cloning of the sox genes from Corynebacterium sp. P-1 and the expression of recombinant protein in E.coli revealed an interesting property of the Corynebacterial enzyme (Chlumsky et al., 1993). The recombinant enzyme was isolated as the complete heterotetramer and shown to have a flavin content similar to
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the native enzyme, but had significantly different spectral properties. These spectral changes were attributed to half the flavin forming a reversible covalent 4a-adduct with a cysteine residue (Chlumsky et al., 1993). The adduct was shown to dissociate upon oxidation of the cysteine residue by H₂O₂ or during aerobic turnover with sarcosine. Further evidence came from a steady-state peroxidase-coupled assay in which a lag phase was observed in the reaction catalysed by the recombinant enzyme, but not with the native enzyme. This lag phase could be eliminated by prior treatment of the recombinant enzyme with H₂O₂. The conclusion was that the 4a-thiolate adduct was an inactive form of TSOX that could be reactivated by reaction with sarcosine.

1.2.3 Structure of MSOX

At present there is no structural data available for TSOX. However, a X-ray crystal structure for MSOX from Bacillus sp. B-0618 has recently been solved at 2.0 Å resolution (Trickey et al., 1999). MSOX comprises two domains with an overall topology similar to D-amino acid oxidase (DAAO; Mattevi et al., 1996). As the MSOX from Bacillus sp. B-0618 displays strong sequence identity (~23%) with the β subunit of TSOX, the structural data may provide relevant information on substrate and flavin binding in TSOX.

MSOX from Bacillus sp. B-0618 has a molecular mass of 43.8 kDa, an N-terminal ADP binding motif and contains one mol of FAD covalently bound to Cys 315 through an 8α-S-cysteiny1 linkage. This cysteine is conserved in MSOX enzymes from other sources, MTOX and PIPOX, but not in TSOX. The flavin ring of the 8α-S-cysteiny1 FAD is located in a very basic environment unique to known flavoprotein structures, and is a feature that is likely to raise the flavin redox potential (Trickey et al., 1999). The active site residues likely to be involved in sarcosine binding or in the oxidation chemistry have been identified based on structures with bound inhibitors. Importantly, these residues are conserved in other MSOX enzymes, MTOX, PIPOX and βTSOX.

The two-domain structure of MSOX (Figure 1.6) belongs to the structural class of flavoenzymes first observed for p-hydroxybenzoate hydroxylase (PHBH; Schreuder et al, 1989). One domain contains a classic FAD-binding motif common to the PHBH and glutathione reductase (GR) class of flavoproteins (Karplus & Schulz, 1987), comprising a five stranded parallel β sheet flanked by two α helices, and
followed by two sets of three-stranded anti-parallel β strands. The FAD domain is interrupted twice by regions of the catalytic domain comprising a two part, eight-stranded anti-parallel β sheet. The two-part nature of the second (catalytic) domain is also characteristic of the PHBH flavoprotein class, but not the GR class. The covalent FMN attachment site in βTSOX (His173) aligns with Ser149 in MSOX; the hydroxyl group of this serine residue is located in a small depression on the protein surface, above the si face of the FAD ring about 8 Å from the flavin N(5) atom (Trickey et al., 1999). This positions Ser149 on the side of the protein opposite to the substrate entry channel, leading to the substrate-binding cavity on the re face of the FAD isoalloxazine ring.

Crystal structures of MSOX bound to two competitive inhibitors [methylthioacetic acid (MTA) and pyrrole-2-carboxylic acid (PCA)] suggest the location of the sarcosine binding site (Figure 1.6). Both inhibitors are bound on the re face of the flavin ring with one carboxylate oxygen hydrogen bonded to the side chain of Lys348. Arg52 moves into the active site on inhibitor binding and forms hydrogen bonds with the carboxylate groups of the inhibitor. Movement of Arg52 displaces five solvent molecules from the active site and induces conformational changes in a flexible loop comprising a glycine and two glutamate residues. This conformational movement makes the active site inaccessible to further solvent. The pyrrole nitrogen atom of PCA and the sulphur atom of MTA, both of which correspond to the nitrogen atom of the substrate, are positioned above the C(4a) atom of the flavin ring. The methyl group of MTA and the corresponding C(5) atom of PCA are positioned over the N(5) atom of the flavin ring and are close to His269 (~3.5 Å) making it a likely candidate for an active site base.
Figure 1.6. Structure of MSOX from *Bacillus* sp. B-0618. (A) Ribbon diagram of MSOX showing the two domain structure: catalytic domain in blue, FAD binding domain in red, FAD in yellow. (B) Active site structure of MSOX in the presence of inhibitors MTA and PCA, showing the important catalytic residues (taken from Trickey *et al.*, 1999).
1.2.4 Cloning of the *Arthrobacter* sp. 1-IN TSOX Genes

An *Arthrobacter* sp. (sp. 1-IN) producing TSOX has been isolated from soil samples using indicator plates (Meskys *et al.*, 1996). This *Arthrobacter* sp. was shown to produce TSOX since production of formaldehyde was detected when cell extracts were assayed using the Nash procedure (Suzuki, 1981). Non-denaturing PAGE identified the presence of a single protein band for purified enzyme. However, when subjected to SDS-PAGE, four protein bands were observed with apparent molecular weights of 106, 43, 24 and 15 kDa; these were very similar to the subunits of the *Arthrobacter ureafaciens* and *Corynebacterium* enzymes. The genes encoding TSOX from *Arthrobacter* sp. 1-IN have been cloned (Meskys *et al.*, 1996). The genes were isolated as part of a 12.5 kb genomic DNA fragment. Restriction mapping was used to determine the location of the *sox* genes within this fragment. It was shown that only 7.5 kb of the sequence was necessary to obtain full enzyme activity and it is this 7.5 kb DNA fragment which is the starting point for the work described in this thesis.

1.3 Quantum Processes in Enzymatic Small Particle Transfer

This thesis is concerned with the mechanism of small particle transfer (i.e. transfer of the electron and hydrogen nucleus) in TSOX. Quantum mechanisms of electron transfer are well established in enzyme molecules (DeVault & Chance 1966; Marcus & Sutin, 1985). Quantum mechanisms of H-transfer are less well appreciated, but recent work has identified strong parallels between the mechanisms of electron and hydrogen transfer in enzymes (Sutcliffe & Scrutton, 2000; Scrutton *et al.*, 1999; Basran *et al.*, 1999). The following section reviews briefly the state of our theoretical understanding of biological electron transfer. There then follows a more in-depth account of the theory of H-transfer, which conceptually is similar to electron transfer theory. However, the approaches used in studying quantum H-transfer in enzymes are sophisticated making use of isotopic substitution and the measurement of kinetic isotope effects (KIEs).
1.3.1 Electron Transfer

In chemical reactions, electron transfer occurs because of overlap between electron orbitals associated with the donor and acceptor molecules. In biological electron transfer, the redox centres are embedded in an electrically insulating, protein environment, up to ~20 Å apart, and consequently electron orbital overlap is small. However, electrons can travel over large distances (10-30 Å) at relatively fast rates (Devault, 1980). The idea of electrons passing through regions of electrically insulating protein at fast rates can be understood in terms of the wave-like properties of the electron. The wave-like properties enable the electron to pass, by quantum tunnelling, through areas where it would be excluded by its particle-like nature. The equation for predicting the rate of biological electron transfer, \( k_{e_t} \), is referred to as 'Fermi's Golden Rule' (Equation 1.1; Devault, 1980):

\[
 k_{e_t} = \frac{2\pi}{h} H_{ab}^2 (FC)
\]

Equation 1.1

where \( h \) is Planck's constant divided by \( 2\pi \) and \( H_{ab}^2 \) is the 'electronic factor' representing the electronic coupling between the reactant and the product wave-functions. This gives the probability of electron transfer and can only be understood in quantum mechanical terms. The Frank-Condon factor (FC), is the 'nuclear factor', which can be understood in classical terms, providing that the frequency of nuclear motions coupled to electron transfer is not too high (Marcus & Sutin, 1985).

1.3.1.1 The Nuclear Factor

The Frank-Condon (FC) factor is the reorganisational energy that accompanies electron transfer, as a charge is transferred within the system causing the equilibrium position of polar groups to change. This can be understood in energy terms using a potential energy diagram for the reactants and products (Figure 1.7). The energy of the reactants is represented as a single harmonic potential energy curve, the products are represented by a similar curve, whose equilibrium position has changed and has lower minimum energy as a result of the transfer reaction. The rate of electron transfer is defined by the free energy change, \( \Delta G^\circ \), associated with electron transfer (vertical displacement between equilibrium positions), and the reorganisation energy, \( \lambda \), (the free energy required to alter the equilibrium geometry
without removing the electron. Experiments show that $\lambda$ increases as a function of the polarity of the environment of the redox centres. $\Delta G^*$ is the activation free energy and represents the minimum energy required by the reactant before crossing over to the product curve.

Electron transfer only occurs when the nuclear co-ordinates are such that the potential energies of reactant and product are equal. In long-range electron transfer reactions where coupling is weak, the reactant will oscillate past the point of intersection many times before electron transfer takes place. The reaction is therefore non-adiabatic, as the reaction does not always proceed once the intersection point is reached.

In the late 1950s Marcus derived an equation which related the activation free energy, $\Delta G^*$, to the driving force of the reaction, $\Delta G^0$, and the reorganisational energy, $\lambda$. The relationship is known as the ‘Marcus gap law’ (Equation 1.2; Marcus, 1956)

$$\Delta G^* = \frac{(-\Delta G^0 + \lambda)^2}{4\lambda}$$

**Equation 1.2**

This equation has two effects on the rate of electron transfer. First, as $-\Delta G^0$ increases, the activation energy decreases, and consequently the electron transfer rate will increase until $-\Delta G^0$ is greater than $\lambda$ at which point the rate will begin to decrease. Second, when $-\Delta G^0 = \lambda$, this results in $\Delta G^* = 0$, and this is known as the Marcus optimum. Equations 1.1 and 1.2 can be combined in the form of the Arrhenius equation which relates the rate of electron transfer to the thermal fluctuations of the protein (Equation 1.3)

$$k_{et} = \frac{4\pi^2 H_{AB}^2}{h(4\pi\lambda k_B T)^{\frac{1}{2}}} e^{-\frac{\lambda + \Delta G}{4\lambda k_B T}}$$

**Equation 1.3**

Under conditions where $-\Delta G = \lambda$ the exponential term in Equation 1.3 becomes unity and the electron transfer rate becomes almost temperature independent. This is known as non-tunnelling temperature independence (Devault, 1984), and the rate of electron transfer is solely dependent on the electronic factor $H_{AB}^2$.  

25
Figure 1.7. Potential energy diagram of the reactant and product curves used to explain the Frank-Condon Factor. $\Delta G^*$ is the activation energy, $\Delta G^0$ is the driving force between redox centres, $\lambda$ is the reorganisation energy and $Q_R$ and $Q_P$ are the equilibrium minima for reactant and product respectively.
1.3.1.2 Quantum Mechanical Application of the Nuclear Factor

The classical equation is accurate at physiological conditions, but it was first shown to breakdown when applied to the reaction catalysed by light induced cytochrome oxidation in *Chromatium vinosum* at very low temperatures (Chance & Nishimura, 1960). Chance & Nishimura were able to measure electron transfer at 77K, despite the fact that nuclear vibrations would be frozen at the ground state and reaching the transition state by classical vibrations would be impossible. This was explained in terms of quantum mechanical tunnelling of nuclear wave-functions. In quantum mechanics the energy levels of an oscillator are quantised. For biological electron transfer the nuclear vibrations are approximated to a simple harmonic oscillator, whose energy levels are quantised with spacing equal to $\hbar \omega$ and the ground state at $\frac{1}{2} \hbar \omega$. When $\hbar \omega \ll k_B T$, many vibrational levels are populated and a quantum mechanical treatment gives the same result as a classical treatment. At low temperatures when $\hbar \omega \gg k_B T$, the observed electron transfer rate can be explained in terms of nuclear wave-functions. The wave-functions of the reactant state tunnel through the potential energy barrier to overlap with the nuclear wave-functions of the product state (Figure 1.8).

Figure 1.8. Potential energy diagram to illustrate the nuclear wave-function overlap due to quantum mechanical tunnelling of electrons between the reactant and product states (taken from Devault, 1984).
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1.3.1.3 The Electronic Factor

The electronic factor \( H_{\text{el}}^2 \) in Fermi’s Golden Rule represents the electronic coupling between reactant and product wave-functions, and determines the probability of the system jumping from the reactant curve to the product curve once the transition point has been reached. In biological systems where orbital overlap is small, the electron transfer probability at the intersection point is small and reactants rarely jump to the product curve. The ‘slowness’ of electron transfer at the intersection indicates that there is still a potential energy barrier to the movement of an electron from reactants to products. This energy barrier is traversed by electron tunnelling. The rate of tunnelling is determined by Gamow’s tunnelling law (Equation 1.4):

\[
H_i^2 = H_0^2 e^{-\beta}
\]

Equation 1.4

where \( H_0 \) is the electronic coupling between two redox centres in van der Waals contact and \( \beta \) is the electronic decay factor. The electron transfer rate is predicted to depend on the electron transfer distance, \( r \), by the following relationship: (Equation 1.5; Marcus & Sutin, 1985):

\[
k_{\text{et}} = k_0 e^{-\beta(r-r_0)} e^{-(\Delta G + 2B)^2 / 4kT}
\]

Equation 1.5

where \( k_0 \) is the characteristic vibrations of the nuclei, assigned a value of \( 10^{13} \) s\(^{-1} \) (Marcus & Sutin, 1985) and \( r_0 \) represents the van der Waals distance (3 Å). From results published by Moser et al. (1992) it was shown that the electron transfer rate \( (k_{\text{et}}) \) is greatly affected by the distance between the redox centres, \( r \), and the nature of the media between them, \( \beta \).

The energy dependence of the electron transfer process is due to the distortion of the nuclear geometry of the reactant state and is aided by the thermally activated, breathing of the protein molecule. Thermal activation of the reactant state leads to overlap of the reactant and product potential energy curves. The point of overlap is the nuclear geometry compatible with electron tunnelling. At this intersection point there is an energy barrier through which the electron tunnels to the product. Recent work has demonstrated a similar role for protein dynamics in H-tunnelling (Basran et al., 1999, Scrutton et al., 1999, Kohen & Klinman, 1999), and is discussed in the following section (Section 1.3.2).
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1.3.2 Protein Dynamics in the Quantum Transfer of the Hydrogen Nucleus in Enzymes

1.3.2.1 Introduction

Enzyme catalysis has been modelled on the premise that enzymes are able to achieve high catalytic rates by evolving complementarity with the transition state of the reaction. A popular approach has been to visualise an energy barrier that has to be surmounted for the reaction to proceed from reactants to products. The greater the height of this barrier, the slower the rate of reaction. Enzymes reduce the amount of energy needed to surmount the barrier and consequently increase the rate of reaction. The transition state is the structure of the unstable reactant at the top of the barrier, and the energy required to pass over the barrier is the activation energy, and is supplied by the thermal excitation of the substrate. This classical treatment of enzyme catalysis is known as the transition state theory (TST) and has been applied to enzyme catalysed reactions since its conception over 50 years ago (Glasstone et al., 1941). However, when considering enzyme reactions involving the transfer of the hydrogen nucleus (e.g. during the cleavage of a stable C-H bond) this theory is flawed. TST fails to account for the role played by protein dynamics and the quantum properties of the small hydrogen particle.

Enzymes are dynamic structures (Karplus et al., 1986) that can undergo large conformational changes when substrate is bound. These large conformational changes can activate the enzyme, accommodate the binding of larger substrates, prevent solvent entering the active site or prevent reactants leaving until catalysis is complete. The role of these large-scale structural changes in enzyme catalysis is understood and accepted, but the roles of intrinsic, small-scale, low frequency vibrations (i.e. 'breathing') of the protein have not been fully realised (Gutfreund et al., 1995; Karplus et al., 1986). A protein in solvent has been described as "resembling a highly associated viscous liquid" (Gavish et al., 1986), which undergoes a range of small motions on sub milli-second time-scales. These would include movement of individual amino acids, twisting of the polypeptide chain and vibrations of individual bonds which can lead to localised areas of viscoelasticity within the protein scaffold. The link has been made between protein dynamics and enzyme function (Rasmussen et al., 1992; Rudd et al., 1994) but as yet, these...
observations have not yielded a complete model of enzyme catalysis which incorporates protein 'breathing'.

Recently the dynamic properties of enzymes have been shown to facilitate the cleavage of C-H bonds (Basran et al., 1999; Kohen et al., 1999). In these studies, the transfer of hydrogen from reactants to products is proposed not to proceed via a transition state on a potential energy barrier. Instead, the hydrogen is transferred through the barrier by the process of quantum tunnelling, which can occur due to the quantum mechanical properties of small particles such as hydrogen. The probability of hydrogen tunnelling (H-tunnelling) is dependent on the distance the hydrogen is transferred between donor and acceptor groups. The intrinsic protein dynamics are utilised by increasing the probability of tunnelling by reducing the distance the hydrogen is transferred (i.e. reducing the barrier width), and by equalising the energy states of reactants and products. The cleavage of stable C-H bonds by enzymes is key to many biological processes and the mechanism of H-tunnelling aided by protein fluctuations is an attractive explanation for these energetically unfavourable reactions.

The theory of H-tunnelling will be explained in greater detail in the following sections and an explanation of the importance of protein dynamics in this process will be investigated. There have been various studies which provide direct evidence for H-tunnelling enhanced by protein fluctuations and recently several reviews of the topic have been written (Kohen & Klinman, 1999; Scrutton et al., 1999; Sutcliffe & Scrutton, 2000). The theoretical approaches, which have led to the development of dynamic tunnelling theories for hydrogen transfer during enzyme catalysis, will be discussed. This will provide a useful background to the work on C-H bond cleavage in TSOX presented in Chapter 4.

1.3.2.2 Classical Approach to Enzyme Catalysis

Enzyme catalysis has exclusively been modelled using TST and it is the accepted explanation for an enzyme's ability to enhance the rate of a chemical reaction (Glasstone et al., 1941; Kraut, 1988). TST is popular, probably due to the simple mathematical relationships involved, and the ease by which one visualises a static energy barrier (Gutfreund et al., 1995). The basic idea of TST is that a reaction from substrate to product proceeds via a high energy state at the top of a static potential energy barrier (Figure 1.9). The rate of this two-step reaction, $k$, is
dependent on populating the transition state and a simple mathematical relationship relating $k$ and the activation energy, $\Delta G^\ddagger$, can be defined in the simple reaction:

$$ S \leftrightarrow X^\ddagger \rightarrow P $$

$X^\ddagger$ is the transition state or 'activated complex' representing the highest-energy structure that occurs on the conversion of substrate to product. The transition state, $X^\ddagger$, is treated as being in quasi-equilibrium with the ground state of the substrate and a quasi-equilibrium constant, $K^\ddagger$, can therefore be defined:

$$ K^\ddagger = \exp(-\Delta G^\ddagger/RT) $$  \hspace{1cm} \text{Equation 1.6}

where $\Delta G^\ddagger$ is the Gibbs free energy that is required to pass over the barrier (the energy difference between reactant and the transition state), $T$ is the absolute temperature and $R$ is the gas constant. The first order rate constant for this reaction, $k$, is related to the extent by which the transition state is populated and can be written in its simplest form as follows:

$$ k = (k_B T/h) \exp(-\Delta G^\ddagger/RT) $$  \hspace{1cm} \text{Equation 1.7}

where $k_B$ is the Boltzmann constant, and $h$ is Planck's constant. The Gibbs energy of activation can be separated into enthalpic and entropic terms by using an equilibrium thermodynamics relationship:

$$ \Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger $$  \hspace{1cm} \text{Equation 1.8}

where $\Delta H^\ddagger$ is the enthalpy and $\Delta S^\ddagger$ the entropy of activation. The rate constant can thus be expressed as:

$$ k = (k_B T/h) \exp(S^\ddagger/R) \exp(-\Delta H^\ddagger/RT) $$  \hspace{1cm} \text{Equation 1.9}

Equation 1.9 implies that the rate of reaction is related to the height of a static reaction barrier. TST has fashioned the idea that enzymes are complementary to the transition state of the reaction, rather than the ground state conformations (Kraut, 1988). An enzyme is able to speed up the rate of a reaction by stabilising the transition state relative to the ground state thus increasing the degree by which the transition state is populated and effectively reducing $\Delta G^\ddagger$. 


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Figure 1.9. Potential energy diagrams representing (a) the TST and (b) Kramers’ theory. Both involve the conversion of substrates, S, to products, P, over a potential energy barrier, requiring an activation energy $\Delta G^\dagger$ or $\Delta U^A$, respectively. In TST the activated complex, $X^\dagger$, is in equilibrium with S, which moves unidirectionally to P. In Kramers theory a particle diffuses over the potential energy barrier driven by structural fluctuations, $\tau$, of the substrate and product states.
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TST has been the model for improving enzyme catalysis and achieving 'catalytic perfection'. Techniques for the rational redesign of enzymes include site-directed mutagenesis, the grafting of catalytic function into existing molecules and gene shuffling. These techniques have produced the occasional success story (Clarke et al., 1989; Scrutton et al., 1990) but on the whole have been unsuccessful due to lack of a complete picture of the catalytic power of enzymes. Recently, random methods of redesign have lead to some success. This is achieved by mimicking evolution, by identifying improved enzymes from a large collection of randomly modified enzymes contained in an enzyme library (Stemmer, 1994). Structural analysis of substrates bound in active sites and the use of transition state analogues as inhibitors of enzymes has given some credence to the TST.

TST is probably an over-simplification when applied to enzyme catalysis as it was originally developed to account for gas-phase reactions (Sutcliffe & Scrutton, 2000). Problems with its application to enzymes have been raised by several authors (e.g. Gutfreund et al., 1995; Scrutton et al., 1999; Sutcliffe & Scrutton, 2000; Benkovic and Bruce, 2000). These authors have recognised the role of protein and solvent dynamics as originally proposed by Kramers for enzyme systems (Kramers, 1940). Kramer's theory can also be viewed in terms of a reaction co-ordinate diagram, but a reaction from substrate to product proceeds via a process of diffusion over a potential energy barrier (Figure 1.9). The energy required for this process comes from the thermally-generated, structural fluctuations of the protein, which are translated into movement of the substrate. This kinetic energy is dissipated by a viscous frictional force with the surroundings and causes a degree of strain in the substrate enabling it to proceed along the reaction co-ordinate and over the potential energy barrier. In this 'transient-strain' model of enzyme catalysis, the rate constant, \( k \), is related to the height of the potential-energy barrier, \( \Delta U \), by the following equation:

\[
\begin{align*}
  k &= \frac{1}{\tau} \exp(-\Delta U^f/RT) \\
  &\text{Equation 1.10}
\end{align*}
\]

\( \tau \) is the time constant of structural fluctuations and is proportional to the viscosity of the medium. This equation is the same form as the Arrhenius equation (Equation 1.11):

\[
\begin{align*}
  k &= A \exp(-\Delta E/RT) \\
  &\text{Equation 1.11}
\end{align*}
\]
where $A$ is the pre-exponential factor and $\Delta E$ is the activation energy. However, the dynamic nature of molecules is incorporated into $\Delta U$, but not $\Delta E$ (Sutcliffe & Scrutton, 2000).

An important conceptual point is that $k$ depends on local viscosity and is thus related to effects due to structural fluctuations and is not dependent on the diffusion of substrate into the active site of the enzyme (Scrutton et al., 1999). Kramer's theory acknowledges the dynamic nature of protein molecules and it has provided the framework for developing theories of hydrogen tunnelling in enzymes (Bruno & Bialeck, 1992).

1.3.2.3 Kinetic Isotope Effects (KIEs) and Their Use in Investigating Hydrogen Tunnelling

Kinetic isotope effects (KIEs) are very useful tools for investigating the mechanism of C-H bond cleavage. Measurement of KIEs involves the replacement of hydrogen with its heavier isotopes deuterium ($^2$H) or tritium ($^3$H) (Klinman et al., 1978; Northrop et al., 1982; Cleland, 1999). The incorporation of these heavier isotopes into substrates, cofactors, solvents or the enzyme itself causes measurable differences in the rate of reaction compared to reactions with a lighter isotope. The KIE is the ratio of the rate of reaction with one isotope to the rate of reaction with a heavier isotope ($k_H/k_D$). KIEs can be used to determine the importance of the C-H bond cleavage step in a complex enzyme reaction, or to determine the nature of intermediates in chemical transformations involving multiple bond cleavages (Klinman, 1978).

The application of hydrogen KIEs is widely used in exploring enzyme mechanisms, both in the presteady-state and steady-state since the mass ratio of its isotopes is much larger than with other elements, resulting in relatively large KIE values (Kohen & Klinman, 1999). Isotope effects are useful as mechanistic probes since isotopic substitution does not alter the structure of the transition state (Klinman, 1978). This is because the potential energy curves and surfaces in which the nuclei move are not affected by substitution of one nucleus by its isotope. Interatomic and intermolecular forces are dependent on attractions or repulsions between the charges on electrons and nuclei and not on the masses of the nuclei (Bell, 1980).

Isotopic substitution affects only the mass and moments of inertia, which consequently affect the translational, rotational and vibrational modes of a substrate.
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(the degrees of freedom of the molecule). The changes in translational and rotational modes are very limited between isotopes and can be considered to be negligible; by far the most important factor determining the size of a KIE is the effect of isotopic substitution on vibrational energy levels. More thorough explanations of the origin of KIEs have been made, which include functions of rotational and translational movements, and take into account the masses and moments of inertia of reactants and the transition state (Van Hook et al., 1971; More O’Ferrall. 1975; Bell, 1980). However, these functions will cancel out to give an approximation of hydrogen isotope effects where the vibrational movements produce the greatest effects. The KIE can mainly be attributed to the differences in zero-point energies between isotopically different bonds. A C-H bond and a carbon-deuterium (C-D) bond will occupy the zero-point energy vibrational state at physiological temperatures (Figure 1.10). However, the zero-point energy occupied by either hydrogen or deuterium is not at the bottom of a potential energy curve, as this would mean that the position and momentum of these atoms would be known with respect to the carbon atom which is in violation of the Heisenberg Uncertainty Principle. Therefore, the zero-point energy is always above the electronic potential minimum and its energy, \( E \), is estimated by its de Broglie wavelength:

\[
E = \frac{\hbar v}{2}
\]

**Equation 1.12**

where \( \hbar \) is Planck’s constant and \( v \) is the vibrational frequency of the bond. This is shown in the expression:

\[
v = \frac{1}{2} \pi \sqrt{k/m}
\]

**Equation 1.13**

where \( k \) is the force constant, or ‘stiffness’ of the bond, and \( m \) is the reduced mass \( (mI \times mC)/(mC + mI) \), with \( mI \) being the mass of the isotope (proton or deuteron) and \( mC \) is the mass of the carbon nucleus. The vibrational frequencies have been measured using infrared spectroscopy and are 2900 cm\(^{-1}\) for the C-H bond and 2100 cm\(^{-1}\) for the C-D bond. Therefore, the zero-point energy for a C-H bond is \( \sim 17.4 \text{ kJ-mol}^{-1} \) and \( \sim 12.6 \text{ kJ-mol}^{-1} \) for a C-D bond.
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Figure 1.10. Potential energy diagram to demonstrate the effect of zero-point energies on the activation energies for the cleavage of C-H, C-D and C-T bonds. A-H is the substrate-hydrogen bond and B the acceptor of the hydrogen nucleus (either a residue in the active site or a second substrate molecule).
According to TST, all of this vibrational energy in the transition state is converted into translational energy along the reaction co-ordinate. Therefore if the transition state is symmetrical and linear, a C-H bond requires 4.8 kJ·mol⁻¹ less free energy of activation than the equivalent C-D bond to reach the transition state (Fersht, 1985).

The KIE for cleavage of a C-H bond compared to a C-D bond can be predicted by TST, which relates the difference in the isotopic rates (i.e. the KIE) to the difference in free activation energy ($\Delta A G'$):

$$\frac{k_u}{k_D} = \exp\left(-\frac{\Delta A G'}{RT}\right) = 7$$

Equation 1.14

Therefore TST would place an upper limit of 7 for the KIE between a reaction in which the C-H or C-D bond is cleaved.

Experimental observations of KIEs often show deviations from the maximum value predicted by the semi-classical treatment of TST (Westheimer, 1961). It is these deviations which make the use of isotopic substitution so useful, as a variation in isotope effect can be used as a guide to changes in transition state structure, or as evidence for the quantum mechanical tunnelling of hydrogen. There are a number of reasons for changes in the hydrogen isotope effect. These include (i) H-tunnelling, which leads to higher than expected KIEs, (ii) production of an asymmetric transition state or (iii) non-linear transfer of hydrogen which leads to reduced KIEs.

An association between the symmetry of the transition state structure and KIEs has been made by Westheimer (Westheimer, 1961). The 'Westheimer effect' is caused by an asymmetric transition state that can be visualised using a three-centre model for the transition state:

$$DH + A \rightarrow D-----H-----A \rightarrow D + HA$$

where D and A are atoms or molecules which are donating and accepting a proton respectively and D-----H-----A is a linear transition state. This state occurs as the cleavage of a C-H bond rarely involves a simple dissociation of a proton and in most cases hydrogen is transferred between two atoms. The transition state has two stretching vibrations, symmetric and asymmetric:

- D-----H-----A
  - Symmetric stretch
- D-----H-----A
  - Asymmetric stretch
If the transition state is symmetrical, the stretching vibrations of D and A are opposite and equal, the H-nucleus will remain motionless and the vibrational frequency will be independent of the isotope. Therefore zero point energy differences will be dominant and the full KIE effect will be expressed. However, when the transition state for H-transfer becomes 'asymmetric' i.e. the proton is associated with the donor or acceptor molecule, a reduction in the observed KIE is expected. This is because the symmetric stretching component of the transition state is now effected by isotopic substitution (More O'Ferrall, 1975), and leads to cancellation of ground state frequencies by transition state vibrational frequencies producing smaller isotope effects (Klinman, 1978). Therefore, the 'Westheimer model' predicts a large variation in the observed isotope effect and dependence of the KIEs on the position of hydrogen in the transition state.

The Westheimer effect can be understood in terms of the Hammond postulate, which relates the structure of the transition state to the thermodynamic driving force of the reaction. For example, when the transition state resembles the structure of the reactant or the product, (ΔG > 0 or ΔG < 0 respectively) there is a reduction in the value of the KIE. However, in isoenergetic reactions (ΔG = 0) the transition state is predicted to be symmetrical and the maximum value for the KIE is expressed (Klinman, 1978). The Westheimer model is based on the assumption that hydrogen transfer is linear, but if transfer is non-linear, the KIE is predicted to be lower (Bell, 1980). Some enzyme reactions involve the transfer of hydrogen where the acceptor and donor are not linear and produce low isotope effects because of rearrangements in the substrate which accompany proton transfer (van Bastelaere, 1995).

1.3.2.4 Commitment to Catalysis

The framework within which KIEs can be understood in biology is complicated by the nature of enzyme catalysis. Although non-enzymatic reactions tend to provide full expression of the isotope effect on measured rates of reaction, this is not the case for enzymes (Klinman, 1978). Enzyme-catalysed reactions take place within multiple steps in the reaction co-ordinate, often with similar energetic barriers. Consequently, the chemical step which gives the intrinsic KIE could be buried between enzymatic steps that include forward and reverse commitment, rate-limiting product release, or rate-limiting enzymatic conformational changes (Albery &
Knowles, 1976; Cleland \textit{et al.}, 1999; Schramm \textit{et al.}, 1999). The combined rate constant for the overall reaction usually includes the rate constants for these steps (Kyte, 1995).

A consequence of the kinetic complexity of enzymatic reactions, relevant to the use of KIEs as probes of reactions, is that the observed KIE might not have the same value as the KIE on the isotopically sensitive step of a reaction, known as the intrinsic KIE (Bahnson & Klinman, 1995). In using KIEs to study C-H bond cleavage, for example, it is therefore vital to be able to relate the intrinsic KIE to the observed KIE of a reaction, or to ensure that the KIE being measured directly reflects C-H bond cleavage (Cha \textit{et al.}, 1989; Grant & Klinman, 1989).

The concept of commitment to catalysis can be illustrated by a reaction coordinate diagram for an enzyme-catalysed reaction, consisting of a number of energetic barriers. The rate-limiting step for the reaction corresponds to the step with the highest activation barrier in the energy profile. If the isotopically sensitive chemical step is essentially rate-limiting, by being several kJ·mol$^{-1}$ above all others, the isotope effect will be fully expressed. However, if the C-H bond breakage step does not have the highest barrier, isotope effects are suppressed by commitments to catalysis. A commitment is defined as “the ratio of the rate constant for the isotopically-sensitive step to the overall rate constant for the first irreversible effect in the opposite direction from this step”, e.g. the release of substrate or product or a chemically irreversible step (Schramm \textit{et al.}, 1999). Hence, there are both ‘forward’ or ‘reverse’ commitments to catalysis. Forward commitments precede the isotopically-sensitive step, reverse commitments follow it.

The presence of a forward commitment in a reaction represents a tendency for the ES species to go on to react to form product, rather than dissociate to form precursors. As the forward commitment to a reaction increases from negligible to dominant, observed KIEs will vary between intrinsic and unity, i.e., for a reaction that always proceeds to product formation following substrate binding, no KIE will be observed. Reactions that are energetically reversible often exhibit reverse commitments, in which enzyme-bound products revert to form enzyme-bound substrate before product release occurs. Observed KIEs in the presence of reverse commitments can vary in value between the intrinsic KIE and the equilibrium effect that the heavier isotope has on the reaction (Kyte, 1995). The full isotope effect will only be seen if both commitments are small. Unfortunately, this almost never occurs.
and commitments have to be determined experimentally (Klinman, 1978; Northrop et al., 1981; Kyte, 1995) or removed by changing experimental conditions (Bahnson et al., 1993). However, it should be noted that techniques used to determine intrinsic KIEs may not be applicable in the presence of quantum tunnelling of hydrogen.

1.3.2.5 Quantum Tunnelling of Hydrogen

The main phenomenon that increases predicted values for KIEs is the tunnel effect (Bell, 1980; Atkins, 1982). Tunnelling arises from a consideration of quantum mechanics in which particles have wave-like properties. This allows particles to penetrate into areas that are forbidden by classical physics i.e. ‘tunnelling’ through a potential energy barrier. Every particle possesses a finite probability that it can appear on the other side of a potential energy barrier without possessing the energy that is required to surmount the barrier based on classical physics. The probability that a particle will be able to tunnel is greatly influenced by its size. Using a rectangular potential energy barrier model, the tunnelling probability, \( P_{\text{tunnel}} \), is related to the mass of the tunnelling particle by the following relationship:

\[
P_{\text{tunnel}} \propto \exp \left( -\frac{2S}{h} \right)
\]

Equation 1.15

\( S \) is the Wentzel-Kramers-Brillouin (WKB) action (Miller et al., 1986), and can be understood in terms of the ‘resistance’ to barrier penetration. It is related to the mass of the transferred atom and the potential energy barrier between reactant and product states. This occurs in such a manner that the transmission probability decreases exponentially with the width of the barrier, and with increasing particle mass. Hence, particles with low mass are more likely to be able to tunnel through barriers than heavier particles and particles are more likely to tunnel through thin barriers.

Electron tunnelling is already a well-established phenomenon in biology and has been shown to occur over large distances, up to 25 Å (Section 1.3.1). In terms of particle size, hydrogen is the second most likely particle to tunnel after the electron, during a chemical reaction. The mass of the H nucleus is 1840 times larger than that of an electron, which means that the probability of an electron tunnelling up to 25 Å is equivalent to the probability that protium will tunnel up to a distance of 0.58 Å. The latter distance is similar to the length of a reaction co-ordinate (Sutcliffe & Scrutton, 2000). The larger masses of deuterium and tritium lead to transfer distances of 0.41 Å and 0.34 Å, respectively.
1.3.2.6 Detection of H-Tunnelling using KIEs and Temperature Dependence

1.3.2.6.1 Kinetic Isotope Effects as a Probe of Tunnelling

The size of the KIE has been a useful probe for tunnelling in a chemical reaction, since a $k_{\text{H}}/k_{\text{D}}$ value greater than 7 (the classical theoretical upper limit; Equation 1.14) provides evidence for tunnelling (Bell, 1980). It has also been shown that, in the absence of tunnelling, $k_{\text{H}}/k_{\text{D}}$ and $k_{\text{H}}/k_{\text{T}}$ are related by the Swain-Schaad relationship (Equation 1.16; Swain, 1958):

$$\left(\frac{k_{\text{H}}}{k_{\text{D}}}\right)^{1.44} = \frac{k_{\text{H}}}{k_{\text{T}}}$$  \hspace{1cm} \text{Equation 1.16}

The Swain-Schaad relationship results in the TST placing an upper limit of $\sim 16.5$ for the tritium isotope effect. Saunders (1985) demonstrated an important extension of the Swain-Schaad relationship from which they devised a relationship between the two isotope effects $k_{\text{H}}/k_{\text{D}}$ and $k_{\text{H}}/k_{\text{T}}$ in terms of reduced-mass considerations Equation 1.17:

$$\left(\frac{k_{\text{H}}}{k_{\text{T}}}\right)^{3.26} = \frac{k_{\text{H}}}{k_{\text{T}}} \hspace{1cm} \text{Equation 1.17}$$

This relationship differs from the Swain-Schaad relationship in that tritium, and not protium, is the common atom connecting both sides of the equation. Equation 1.17 has led to the development of so-called ‘competitive labelling’ experiments to detect tunnelling (Bahnson & Klinman, 1995) in which the H/T and D/T isotope effects on a reaction are measured in parallel (Cha et al., 1989). In a protium versus tritium experiment, the experimentally measured value for H/T $[(k_{\text{H}}/k_{\text{T}})_{\text{obs}}]$ is determined. In a parallel experiment, the D/T effects for the reaction are measured and used to calculate a value for the H/T effects $[(k_{\text{H}}/k_{\text{T}})_{\text{calc}}]$. If H-tunnelling is a feature of a reaction, $(k_{\text{H}}/k_{\text{T}})_{\text{obs}}$ will be greater than the semi-classical limits, and a comparison of $(k_{\text{H}}/k_{\text{T}})_{\text{obs}}$ to $(k_{\text{H}}/k_{\text{T}})_{\text{calc}}$ yields the following inequality:

$$\left(\frac{k_{\text{H}}}{k_{\text{T}}}\right)^{3.26} = (k_{\text{H}}/k_{\text{T}})_{\text{calc}} < (k_{\text{H}}/k_{\text{T}})_{\text{obs}} \hspace{1cm} \text{Equation 1.18}$$

This relationship has been used to reveal H-tunnelling in enzyme-catalysed H-transfers (Cha et al., 1989; Klinman et al., 1989; Bahnson & Klinman, 1995).

1.3.2.6.2 Temperature Dependence Studies as a Probe of Tunnelling

Another common probe of tunnelling can be derived from studies of the temperature dependencies of reaction rates (Bell, 1980). The Arrhenius equation is normally used to analyse the temperature dependence of a reaction, but is only useful over a limited range of temperature:
In Equation 1.19, \( A \) is the pre-exponential factor and \( \Delta E_a \) is the apparent activation energy of the reaction. Reaction rates are measured over a range of temperatures and plotted as \( \ln k \) versus \( 1/T \), enabling the activation energy of the reaction to be calculated from the slope of such a plot, \((\Delta E_a/R)\); \( A \) can be calculated from the point of intersection on the ordinate axis. Tunnelling is investigated by carrying out the temperature dependence of an enzyme catalysed reaction with both protium and deuterium substrates. Factors that provide evidence for tunnelling include:

(i) Curvature of Arrhenius plots (Stern, 1974; Bell, 1980). Although curvature in Arrhenius plots has been observed for some chemical systems (Brunton et al., 1976; Wang & Williams, 1972), the denaturation of proteins at high temperatures makes it impractical to study enzyme-catalysed reactions over a sufficient temperature range to detect curvature. It should also be noted that other reasons for non-linearity in Arrhenius plots can occur; e.g. temperature-induced interconversion of two forms of an enzyme (Gutfreund et al., 1995).

(ii) Ratios for the Arrhenius pre-exponential factors with protium and deuterium (\( A_H \) and \( A_D \) respectively) \( A_H:A_D < 1 \) (Bell, 1980). The link between tunnelling and its effects on the ratio of Arrhenius pre-exponential factor is illustrated in Figure 1.11.

(iii) Large differences in the activation energies between protium and deuterium transfer (Bell, 1980). The calculated maximum difference between \( \Delta E_{aH} \) and \( \Delta E_{aD} \) for classical transfer based on differences in zero-point energies is \(~5.4\) kJ-mol\(^{-1}\). Values for \( \Delta E_{aD}-\Delta E_{aH} > 5.4\) kJ-mol\(^{-1}\) would indicate that protium was being transferred by H-tunnelling.

1.3.2.7 Hydrogen Tunnelling in Enzyme Systems

H-tunnelling was not described in enzyme-catalysed H-transfer reactions until 1989. Unusually high hydrogen isotope effects had been observed in some enzyme systems prior to 1989, but had not been attributed to a tunnelling phenomenon (Klinman, 1978; Reinsch et al., 1980; Palcic et al., 1983). This was primarily due to interpretation of KIEs and Arrhenius plots in the context of the semi-classical theory and the misconception that the large mass of hydrogen prevented tunnelling (Scrutton et al., 1999). Also, tunnelling was thought to occur only at low (cryogenic) temperatures (Ringe et al., 1999). More recently studies of the variation
in KIE as a function of temperature have been used to investigate H-tunnelling in enzyme systems and this is discussed in the following section.

1.3.2.8. KIEs Measured in Enzyme Systems

H-tunnelling was first demonstrated in yeast alcohol dehydrogenase (YADH) (Cha et al., 1989). In the YADH reaction the conversion of benzyl alcohol and NAD$^+$ to benzaldehyde and NADH is rate-limiting. H-tunnelling was investigated using a competitive, double-labelling protocol (Klinman et al., 1989; Bahnson & Klinman, 1995) and the data analysed using Equation 1.18. These experiments compared rates of hydrogen-isotope transfer to NAD$^+$, from a reaction mixture containing two forms of substrate: tritiated benzyl alcohol and $[^{14}$C]-labelled benzyl alcohol (ring-labelled). Scintillation counting was used to determine the ratio between the amount of $^3$H incorporated into NAD to the amount of $[^{14}$C]-labelled benzaldehyde. Data were then analysed using Equation 1.18 and the relationship between calculated and observed isotope effects was found to be consistent with H-tunnelling. Values of $(k_{H}/k_{T})_{calc}$ were 5.91, compared to values for $(k_{H}/k_{T})_{obs}$ of 7.13, the observed value was greater indicating H-tunnelling. Secondary KIEs showed a relationship of $(k_{H}/k_{T})_{calc} = 1.11$ versus $(k_{H}/k_{T})_{obs}$ of 1.35, again conducive of H-tunnelling. The values of the pre-exponential factors calculated from the D/T and H/T isotope effects were 3.58 and 10.2, respectively, both of which are well outside the theoretical upper limit of 3.34 for isotope effects derived purely from a semi-classical consideration (Klinman et al., 1989).

Following this first demonstration of H-tunnelling in an enzyme reaction, several other enzyme systems were investigated using the same competitive labelling technique. Bovine serum amine oxidase (BSAO; Grant & Klinman, 1989) was the second example. This enzyme catalyses the conversion of amines to aldehydes and had previously been shown to have inflated isotope effects (Palcic et al., 1983). Primary H/T and D/T isotope effects determined by stopped-flow techniques were 35.8 and 3.07, respectively. The Arrhenius plot of the primary isotope effect resulted in an Arrhenius pre-exponential factor ratio, $A_{H}/A_{T}$ of 0.12, well below the semi-classical limit of 0.6 (Bell, 1980). In addition, the $A_{D}/A_{T}$ value of 0.51 showed that deuterium was also tunnelling since the semi-classical lower limit for this ratio was 0.9.
A few years later H-tunnelling was discovered in horse liver alcohol dehydrogenase (LADH), again using the competitive labelling approach (Bahnson et al., 1993). In LADH, however, the rate of oxidation of benzyl alcohol is limited by product dissociation and this commitment to catalysis masks the intrinsic KIE. Tunnelling was therefore revealed by the introduction of mutations into the enzyme, which increased or decreased the size of the alcohol binding pocket and consequently affected the rate of product dissociation. Increasing the size of the binding pocket had little effect on the kinetic complexity and produced no change in isotope effects or the exponential relationship between $k_{r}/k_{\tau}$ and $k_{r}/k_{\tau}$. However, the mutations Leu57Phe and Phe93Trp when introduced separately into the enzyme to decrease the size of the binding pocket, provided evidence of protium tunnelling; secondary KIEs became significantly elevated from 4.1, in wild-type enzyme, to 8.5 and 6.3 in the L57F and F93W mutants respectively. A temperature dependence study of the F93W mutant produced Arrhenius pre-exponential ratio $(A_{H}/A_{T})$ of 0.49, which is strongly suggestive of protium tunnelling.

H-tunnelling has also been shown to occur in monoamine oxidase B (MAOB; Jonsson et al., 1994), a flavoprotein that catalyses the oxidation of amines. Tunnelling was demonstrated using competitive labelling of the substrate $p$-methoxybenzylamine. The MAOB reaction also contained commitments to catalysis, which reduced the KIE values and the exponents relating $k_{r}/k_{\tau}$ and $k_{r}/k_{\tau}$ were also below semi-classical values. However, these commitments were shown to be constant over the temperature range and did not significantly affect the Arrhenius plot. The ratio of the Arrhenius pre-exponential factors, $A_{H}/A_{T}$ and $A_{D}/A_{T}$, had values of 0.13 and 0.52 respectively, providing evidence for H-tunnelling. As with BSAO, the MAOB reaction was also shown to involve the tunnelling of protium and deuterium.

1.3.2.9 Use of Arrhenius Plots to Provide Evidence for H-tunnelling

Jonsson et al. (1996) characterised the H-transfer reaction catalysed by soybean lipoxygenase (SBL) in the steady-state and produced the largest deuterium isotope effects reported for an enzyme system to date: $k_{H}/k_{D} = 48$ at 25 °C (Glickman et al., 1994). Temperature dependence studies of $k_{cat}$ for the steady-state oxidation of linoleic acid (LA) revealed an isotope effect of 56 at 32 °C. Arrhenius plots showed almost horizontal, parallel lines for C-H and C-D bond breakage, with enthalpies of activation of 5.0 kJ·mol$^{-1}$ and 6.7 kJ·mol$^{-1}$ for H-LA and D-LA, respectively. The ratio
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of the Arrhenius pre-exponential factor showed a large deviation from classical behaviour: $A_H/A_D = 27$ in the steady-state and 50 using rapid mixing techniques.

New criteria were developed for interpreting Arrhenius plots based on the behaviour of SBL and other enzymes (Jonssen et al., 1996). The authors proposed a description of a thermally activated process, where deviations from Arrhenius behaviour arise under conditions of activationless quantum mechanical behaviour. This kind of behaviour is difficult to observe in enzyme systems, due to the limited range of temperature available for studying enzyme reactions. However, the authors proposed a classification of the Arrhenius plots for a light and heavy isotopes by defining four regions or regimes based on the extent of H/D tunnelling (Figure 1.11). Region I corresponds to classical behaviour, with large enthalpies of activation and an extrapolated value for $A_H/A_D = 1$. Region II represents moderate protium tunnelling as seen for BSAO and MAOB, in which isotope effects may be inflated and extrapolated Arrhenius prefactor ratios become less than unity; this reflects a higher degree of tunnelling, and produces curvature in the Arrhenius plot for the lighter isotope. Extending the Arrhenius plots to temperature ranges where significant tunnelling of all isotopes of hydrogen occurs, leads to a process that is nearly activationless for both H and D transfer (Region IV). These horizontal Arrhenius plots (Region IV) predict enthalpies of activation close to zero and may result in large isotope effects, which are close to the Arrhenius prefactor ratios. This is the situation observed for the SBL reaction. This description of the Arrhenius plots (Figure 1.11) predicts that systems will be found that operate in regions II, III and IV and that the enthalpy of activation, $\Delta H^\ddagger$, will decrease as tunnelling becomes more prominent.
Figure 1.11. (a) The different regions of an Arrhenius plot based on the extent of tunnelling (Jonsson et al., 1996) (b) Static barrier model of H-tunnelling. Regime I, classical over-the-barrier transfer, regimes II and III, moderate tunnelling of protium and minor tunnelling of deuterium. Regime IV, pure quantum tunnelling of protium and deuterium.
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Region III, represents intermediate tunnelling behaviour, and would have properties that are hard to predict as small changes in rate could lead to values of $A_H/A_D$ which are either normal or inverse. Studies of glucose oxidase suggested that it may operate in Region III, as small changes in $k_{cat}$ for this enzyme correspond to a significant shift in the value of $A_H/A_D$ from inverse to well above unity (Kohen et al., 1997). Additional evidence for a decrease in the enthalpy of activation ($\Delta H^f$) as tunnelling becomes more prominent, is seen among the enzyme systems previously mentioned and studied by Klinman and co-workers (BSAO, MAO-B, GO and SBL), where a change in Arrhenius prefactor ratios from inverse values to large values corresponds to a decrease in $\Delta H^f$.

1.3.2.10 Evidence for Hydrogen Tunnelling Driven by Protein Dynamics

The relationship between the Arrhenius plots and the extent of tunnelling illustrated in Figure 1.11 presented a description with which to interpret temperature-dependence data. One of the predictions from the plot, that $\Delta H^f$ decreases to zero as tunnelling becomes more prominent, appeared to apply to the enzyme systems studied so far. However, recent work on the reaction catalysed by methylamine dehydrogenase (MADH) from *Methylophilus methylotrophus* (Basran et al., 1999), demonstrated that this relationship no longer holds. MADH catalyses the oxidative demethylation of methylamine to formaldehyde and ammonia, involving the cofactor tryptophan tryptophylquinone (TTQ). The reduction of TTQ can be measured by stopped-flow spectroscopy and provides a direct measurement of the rate of C-H bond cleavage. Temperature dependence studies of the MADH reaction revealed a large, temperature independent KIE of $\sim 17$. Temperature dependence plots for protiated and deuterated methylamine were parallel but not horizontal (Figure 1.12), so that the prefactor ratio, $A_H/A_D$ was identical to the KIE value for the reaction. These results indicated that extensive tunnelling of both protium and deuterium occurred in the cleavage of the substrate C-H and C-D bond. Therefore, MADH was shown to be operating in Region IV (Figure 1.11). No difference in activation energy between the two isotopes was observed, so tunnelling of protium and deuterium was occurring from the ground state.
Figure 1.12. Temperature dependence and KIE data for MADH. Temperature dependence plot for MADH with methylamine (filled circles) and deuterated methylamine (open circles). Note the parallel plots for C-H and C-D bond breakage. Inset: Plot of ln KIE vs. 1/T showing no change in KIE over the temperature range studied. Data taken from Basran et al. (1999).
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In the description developed by Jonsson and co-workers (1996), the reaction rates should be independent of temperature in region IV (Figure 1.11). However, the plots for MADH clearly indicated that the reactions were strongly temperature dependent, requiring an activation energy of approximately 45 kJ·mol⁻¹ for both protiated and deuterated substrate. The observed activation energy for MADH could only be explained by invoking a fluctuating, temperature-dependent potential energy surface that drives proton abstraction by quantum mechanical tunnelling of hydrogen (Basran et al., 1999). The source of these fluctuations is from the protein itself due to the intrinsic ‘breathing’ of the enzyme molecule (Scrutton et al., 1999).

Following the work on MADH a role for protein dynamics was also demonstrated for the reaction catalysed by thermophilic alcohol dehydrogenase (ADH-hT) from Bacillus stearothermophilus strain LLD-R (Kohen et al., 1999). The optimum temperature for the ADH-hT reaction is 65°C, which allows temperature dependence studies to be carried out over an extensive temperature range. Kohen et al. (1999) studied hydrogen tunnelling as a function of temperature in ADH-hT, examining how protein mobility affected the contribution that hydrogen tunnelling made to the reaction rate. They proposed that if protein mobility was important, a decreased contribution of tunnelling to the reaction rate would be expected, due to decreased protein motility at lower temperatures. This would be different to descriptions of H-tunnelling through a rigid barrier, which predicted an increased contribution of tunnelling to the reaction rate with decreased temperature (Goldanskii et al., 1979).

The temperature dependence of the ADH-hT-catalysed reaction was studied using the same approach described for YADH (Cha et al., 1989). The contribution of tunnelling to the reaction rate indicated by the value of the exponent relating $k_H/k_D$ and $k_O/k_T$, was found to decrease as the temperature fell below 30°C. KIEs were shown to be essentially temperature independent between 65 and 30°C, but the value was found to rise steeply below 30°C. This indicated that a physical change in the behaviour of ADH-hT at temperatures below 30°C was affecting the Arrhenius plots for both hydrogen and deuterium transfer. Between 65°C and 30°C, the plots for protium and deuterium substrates were almost parallel at elevated temperatures, with $A_H/A_D >1$. Below 30°C, the slopes for protium and deuterium substrates diverge, with increased values for enthalpy of activation and $A_H/A_D <1$. The source of the decreased tunnelling contribution in ADH-hT at 30°C and below was attributed to a
temperature-dependent transition in the behaviour of the protein which impairs H-tunnelling (Kohen et al., 1999).

As with MADH (Basran et al., 1999; Scrutton et al., 1999), in the physiological temperature range for ADH-hT (30-65°C), the $\Delta H^\ddagger$ for the deuterated substrate was equivalent to that for the protium substrate and thus values for $\Delta H^\ddagger$ were comparatively large ~ 21 kJ·mol$^{-1}$. Consequently, tunnelling from the ground state of reactants was shown to require an activation energy, which can only be attributed to thermally activated motions of the protein scaffold.

1.3.2.11 Development of a Description of H-tunnelling Involving Protein Dynamics

TST proposes that the rate of an enzyme catalysed reaction is governed by the height of a static potential energy barrier, and that an enzyme increases the reaction rate by stabilising a transition state and effectively lowering the height of the barrier. H-tunnelling proposes an alternative where the rate of a reaction is determined by barrier width and not barrier height (Bruno & Bialeck, 1992). Early investigations of H-tunnelling were interpreted using semi-classical approaches (Jonssen et al., 1996) where large KIEs were used as evidence for H-tunnelling. In these systems, tunnelling could only occur when there was enough energy for reactants to progress up a potential energy barrier, to a point where the barrier was narrow enough for tunnelling to occur. For MADH and ADH-hT, H-tunnelling occurs from the ground state of the reactants, but these systems still require an input of energy as their reactions are temperature-dependent (Basran et al., 1999; Kohen et al., 1999). This paradox was explained by including thermal fluctuations of the protein in the description of H-tunnelling. The thermal excitation of the protein causes an increased frequency of vibrations, which can explain how enzymes reduce barrier width in a reaction, and why there is a requirement for activation energy in a reaction occurring from the ground state. Protein dynamics reduce barrier width by reducing the distances between donor and acceptor atoms, and the apparent enthalpy of activation is actually the energy required to produce a configuration in the protein structure that is conducive for H-transfer to occur by quantum mechanical tunnelling (Scrutton et al., 1999).

Molecular dynamics studies have proposed a dynamic role for the protein molecule in enzymatic H-tunnelling (Bala et al., 1996; Hwang et al., 1991; Hwang &
Warshel, 1996), and some theoretical studies have recognised the role of thermal motion in H-tunnelling (Sumi & Ulstrup, 1988; Dognadze et al., 1977; Bruno & Bialek, 1992; Benkovic & Bruice, 2000). Thus through experimental and theoretical studies the use of TST in enzyme catalysis has been brought into question owing to the importance of protein dynamics (Gavish et al., 1986; Gutfreund et al., 1995; Scrutton et al., 1999). Kramer’s view of catalysis is thus more appropriate, in which Brownian motion ‘energises’ the reactants to proceed over a potential energy barrier. As we have seen, Kramer’s ideas are readily extended to theories of enzymatic H-tunnelling.

1.3.2.12 Vibrationally Enhanced Ground State Tunnelling (VEGST)

The only theoretical treatment to explicitly recognise the role of protein dynamics and relate this to the observed KIE was described by Bruno and Bialek (1992). As explained by Scrutton et al. (1999), the data for the MADH-catalysed reaction fits to the model of ‘vibrationally enhanced tunnelling’, developed by Bruno and Bialek (1992). This theory has been termed vibrationally enhanced ground state tunnelling theory (VEGST) (Bruno & Bialeck, 1992; Figure 1.13). A prediction of this theory is that H-tunnelling can occur even when the value of the H/D KIE falls within semi-classical limits (i.e. <7), indicating that KIEs might be poor indicators of quantum tunnelling in enzymes.

Bruno and Bialek analysed the data for the BSAO reaction (Grant et al., 1989) using VEGST and found it to be consistent with, but not a verification of, VEGST theory. In VEGST theory, thermal fluctuations of the protein reduce the distance the hydrogen must tunnel, and the tunnelling probability is decreased when heavier isotopes are transferred giving rise to a KIE > 1. Tunnelling is assumed to occur from the vibrational ground states as vibrational quanta are comparable to barrier height and the relationship between the KIE and temperature is described by an expression that predicts a parabolic dependence on temperature.

VEGST can be used to describe the MADH reaction, but it does not apply to reactions where tunnelling occurs just below the saddlepoint of a potential energy barrier to a reaction. What it does provide is a starting point from which qualitative descriptions for the role of protein dynamics in H-tunnelling can be derived. Such descriptions have been provided by Scrutton et al. (1999); Sutcliffe & Scrutton (2000) and Kohen et al. (1999). In their descriptions, H-transfer from reactant to
product states is treated as a double-well system across the reaction co-ordinate, with a potential energy barrier in between (Figure 1.13). Tunnelling occurs when the probability distribution for a particle in the reactant well overlaps with the probability distribution of finding a particle in the product well. The extent of tunnelling between the two bound states is determined by the shape of the reactant and product wells and the barrier. The thermally induced breathing of the protein molecule may pass the nuclear geometry through the reactant and product intersection many times before H-tunnelling occurs. If the potential surface is rigid, and tunnelling takes place between two vibrational ground states of equal energy, the particle will simply tunnel back and forth between the reactant and product wells (oscillate coherently). To obtain a net reaction rate coherency has to be destroyed by achieving a fluctuation in the system that alters the relative energies of reactant and product (i.e. by coupling to other degrees of freedom in the system). Fast destruction of coherence leads to a well-defined transfer rate.

There are two types of fluctuation which will effect the tunnelling probability; one influences the symmetry of the system by equalising the energy states between reactant and product, and the other brings the two wells close enough together for tunnelling to occur. The latter is essential because bound substrates are often too far apart to support tunnelling, e.g. in YADH a H-transfer distance of 3.4 to 3.6 Å is indicated from the crystal structure of the enzyme. The temperature dependence of KIEs is a result of the degree of coupling of the symmetric vibration to the reaction co-ordinate as only the distance between donor and acceptor is sensitive to isotopic labelling. H-tunnelling is improved in enzymes which have evolved to stabilise the "transition state", and also those which bring the donor and acceptor atoms closer together and alter the energy levels of reactant and product. As recent studies have verified the use of VEGST theory in H-tunnelling, more work will need to be carried out to explore whether ground state tunnelling can be observed in other systems. This dynamic barrier approach to enzyme catalysis has major implications for how H-transfer and other reactions are treated and may affect the application of the TST to any form of enzyme catalysis (Kohen et al., 1999).
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Figure 1.13. Vibrationally Enhanced Ground State Tunnelling. (A) Reactant and Product curve to illustrate the origin of activation energy seen in ground state tunnelling. (B) Predicted temperature dependence seen in tunnelling of protium and deuterium from the ground state (Regime IV).
1.4 Aims

The work presented in this thesis explores different aspects of the enzymology of heterotetrameric sarcosine oxidase from *Arthrobacter* sp. 1-IN. The first task was to sequence the genes and develop an expression and purification protocol for the enzyme (Chapter 3). With this goal achieved a more general characterisation of TSOX and comparison with similar enzyme systems was performed. As the reaction catalysed by TSOX was poorly understood and little work had been performed by transient kinetic methods, stopped-flow approaches were used to investigate the reductive half-reaction (i.e. reduction of the flavin by substrate; Chapter 4) and also to explore the mechanism of inter-protein electron transfer between the flavins (Chapter 5). Owing to the recent findings that H-transfer in MADH occurs by quantum tunnelling from the substrate ground state, evidence for a similar mechanism of H-transfer in TSOX was sought (Chapter 4). To facilitate studies of interflavin electron transfer in TSOX the redox potentials were determined by anaerobic reduction and oxidation of TSOX, in the presence of redox mediators using facilities in the Department of Chemistry at the University of Edinburgh (Chapter 6). The redox potential measurements in conjunction with pH-jump experiments indicated unexpectedly that interflavin electron transfer in TSOX occurs by endergonic electron tunnelling in the physiological direction. This analysis provides rare (if not unique) evidence for the existence of physiological, endergonic electron tunnelling regimes in enzyme molecules.
Chapter 2

Materials and Methods
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2.1 Materials

2.1.1 Chemicals and Reagents

α-dianisidine, 5-bromo-4-chloro-3-indolyl-β-galactoside (x-gal), isopropyl-β-D-thiogalactopyranoside (IPTG), sarcosine, creatine, creatinine, N,N-dimethylglycine, ampicillin, NAD⁺, FAD, FMN, D₂O and horseradish peroxidase were from Sigma chemical company. N-methyl-D₃-glycine HCl (perdeuterated sarcosine) was from CK Gas Products Ltd. and ‘Timentin’ (15:1 w/w ticarcillin sodium:potassium clavulanate) was from Beecham Research Laboratories. Sodium dodecyl sulphate (SDS) was from BDH chemicals and ethidium bromide was from Bachem. ‘Protogel’ (37.5:1 w/v acrylamide: bisacrylamide) was from National Diagnostics. [³⁵S]dATP-αS triethylammonium salt for DNA sequencing (1000 Ci/mmol) was supplied by Amersham Life Science and Pharmacia supplied the T7 DNA sequencing kit. Ultra pure agarose was purchased from Life Technologies Inc. and low melting point agarose was obtained from Sigma. All other chemicals were supplied by Sigma-Aldrich, Fisher or BDH and were of analytical grade wherever possible. Water used was glass-distilled and deionised.

2.1.2 Bacterial Strains and Media

Arthrobacter sp. 1-IN was a kind gift from Dr Rolandas Meškys (Laboratory of Bioanalysis, Institute of Biochemistry, Mokslininku, Vilnius, Lithuania). Bacterial E. coli strains employed were JM109 (Stratagene; r⁻, m⁺, rec A1, sup E, end A1, hsd R17, gyr A96, rel A1, thi, (lac-pro AB)/F' [tra D36, pro A'B⁺, lac I', lac Z M15]), JM109 DE3 (Promega; r⁻, m⁺, rec A1, sup E44, end A1, hsd R17, gyr A96, rel A1, thi, (lac-pro AB)/F' tra D36, proA'B⁺, lac I', lac Z M15, (DE3)), BL21 DE3 (Stratagene; E.coli BF' dcm ompT hsdS (r⁻m⁻) galA (DE3)), DH5α (Stratagene; end A1 hsd R17 (r⁺ m⁻) sup E44 thi-1 rec A1 gyr A (Nal') rel A1 (lac IZYA- arg F) U169 deo R (φ80 d lac (lac Z) M15) and TG1 (Amersham; sup E, hsd D5, thi, (lac-pro AB) F'[tra D36, pro A'B⁺, lac I', lac Z M15]).

E.coli strains were grown in 2x YT media, which contains, per litre: 16 g tryptone, 10 g yeast extract and 5 g NaCl. Solid media used was LB (Luria-Bertani) medium which contains, per litre: 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g bacteriological agar. All media components were of laboratory standard supplied by OXOID Ltd., Basingstoke, Hampshire. Solid media was produced by the addition of
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1.5 % bactoagar (w/v) to the liquid media. Top agar was 0.7 % bactoagar in water. Transformed bacteria were grown in 2xYT media supplemented with 50 μg/ml ampicillin. Glycerol stocks were made by adding 0.5 ml of a late exponential phase culture to 0.5 ml sterile glycerol. The stocks were stored at -20°C or -70°C.

Electroporation of *E. coli* cells required the use of SOC media (20 g bacto-tryptone, 5 g yeast extract and 1.8 g glucose per litre). *E. coli* strain TG1 was maintained on M9-glucose minimal media plates (6 g anhydrous Na₂HPO₄, 3 g KH₂PO₄, 1 g NH₄Cl and 0.5 g NaCl per litre), supplemented with 1 mM MgSO₄, 1 mM thiamine HCl, 0.1 mM CaCl₂ and 0.2% glucose.

*Arthrobacter sp.* 1-IN was grown on various liquid media (Meškys et al., 1996); SMT1 consisted of (g/l): sarcosine (5), Na₂HPO₄ (5.24), KH₂PO₄ (2.77), MgSO₄.7H₂O (0.25); SMT2 medium was made by adding 2 g yeast extract and 2 g NH₄Cl to 1 l SMT1 medium. KMT media was the same as SMT2 media except that creatinine was used instead of sarcosine. GMT medium consisted of (g/l): glycerol (8), NH₄Cl (2), yeast extract (2), KH₂PO₄ (1), K₂HPO₄ (2), MgSO₄.7H₂O (0.25). All media were autoclaved before use; concentrated solutions of the substrates sarcosine and creatinine were adjusted to pH 7 before being filtered using a 0.2 μm filter into the medium. Indicator plates were prepared by adding 10 ml horse radish peroxidase solution (400U/ml) and 10 ml o-dianisidine solution (10 mg/ml) to 1 l of precooled <45°C SMT1 medium containing 15 g agar/l.

The pXOR9 plasmid construct containing the cloned *sox* genes was a kind gift from Dr Rolandas Meškys (Laboratory of Bioanalysis, Institute of Biochemistry, Mokslininku, Vilnius, Lithuania) and contained a 12.5 kb genomic DNA fragment cloned into the pTZ18R plasmid vector using the Hin dIII and Xba I restriction sites (Figure 2.1).

2.1.3 DNA Modifying Enzymes

Restriction endonucleases *Eco RI*, *Hin dIII*, *Kpn I*, *Sma I*, *Sac I*, *Pst I*, *Xba I*, *Nco I*, and *Bam HI* were supplied by Pharmacia and Amersham Life Sciences. T4 DNA ligase and calf-intestinal alkaline phosphatase were obtained from Pharmacia, and T4 polynucleotide kinase was from Amersham Life Sciences.
2.1.4 Chromatographic Media and Membranes

Pre-swollen microgranular diethylaminoethyl cellulose (DE-52) anion exchange media was from Whatman Biosystems Ltd. Phenyl sepharose® high performance hydrophobic interaction media, Q sepharose, hydroxyapatite media and EAH sepharose-4B activated affinity resin were obtained from Pharmacia. For N-terminal sequencing, Problott™ membrane was supplied by Applied Biosystems Inc. Cellulose 100 kDa diaflo ultrafiltration membranes were supplied by Orange Scientific. PM30 Diaflo ultrafiltration membranes, Centricon 10 and Centricon 100 ultrafiltrators were supplied by Amicon Inc. Visking dialysis tubing (width 2.2 cm, bore 1.43 cm, molecular weight cut-off 12,000-14,000 Da) was obtained from Medicell International Ltd.

2.1.5 Oligonucleotides

Oligonucleotides were synthesised by the Protein and Nucleic Acid Laboratory (PNACL), University of Leicester.
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Figure 2.1. Plasmid pXOR9 as supplied by Lithuanian collaborators. The plasmid consists of a 12.5 kb fragment of *Athrobacter sp.* 1-IN genomic DNA known to contain the sox genes cloned into the plasmid vector pTZ18R.
2.2 Molecular Biology Methods

2.2.1 DNA Purification

For small-scale plasmid DNA isolation (miniprep), a single *E. coli* colony was picked from an agar plate and used to inoculate 5 ml or 10 ml of 2x YT medium, containing 50 μg/ml ampicillin. For large-scale plasmid isolation (maxiprep), 5 ml of an overnight culture was used to inoculate 500 ml of 2x YT medium containing 50 μg/litre ampicillin. Cultures were grown overnight at 37°C with vigorous shaking. Minipreps were carried out using the Wizard DNA Purification kit (supplied by Promega) or the Hybaid Recovery system. Maxipreps were carried out using the Wizard DNA Purification kit supplied by Promega. Purification of DNA isolated from low melting point agarose gels was carried out using the PCR Purification kit supplied by Promega.

2.2.2 Transformation of *E. coli* Cells with DNA

Competent cells were prepared using a modified version of the 'rubidium chloride' method described by Sambrook *et al.*, (1989) and kindly supplied by Dr J. Basran, University of Leicester. Competent cells were removed from -70°C storage, and allowed to thaw on ice. Plasmid DNA (approximately 1-5 μg in 5-10 μl) was introduced into the cell suspension with gentle mixing, and the cells left on ice for 30 min. The cells were then heat-shocked at 42°C for 90 s before being returned to ice for 2 min. One ml of 2xYT media was then added to the cells, which were then incubated at 37°C for 1 h with gentle shaking. Aliquots of 10 μl to 200 μl cells were plated on to LB agar plates containing 50 μg/ml ampicillin. The plates were allowed to stand on the bench for 15 min and were then inverted and incubated at 37°C overnight.

Cells for electroporation were prepared from a 1 l culture of untransformed *E. coli* cells grown on 2x YT media in a shaking incubator at 37°C until an optical density at 600 nm of 0.6 was reached. The cells were then harvested in a cold rotor at 5000 g for 15 min. the supernatant was discarded and the cells were resuspended in 500 ml of sterile, ice-cold 10% glycerol solution. The cells were repelleted by centrifugation as described. Further resuspensions and centrifugations were performed with volumes of 400 ml and then 20 ml 10% glycerol and the cell pellet
was finally resuspended in 2-3 ml of ice cold 10% glycerol solution. The cell suspension was aliquoted (200 µl) and snap-frozen.

Electroporation and preparation of recipient cells was carried out according to the method of Heery & Dunican (1989). An E. coli Pulser (Bio-Rad Labs) was employed using 0.2 cm gap disposable cuvettes. The electroporation-competent cells were pulsed at 2.0 kV with a time-constant of 5 ms.

2.2.3 Enzymic DNA Modifications

Phosphorylation of the 5' end of DNA fragments was achieved using T4 polynucleotide kinase. After incubation for 30 min at 37°C, the reaction was terminated by heat inactivation (65°C for 10 min). The terminal 5' phosphate of DNA fragments was removed by treatment with calf intestinal alkaline phosphatase. The reaction was terminated after 1 h by extracting the protein using phenol/chloroform (1:1 v/v). For DNA ligation, the reaction mixture contained alkaline phosphatase-treated vector DNA, the fragment DNA to be cloned into the vector and T4 DNA ligase; this was then incubated for 18 h at 16°C.

Reactions were carried out using the buffers and instructions supplied with each enzyme. DNA restriction digests were carried out using 1-10 µg of DNA, 1x appropriate enzyme buffer, made up to a final volume of 25 µl with sterile water. The reaction was incubated at the manufacturers recommended temperature (usually 37°C) for 3-4 h, then terminated by heat inactivation (65°C, 5 min) if necessary.

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out according to the method of Sambrook et al., (1989). Agarose gels (0.8% w/v, 11 cm x 12 cm) containing 0.6 µg/ml ethidium bromide were cast, and electrophoresis was performed in 1x TAE buffer (50x TAE buffer is 242 g Tris base, 18.6 g EDTA and 57.1 ml glacial acetic acid in 1 litre), at 150 V for 40 min. Samples (20-25 µl) were loaded onto the gel in 1x loading buffer (6x loading buffer is composed of 0.25% bromophenol blue, 0.25% xylene cyanol and 30% v/v glycerol in water). DNA markers (1 kbp ladder) were also loaded onto the gel. Band sizes were (in base pairs): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, and 500. DNA was visualised by exposure to long wavelength UV light on a UVP transilluminator.
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2.2.5 DNA Sequencing

Dideoxynucleotide DNA sequencing (Sanger et al., 1980) was used to sequence the sox genes and was carried out both manually, using the T7 system supplied by Pharmacia, and via automated sequencing using the facility at the Protein and Nucleic Acid Laboratory, University of Leicester.

2.2.5.1 Manual Sequencing

When sequencing plasmid DNA, template denaturation was accomplished by the addition of 8 µl of freshly made 2 M NaOH to about 2 µg of DNA contained in 32 µl water or TE buffer. Primer (4 µl of a 3.0 OD\textsubscript{260} nm/ml stock) was added and the mixture incubated at 37°C for 30 min. Neutralisation was carried out with 7 µl of 3 M potassium acetate, pH 4.8. The template DNA was precipitated by addition of 150 µl ethanol and then placed on dry ice for 15 min. Following microcentrifugation (15 min), the pelleted, annealed template-primer material was air-dried and resuspended in appropriately diluted manufacturer's annealing buffer. The manufacturer's instructions were observed from the labelling stage of the procedure onwards.

Completed sequencing reactions were subjected to PAGE through a 40 cm wedge gel comprising of 13.3 ml 'Protogel' acrylamide mix, 25 ml 46.7% (w/v) urea, 5 ml 10x TBE buffer (108 g Tris HCl, 55 g boric acid and 10 g disodium EDTA per litre, pH 8.2) made up to 50 ml with water. After filtering and degassing, polymerization was initiated by the addition of 150 µl 10% ammonium persulfate (APS) and 70 µl N, N', N'-tetramethylethylenediamine (TEMED). The mixture was injected between taped and clamped sequencing plates using a 50 ml syringe. The flat end of a shark tooth-comb was placed at the top of the gel and it was left to polymerise for several hours. Before the sequencing samples were loaded onto the gel, the tape was removed from the bottom of the sequencing plates, and the gel was pre-run for about 1 h in 0.5 x TBE buffer, at 35 mA/1500 V. The shark tooth-comb was inverted and the sequencing samples loaded onto the gel. Gels were typically run for 3 h at 35 mA/1200 V. The gel was then transferred to 3M paper (Whatman), fixed by washing with 10% methanol:10% acetic acid and dried under vacuum on a gel dryer at 80°C for 2 h. X-ray film was exposed to the gel and the film was left to develop for 1-3 days.
2.2.5.2 Automated Sequencing

A large proportion of the 7.5 kb DNA fragment (containing sox genes) was sequenced by automated fluorescent sequencing. This was performed by Mr S. Bayliss on an Applied Biosystems 355 machine with the primers listed below in Table 2.1. The reverse and universal primers were purchased from Pharmacia; all other oligonucleotide primers were synthesised at the Protein and Nucleic Acid Laboratory, University of Leicester. Prior to sequencing, oligonucleotides were purified by ethanol precipitation (Sambrook et al., 1989), and their concentration and purity determined by measuring absorbance at 260 nm and 280 nm.
Figure 2.2. Diagram to show the approximate location and orientation of primers within the 7.5 kb genomic DNA fragment containing the sox genes. The DNA primer sequences and other primer information is given in Table 2.2
### Table 2.1. Sequences of the oligonucleotide primers used to sequence the sox genes.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence 5'-3'</th>
<th>Tm (°C)</th>
<th>%G+C</th>
<th>Size (bases)</th>
</tr>
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<tbody>
<tr>
<td>HF1</td>
<td>CCCCCAATTTTTGGCAGGAG</td>
<td>55.8</td>
<td>60.0</td>
<td>20</td>
</tr>
<tr>
<td>HF2</td>
<td>TTCAGATGTCTGGGCGGAA</td>
<td>53.7</td>
<td>55.0</td>
<td>20</td>
</tr>
<tr>
<td>HF3</td>
<td>GCAGCTCTTGGCGAGCGACCA</td>
<td>57.8</td>
<td>65.0</td>
<td>20</td>
</tr>
<tr>
<td>HF4</td>
<td>CGGGTCGAAACTAGCAGAT</td>
<td>51.7</td>
<td>50.0</td>
<td>20</td>
</tr>
<tr>
<td>HF5</td>
<td>GGAAGGACATGAAACTTGAC</td>
<td>49.6</td>
<td>45.0</td>
<td>20</td>
</tr>
<tr>
<td>HF6</td>
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<td>20</td>
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<tr>
<td>HF9</td>
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<td>55.3</td>
<td>63.2</td>
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<tr>
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<td>64.7</td>
<td>17</td>
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<td>60.0</td>
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<tr>
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<td>60</td>
<td>20</td>
</tr>
<tr>
<td>B6</td>
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<td>20</td>
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<td>48.8</td>
<td>47.4</td>
<td>19</td>
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</tbody>
</table>
2.2.6 Construction of Expression Clone

Over-expression of the *sox* genes was achieved by sub-cloning the whole *sox* operon into the expression vector pET-11d. In this vector the *sox* genes are under the control of the T7 bacteriophage promoter. The *sox* operon had been isolated as a 12 kb fragment which was cloned into the plasmid pTZ18R (Meškys et al., 1995). The operon was released from plasmid pTZ18R by digestion with the restriction enzymes *Hin* dIII and *Kpn* I to produce a 7.5 kb *sox* operon - containing DNA fragment. Information obtained from sequencing data revealed the presence of two *Nco* I sites in the *sox* operon. These *Nco* I sites were located at the position of the initiating methionine in the first gene of the operon (*sox* B) and at the beginning of the *sox* D gene (the next gene in the operon). The 7.5 kb *Hin* dIII/*Kpn* I fragment was therefore partially digested with *Nco* I and the whole *sox* operon cloned into plasmid pET-11d as a 6.5 kb *Nco* I/*Hin* dIII DNA fragment. The resulting construct was designated pSOX and transformed into the *E. coli* strain BL21 (DE3) as described in Section 2.2.2.

2.3 Protein Methods

2.3.1 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS PAGE) was carried out either according to the method described by Laemmli (1970), using 12% or 15% acrylamide gels in a slab gel system. The gel consisted of a 4% stacking gel [1.33 ml 'Protogel', 1.25 ml 1 M Tris-HCl (pH 6.9), 7.26 ml water, 100 µl 10% SDS, 50 µl 10% APS and 10 µl TEMED] and a resolving gel [3.0 ml 1.5 M Tris-HCl (pH 8.8), 100 µl 10% SDS, 50 µl 10% APS and 10 µl TEMED]. The acrylamide mixes for resolving gels were as follows: 12% gel 4.8 ml 'Protogel' and 4.04 ml water; 15% gel 6.0 ml 'Protogel' and 2.84 ml water.

Crude cell extracts for PAGE were prepared as follows: 3-5 ml of cell culture was pelleted by microcentrifugation. Pellets were initially solubilised in 20 µl 8 M urea and then 20 µl of 10% SDS was added. Protein samples were added to an equal volume of 2x 'loading buffer' (20 mM DTT, 2% SDS, 250 mM Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromophenol blue) and placed in a boiling water bath for 5 min, before being loaded on to the gel. Gels were run in 1% SDS running buffer at a constant current of 35 mA until the dye front had reached the base of the gel (about 2
h). The composition of SDS running buffer was 2.9 g glycine, 6.0 g Tris and 1.0 g SDS in 1 litre. Gels were stained using Coomassie Brilliant Blue R250 (0.25% in 5:1:5 v/v solution of methanol: acetic acid: water) for approximately 30 min and then destained in 5:1:5 v/v methanol: acetic acid: water. Dr. H. J. Lee (University of Leicester) produced pre-stained protein molecular weight standards; 83, 63, 45, 29, and 14 kDa.

2.3.2 N-Terminal Sequence Analysis and Electroblotting of TSOX

N-terminal sequencing and electroblotting of TSOX was performed by Dr. K. Lilley using a 476A protein sequencer (Applied Biosystems, Warrington) in the Protein and Nucleic Acid Laboratory (PNACL), of the University of Leicester.

2.3.3 Analysis of TSOX Sequence

DNA and amino acid sequences were analysed using the Genetics Computer Group (GCG) software package (Devereux et al., 1984). Pairwise amino acid sequence alignments were made using the GAP program (Maizel et al., 1981) and multiple sequence alignment results were generated using the PILEUP program (Feng & Doolittle, 1990). The sequence database used to obtain published sequence data was Swissprot. Initial sequence similarities were identified using the BLAST sequence comparison programme on the NCBI website (Altschul et al., 1990).

2.4 Methods for Analysis of Sarcosine Oxidase

2.4.1 Sarcosine Oxidase Purification

2.4.1.1 Native Enzyme Purification

*Arthrobacter sp.* 1-IN was grown in GMT medium in a rotary shaker (110 rpm) at 30°C for 27 h. KMT medium was inoculated with this culture (initial OD<sub>595nm</sub> was 0.6) and grown at 30°C for 17 h. The cells were then harvested by centrifugation, resuspended in a minimal volume of 20 mM potassium phosphate buffer, pH 7.3 and broken by passage through a french press cell at a pressure of between 12-15 psi. The cell debris was removed by centrifugation (10,000 rpm for 20 min) and the cell extract was subjected to (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation with TSOX precipitating in the range of 35-50% saturation. The precipitate was collected by centrifugation (15,000 rpm for 30 min) and dissolved in a minimum volume of 20 mM potassium phosphate buffer, pH 7.3. The protein solution was dialysed against the same buffer overnight (5 l) and
then applied to a DE52 sepharose column (6 cm x 15 cm) equilibrated in the same buffer. The column was washed with 0.15 M KCl in the same buffer and SOX was eluted with a linear gradient of KCl from 0.15-0.6 M. The peak of enzyme activity was eluted between 0.4 and 0.45 M KCl. Active SOX containing fractions were pooled and concentrated by ultrafiltration using a 30 kDa cut-off membrane. The concentrate (10 ml) was adjusted to 3 M KCl and loaded onto a phenyl sepharose column (2.5 cm x 8 cm) equilibrated with 3 M KCl in 20 mM potassium phosphate buffer, pH 7.3. The elution of TSOX was performed with 2 M KCl in the same buffer. SOX-containing fractions were pooled and dialysed extensively against 20 mM potassium phosphate buffer, pH 7.3 and concentrated by ultrafiltration. Finally, the protein sample was applied to a hydroxyapatite column equilibrated with dialysis buffer. The column was washed 20 mM potassium phosphate buffer, pH 7.3. TSOX was eluted with 0.1 M potassium phosphate buffer, pH 7.3, dialysed against 20 mM potassium phosphate buffer, pH 7.3 and concentrated by ultrafiltration using a 30 kDa cut-off membrane. The enzyme could be stored at -20°C in this buffer without any decrease in activity upon thawing.

2.4.1.2 Recombinant Enzyme Purification

*E. coli* BL21-DE3 cells containing the plasmid pSOX were grown in 2x YT media (10 l) containing 50 µg/ml timentin at 21°C until the stationary phase of growth was reached ~48 h. The purification of the recombinant enzyme was as for the native enzyme up until the phenyl sepharose column stage. TSOX-containing fractions were eluted from the DE52 column, were pooled, concentrated to approx. 30 ml by ultrafiltration (100 kDa cut-off membrane) and ammonium sulphate added to a concentration of 1 M. The enzyme was applied to a (3 cm x 10 cm) phenyl sepharose column equilibrated with 20 mM phosphate buffer, pH 7.3, containing 1 M ammonium sulphate. The column was extensively washed with equilibration buffer and the enzyme eluted by applying a gradient of 1 M to 0 M ammonium sulphate contained in 20 mM potassium phosphate buffer, pH 7.3. The fractions containing SOX were pooled and dialysed exhaustively against 20 mM Tris HCl, pH 8.0 and applied to a Q sepharose (HP) fast flow column (2.5 cm x 2.5 cm) equilibrated with 20 mM Tris HCl, pH 8.0. Purified SOX was eluted using a linear KCl gradient (0-0.6 M) contained in 20 mM Tris HCl, pH 8.0. The fractions were checked for purity on a
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12% SDS gel. The pure fractions were pooled, dialysed exhaustively against 20 mM phosphate buffer pH 7.3 and concentrated to approx. 10 mg/ml for storage at -20°C.

2.4.2 Determination of Protein Concentration

For crude extracts, protein concentrations were determined by the method of Bradford (1976). Protein (10-100 µg) in a volume of 0.1 ml was added to 5 ml of Bradford protein reagent (100 mg Coomassie Brilliant Blue G-250, 50 ml 96% ethanol, 100 ml 85% orthophosphoric acid; diluted to 1 l with water). The blue coloration was allowed to develop for at least 2 min and the absorbance at 595 nm was measured in a Hewlett Packard 8452A diode array spectrophotometer. Bovine serum albumin (10-100 µg) was used as the standard protein. A calibration curve was constructed over the range 10-100 µg BSA. A DC Protein assay kit supplied by BioRad was also used to determine the protein concentration. This kit is based on the Lowry assay of an alkaline copper tartrate solution reacting with Folin reagent (Lowry et al., 1951).

The concentration of Pure TSOX could be determined from the flavin absorbance using the extinction coefficient ($\varepsilon_{450} = 12.7 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$) reported for the Corynebacterium enzyme (Kvalnes-Krick & Jorns, 1986). The enzyme concentration was calculated on the basis of flavin content (2 mol of flavin/mol enzyme).

2.4.3 Cofactor Analysis

The presence of the non-covalent cofactors FAD and NAD$^+$ in purified SOX was confirmed by reverse phase HPLC analysis using a Waters Spherisorb S5 ODS2 reverse phase column. The column was equilibrated with 80% 50 mM potassium phosphate buffer, pH 6.5/20% methanol. Samples of enzyme were boiled for 5 min to release the cofactors; protein was removed by microcentrifugation for 15 min, and filtration of the supernatant through a Millipore 0.2 µM protein-binding filter. After loading samples onto the column, the cofactors were eluted using a 20% to 100% methanol gradient. Analytical grade samples of FAD, FMN, AMP, and NAD$^+$ were run on the column under identical conditions as standards.

Covalent linkage of flavin to the β-subunit of TSOX was demonstrated by separating the subunits by 15% SDS PAGE, and detection of flavin fluorescence by exposing the unstained gel to UV light (Scrutton, 1999).
2.5 Kinetic Analysis of Sarcosine oxidase

2.5.1 Steady-state Kinetic Analysis

Steady-state kinetic measurement were performed with a 1 cm light path in 20 mM potassium phosphate buffer, pH 7.3, at 25°C, in a final volume of 1 ml. The reaction mixture consisted of 5 IU horseradish peroxidase, 0.007% w/w o-dianisidine, and enzyme and substrate concentrations as required. Reactions were initiated by microlitre additions of substrate stock solutions, to the assay mixture to obtain the desired final concentration. Initial linear rates of reaction were monitored over 1-5 min as an increase in absorbance at 430 nm due to the oxidation of o-dianisidine (ε\textsubscript{430} = 1.08 x 10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1}). One unit of activity is defined as the amount of enzyme which catalyses the production of 1 μmol of oxidised dye (1 μmol of hydrogen peroxide produced) per min. The rates obtained were fitted to the Michaelis-Menten equation:

\[ v = \frac{V_{\text{max}} [S]}{[S] + K_m} \]

Equation 2.1

where \( K_m \) is the Michaelis constant, [S] is the substrate concentration, and \( V_{\text{max}} \) is the maximal rate. During and after TSOX purification, specific activity assays were performed on enzyme preparations using the peroxidase-coupled assay described above. Conditions were as above except that the concentration of sarcosine was fixed (125 mM). The enzyme was diluted when necessary and 10 μl was used in each assay. All assays were carried out using a Hewlett-Packard 8452A single beam diode array spectrophotometer.

2.5.2 Stopped-flow Kinetic Analysis

Applied Photophysics SF.17MV and SX.18MV stopped-flow spectrophotometers were used to carry out rapid reaction studies. The temperature of the reactions was controlled by attaching the sample-handling unit of the stopped-flow spectrophotometer to a TE-8A Tempette combined pump, thermostat controlled heater unit and refrigerated water bath from Techne Limited. All reactions were carried out at 25°C unless otherwise stated, except for temperature dependence studies where the thermostat was used to alter the temperature of the reaction. The dead-time of the instrument was measured as 1.1 ms using the method of Tonomura et
Rapid-scanning, time-dependent stopped-flow reactions were carried out using a photodiode array and X-SCAN software (Applied Photophysics). Global analysis and numerical integration were performed using PROKIN software (Applied Photophysics). For single wavelength studies, data collected at 450 nm were analysed using non-linear least squares regression, all on an Archimedes 410-1 microcomputer.

Aerobic experiments were carried out at 25°C by mixing TSOX contained in 20 mM potassium phosphate buffer, of different pH values, and sulphite (see Chapter 4) with sarcosine. Sarcosine was at various concentrations in the same buffer. The concentration of substrate used was always at least 10-fold greater than that of the enzyme, ensuring pseudo first-order conditions. Reduction of the flavin was measured as a decrease in absorbance at 450 nm. The rate of formation of a long-wavelength flavin biradical species could be measured at 550 nm. For each substrate concentration at least four replicate measurements were collected, each containing 800 data points, and averaged. The absorbance change monitored was either biphasic or monophasic in nature and fitted to a two exponential expression or single exponential expression, respectively.

\[ A_{450} = C e^{-k_{out}t} + b \]

Equation 2.2

\[ A_{450} = C_1 e^{-k_{out}t} + C_2 e^{-k_{out}t} + b \]

Equation 2.3

Where \( C \) is a constant related to the initial absorbance and \( b \) is an offset value to account for a non-zero baseline. From these fits, rates were obtained by non-linear least squares fitting.

The observed rate constants (\( k_{obs} \)) for monophasic transients and for the fast phase (attributed to flavin reduction) of biphasic transients were found to exhibit hyperbolic dependence on the substrate concentration and the reaction sequence was modelled as shown in Scheme 2.1. Scheme 2.1 involves the establishment of a rapid equilibrium between the ES complex and free enzyme and substrate that forms prior to flavin reduction.
Data where then fitted to obtain related $K_d$ and $k_{\text{lim}}$ values using the Strickland equation (Equation 2.4; Strickland \textit{et al.}, 1975).

$$k_{\text{obs}} = \frac{k_{\text{lim}} [S]}{K_d + [S]}$$

\text{Equation 2.4}

For the pH-dependent study, $k_{\text{lim}}/K_d$ was calculated and the data were fitted using Equation 2.5.

$$k_{\text{lim}} = \frac{T_{\text{max}}}{K_d \left(1 + 10^{(pK_a - pH)} + 10^{(pH-pK_a)}\right)}$$

\text{Equation 2.5}

where $T_{\text{max}}$ is the theoretical maximal value of $k_{\text{lim}}/K_d$.

Kinetic isotope effect (KIE) studies were carried out aerobically at 25°C and pH 8.5 using enzyme in the presence of 20 mM sulphite. The rate of flavin reduction with both sarcosine and N-methyl-D$_3$-glycine (deuterated sarcosine) was studied at various substrate concentrations and a plot of rate versus substrate concentration was used to determine the values of $k_{\text{lim}}$, from which the KIE was obtained. The temperature dependence of the rate of flavin reduction was investigated from 5°C to 35°C with sarcosine and N-methyl-D$_3$-glycine at concentrations of 150 mM (i.e. 10 x $K_d$). The temperature dependence for a unimolecular rate constant is given by Equation 2.6:

$$\ln \frac{k}{T} = \ln \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT}$$

\text{Equation 2.6}

Where $k_B$ and $h$ are the Boltzmann and Planck constants, respectively. Data was fitted to Equation 2.6 from which values of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ were obtained.

Anaerobic rapid kinetic experiments were carried out using a stopped-flow apparatus housed in a Belle Technology anaerobic glove box where oxygen levels were maintained below 5 ppm. All solutions used were made anaerobic by bubbling...
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with argon for at least 2 h and then left to equilibrate in the anaerobic glove box. The enzyme stock was made anaerobic by passing it through a Sephadex column equilibrated in anaerobic buffer of the desired pH. For pH-jump experiments, the enzyme (contained in 10 mM potassium phosphate buffer, 100 mM KCl, pH 7.0) was converted to the biradical form by anaerobic reduction with either L-proline or dithionite. KCl (100 mM) was present in the enzyme solution to ensure that the ionic strength of the solution remained constant throughout the experiment. A jump in pH from 7 to 9 was achieved by rapidly mixing the enzyme biradical form with 100 mM sodium borate buffer, pH 9 and observing the loss of the biradical at 550 nm. Monophasic transients were obtained and the rates of electron transfer were determined by fitting the kinetic data to a single exponential equation (Equation 2.2).

2.5.3. Titration of SOX with Sulphite

The covalent flavin of SOX was titrated with sulphite in the pH range 6.5–10.5 and the absorbance spectrum recorded after each addition. The absorbance change at 450 nm was then plotted as a function of the concentration of sulphite [L] added and the data fitted using the single binding equation (Equation 2.7) to determine the binding constant, $K_d$, for sulphite at each pH value.

$$\Delta A_{450} = \frac{\Delta A_{450\text{max}} [L]}{K_d + [L]}$$

Equation 2.7

2.5.4. Anaerobic Reduction Experiments

Anaerobic reduction of TSOX with L-proline, dithionite and sarcosine was performed by preparing an anaerobic enzyme solution at the desired pH in the anaerobic glove box by passing the enzyme through an anaerobic BioRad DG10 Sephadex column. Enzyme solution (3 ml) was then sealed in a cuvette and transferred to a diode array spectrophotometer to measure the absorbance spectrum. Solutions of the reductant were prepared anaerobically and removed from the anaerobic box in a sealed vessel. Titrations were performed by making 1 μl additions of the reductant to the anaerobic enzyme, using an air-tight 25 μl titrating syringe. Dithionite solutions were standardised by titration with FAD solution under anaerobic conditions.
2.6 Redox Potentiometry

The redox potentials of the flavin cofactors of SOX were measured in the laboratory of Professor Stephen Chapman, Department of Chemistry, University of Edinburgh, with the kind assistance of Dr Andrew Munro. All redox titrations were performed anaerobically at 25°C inside a Belle Technology glove box with levels of oxygen maintained at less than 5 ppm. Enzyme stock solutions were exchanged into anaerobic buffer of the desired pH using a 20 cm x 1 cm Sephadex G25 (coarse) column pre-equilibrated with the same buffer, producing a final anaerobic enzyme concentration of ~ 65 μM. A 10 ml enzyme solution was electrochemically titrated using the method of Dutton (Dutton, 1978) with the following redox mediators; 5 μM 2-hydroxy-1,4-napthaquinone, 2 μM PMS, and 1 μM of both methyl viologen and benzyl viologen. The electrochemical potential of the solution was measured using a CD740 meter (WPA) coupled to a Pt/calomel electrode. The absorbance spectra were obtained using a Shimadzu 1201 UV/vis spectrophotometer with a 1 cm path-length. The enzyme solution was titrated by small additions of sodium dithionite to reduce the flavins, and left to equilibrate before the potential and spectra were measured. This was continued until both the flavins were fully reduced; the flavins were then re-oxidised anaerobically by titrating the solution with potassium ferricyanide. After a range of spectra and redox potentials had been recorded the spectra were corrected to take into account baseline drift due to precipitation and evaporation over the long time course of the experiment. Precipitation was corrected for by the modification of each spectrum using Equation 2.8.

\[ a_1 = \frac{1}{750/\lambda} [a_0 - (A_{750n} - A_{750\text{start}})] \]  

Equation 2.8

where \( a_0 \) and \( a_1 \) are the original and modified absorbances at a given wavelength respectively, \( A_{750n} \) is the absorbance at 750 nm in the spectrum that \( a_0 \) is taken from, \( A_{750\text{start}} \) is the absorbance value at 750 nm of the first spectrum taken before addition of dithionite and \( \lambda \) is the wavelength at which \( a_0 \) was recorded. To correct for increases in absorbance values due to evaporation each spectrum was modified using Equation 2.9:

\[ a_2 = a_1 \left( \frac{n_\lambda - 1}{n_{\text{total}}} A_{\text{FlavinEnd}} \right) A_{\text{FlavinStart}} \]  

Equation 2.9
where \( a_1 \) and \( a_2 \) are the absorbance values modified for precipitation and then evaporation at a given wavelength, \( n_x \) is the spectrum number for each particular absorbance value, \( n_{\text{total}} \) is the total number of spectra taken over the course of the redox determination and \( A_{\text{FlavinStart}} \) and \( A_{\text{FlavinEnd}} \) are the absorbance values of the maximum flavin peak at 454 nm of the first and last spectrum recorded respectively. Plots of absorbance versus the redox potential (measured value +244 mV to standardise against the normal hydrogen electrode) were analysed using the Nernst Equation (Equation 2.10):

\[
E_i = E_0 + \frac{2.303RT}{nF} \log \frac{[\text{oxidised}]}{[\text{reduced}]}
\]

Equation 2.10

\( E_i \) is the potential of the enzyme solution at a given reduction of the enzyme, \( E_0 \) is the mid-point redox potential, \( R \) is the gas constant, \( T \) the temperature (298 K), \( n \) is the number of electrons involved, \( F \) is the Faraday constant and \([\text{oxidised}] / [\text{reduced}] \) is the degree of reduction of the enzyme as determined by the absorbance at 454 nm. For a two-electron transfer at 25 °C, Equation 2.10 can be simplified to Equation 2.11:

\[
E_i = E_0 + 0.295 \log \frac{[\text{oxidised}]}{[\text{reduced}]}
\]

Equation 2.11

as, under these conditions, the term \( 2.303RT/nF \) has a value of 0.295. The Nernst function can be written in a suitable form for graphical analysis for a single-electron (or concerted two-electron) process as a sigmoidal change, Equation 2.12:

\[
A_{454x} = \frac{(A_{454ox} + A_{454red}) (E_o - E_x)}{(E_o - E_x) RTF} \frac{(E_0 - E_x)}{RTF} 1 + 10
\]

Equation 2.12

where \( A_{454x} \) is the absorbance value at 454 nm at the potential, \( E_x \), \( A_{454ox} \) is the absorbance of the fully oxidised enzyme at 454 nm, \( A_{454red} \) is the absorbance of the fully reduced enzyme at 454 nm, \( E_0 \) is the mid-point potential and \( RTF \) is the value for the term \( 2.303 \ RT/nF \) (59mV for a one electron process and 29.5 mV for a two electron process). For the sequential transfer of electrons in which a semiquinone is
formed a double-Nernst function can be used to analyse the data as a double sigmoidal change, (Equation 2.13; Daff \textit{et al}, 1997):

$$A_{454x} = \frac{A_{454ox}10^{(E_x-E_1)} + A_{454sq}10^{(E_2-E_x)}}{1+10^{(E_x-E_1)}} + \frac{A_{454red}10^{(E_2-E_x)}}{1+10^{(E_2-E_x)}}$$

\textbf{Equation 2.13}

where $A_{454ox}$ is the absorbance at 454 nm at the point of maximum formation of the semiquinone while $E_1$ and $E_2$ are the midpoint potentials for the oxidised/semiquinone couple and semiquinone/reduced couple, respectively. In the case of TSOX, which has two flavins and is capable of accepting 4 electrons, the midpoint potentials can be determined using the double Nernst function for two flavins in turn (i.e. $A_{454x} = \text{Equation 2.13 (1st flavin)} + \text{Equation 2.13 (2nd flavin)}$). Alternatively, an equation representing a quadruple-electron process can be derived by extension of the single-electron Nernst equation and the Beer-Lambert law (Equation 2.14; Noble \textit{et al}, 1999):

$$A_{454x} = \frac{a10^{2E_x-E_1} + b10^{E_x-E_2} + c10^{E_1-E_2} + d10^{E_1-E_x} + e10^{E_1+E_2-2E}}{1+10^{2E_x-E_1} + 10^{E_x-E_2} + 10^{E_1-E_2} + 10^{E_1-E_x} + 10^{E_1+E_2-2E}}$$

\textbf{Equation 2.14}

Where a-e are the relative absorbance values at 454 nm of the two flavins of TSOX in each of five non-degenerative oxidation states. The data were analysed using non-linear least squares curve fitting using the Origin 6.0 (Microcal) package. Potentials quoted are those corrected for the standard hydrogen electrode (SHE).

\section*{2.7. Crystallisation of Sarcosine Oxidase}

Pure SOX was dialysed against sterile water (pH 7.0) and concentrated to 6-8 mg/ml. This protein solution was then used to screen for suitable crystallisation conditions using Hampton Research crystal screens I and II. Each sitting drop crystallisation was set up in the same way. Crystallisation solution (100 \mu l) was added to each well in a tray. Enzyme (1 \mu l) was placed in a ledge above the well to which 1 \mu l of the crystallisation solution was added. The well was then sealed with tape and labelled. This was repeated twice for each solution, the trays were then left at 25\degree C or 4\degree C for 5-8 days to allow crystals to form. The conditions under which crystals were
produced were further explored using the hanging-drop method to produce larger crystals of a better quality. Crystallisation reagent (1ml) was added to a well of a vapour diffusion plate. Enzyme (5 µl) was placed on a siliconized cover slide to which an equal volume of crystallisation reagent was added and mixed by gentle aspiration. The cover slide was inverted and sealed on to the well using vacuum grease to prevent evaporation. The plates were then left at 25°C or 4°C for a week or longer. Details of crystallisation conditions are given in Chapter 3.
Chapter 3

Sequencing, Expression and
Characterisation of Sarcosine Oxidase
3.1 Introduction

3.1.1 TSOX Sequencing and Expression

The overall aim of this thesis is to perform a detailed kinetic characterisation of the mechanism of sarcosine oxidation by *Arthrobacter* sp. 1-IN TSOX using stopped-flow methods. However, before any detailed studies of the enzyme were carried out it was necessary to sequence the DNA encoding the 4 *sox* genes and to improve expression and purification of the recombinant enzyme. The pXOR9 plasmid construct, carrying the *sox* operon, was a generous gift from Dr Rolandas Meškys (Laboratory of Bioanalysis, Institute of Biochemistry, Mokslininku, Vilnius, Lithuania). This construct consisted of a 12.5 kb fragment of Arthrobacterial genomic DNA cloned into the multiple cloning site (MCS) of the pUC-derived, pTZ18R plasmid (see Section 2.1.2; Figure 2.1). Restriction mapping of this construct had previously indicated that 5 kb of the cloned fragment did not contain the *sox* genes and could be removed by digestion with restriction enzyme *Kpn* I (Meškys *et al.*, 1996). This *Kpn* I digested construct was capable of expressing recombinant TSOX at a level of 1mg/1 of cell culture in the presence of IPTG. Therefore, the pXOR9 construct was digested with *Kpn* I to remove the non-*sox* coding region and this construct (pKOR9) was used for DNA sequencing and expression of TSOX.

Sequencing of the *sox* genes was carried out using primers which would anneal to plasmid DNA up and down stream of the 7.5 kb DNA fragment containing the *sox* genes. A primer walking strategy was used instead of the nested-deletion method, since the restriction sites required for the technique were also present within the DNA fragment to be sequenced in addition to the MCS of the pTZ18R vector. Those sites that were unique to the MCS were unsuitable for the nested-deletion technique as they did not produce the right DNA ends for protection or digestion. Manual sequencing of 7.5 kb of DNA by primer walking was a slow process, but the sequencing process was accelerated by carrying out automated sequencing in parallel. However, sequencing was hindered by the high G+C content of the genomic DNA. This made primer design difficult; primers which annealed to the template DNA would often only produce short sequence runs terminating after only 200 bases owing to secondary structure in the template DNA.

No TSOX expression could be detected by enzyme assay in extracts of *E. coli* strain JM109 (DE3) transformed with the original pXOR9 plasmid. Poor
expression was also observed with plasmid pKOR9 in the presence of IPTG. Without knowledge of the *sox* DNA sequence the initial approach to improve protein expression was to vary the time and amount of IPTG induction for the pKOR9 construct and to explore the use of additional restriction digests to remove more of the upstream non-*sox* coding DNA. None of these approaches were successful in producing more than 1 mg of TSOX per litre of cell culture. Stopped-flow studies typically require >100 mg of enzyme, therefore expression and purification of native TSOX from *Athrobacter* sp. I-IN was carried out to obtain enough enzyme for preliminary studies. When the sequencing of the *sox* genes was complete, high levels of recombinant TSOX expression was achieved by partial *Nco* I digestion of the 7.5 kb DNA fragment from pKOR9 and ligation into the pET11-d expression vector (pSOX; Section 2.2.6).

3.2 Results

3.2.1 DNA Sequencing of the Arthrobacterial *sox* Operon

The genes coding TSOX from *Arthrobacter* sp. I-IN have been cloned (Meškys et al., 1996), but not sequenced. A similar TSOX from *Corynebacterium* sp. P-1 has already been sequenced (Chlumsky et al., 1995) and provided important clues to the structure and organisation of the Arthrobacterial *sox* operon and nearby genes. Knowledge of the sequence was required to improve expression of the recombinant enzyme by genetic manipulation and to provide necessary information for future structural work.

The nucleotide sequence of the 7.5 kb *Hin* dIII - *Kpn* I fragment of pXOR9 (Figure 3.1), containing the four *sox* genes and flanking genes, was determined using a combination of automated and manual sequencing, and a primer walking strategy. The Auto assembler software (ABI Applied Biosystems) was then used to analyse and edit the sequences before a consensus sequence was compiled (Figure 3.2).
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

Figure 3.1. (A) A diagram to summarise the location, orientation and approximate size of the four sox genes and flanking genes in the 7.5 kb genomic DNA fragment, and the location of important restriction enzyme recognition sites. (B) pXOR9 construct, consisting of a 12.5 kb genomic DNA fragment from Arthrobacter sp. 1-IN ligated into the multiple cloning site (MCS) of pTZ18R.
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

Figure 3.2. Nucleotide sequence of the Arthrobacter sp. 1-IN fragment (6515 bp) containing the sox genes, and the deduced amino acid sequence of the gene products. Putative ribosome binding sites are in bold and underlined, double underlining indicates where N-terminal sequencing agrees with the deduced protein sequence. The covalent flavin binding site in the β subunit of sarcosine oxidase, His 173, is indicated by an arrow (|). The open reading frames are organized in the order glyA, soxB, soxD, soxA, soxG, purU. Bases 1-600 are single strand data, which showed similarity with a serine-hydroxymethyltransferase gene (glyA).

```
1 GCGCAGCTCAGCGACTTCGCGGGCCTGGTGCCTCCGGGGTTGCATCCTTCGCCGGTTCCGCATGCGCAT 60
   A H F A G L V L P G L H P S P V P H A H -
61 GTGGTCACTTGCCAGACCGCAAGCGACCGCGTTGCCGCCGGCGCATCCTGGGCCATGGTGACC 120
   V V T S T T H K T L A G P R G I I L T -
121 AATGATCCTGGAGAAGCTCGGGTTACCTGGCCGGGGTTCGCGCCGTCGCTTGACC 180
   N D A E I A K K L N S P V F P G Q Q G G -
181 CCGTTGAAACTGTGAGTGTACGGGGTTCCGGCGGCGCTGACCGGCGGCCCGCGGAG 240
   P L K H V I A G K A V A F K I A A T A E -
241 TTCAAGGACCGCAAGCGACCGCTTGGCCGGCGATCTGGCCGGAGCGTTGGACC 300
   F K E R Q E R T L A G S R I L A E R L T -
301 CAGGATGATGCGGAGATCGCGAAGAATGCGGACCGGGCGGCCGATGCTGACC 360
   Q D D V A A K G I S V L T G G T D V H L -
361 GTGCTGGTAACTGCGCAAGCGACGGCGGCTGAGTGCCGGCCGCGGATCCTCTGGCCGCG 420
   V L V D L R N S E L D G Q Q A E D L L A -
421 CAGGGGAAATCGCGGATCGCGAAGAATGCGGACCGGGCGGCCGATGCTGACC 480
   Q V E I T V N R N A V P F D P R P P M T -
481 ACCGCGGGTTGCGGATCAGCGGCGCGGCGGGCTGCGGACCGCTTTCGCGAGCGAGCG 540
   T S G L R I G T P A L A T R G F S E A G -
541 TTTGGCAAGGTTGGCCGAGTTATGCGAGGACCTTGGATGCGGCGGCTGCGGCGAATACC 600
   F A E V A I A Q T L I A G A E G N T -
601 GTGGCGCTCCGGAGATTGAAAGCGAGATTCTGGACTGCGGCGCCGACCGCGCCACCTGATAC 660
   G A L P E L R I L A A A H P L Y -
661 CCAACCTGAAAAAGATCGGGAGAAATAGCCATGCTGAGTGGCTGCTGACCCGGAAT 720
   P N L K K I G E * M A D L L P E H P E F -
721 TCCCTCGGGGCCACCGCGGAGCCCAAAGAAGTCTTNTGATGTCGTCATCGTCGGCCGGCCG 780
```
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

L W A N P E P K K S Y D V V I V G G G G -

781 GCCACGGCGGTGCCACCCGCTACTACCTGGAAGAACCACCGCATCACCAACGGTGGCCG 840

H G L A T A Y Y L A K N H G I T N V A V -

841 TCCTGGAAGGGCTGGGCTGGCCGGGAAATGGCCCAGCAACCACACCATCTATCGCT 900

L E K G W L A G G M A R N T T I R S -

901 CCAATACCTGGGACGAGTCTGGCCGCCGCTACTACCTGGAAGAACCACCGCATCACCAACGGTGGCCG 960

N Y L W D E S A G I Y E K S L K L W E Q -

961 AGCTGCCCGAGGAGATTAGAATACGCTTTCCGGTGGCGAGATCTGAACTC 1020

L P E E L D Y D F L F S Q R G V L N L A -

1021 CCCACACCTGGGGCGATGGGCAATCCATTCGCGGGCTGGAAAGCCAAACAGGCTTCAACG 1080

H T L G D V R E S I R R V E A N K P N G -

1081 GCGTGAGGCAGCCCGATGCTAACCGGGAACATGGCCCGAAGGAAATGGCATCAACCGGCT 1140

V D A E W L T P E Q V K E V C P I I N I -

1141 TCATCGGATCGAATCACCCTGGCCTGTGGCAAGGACGAGATCTGCAAGGACGAGATCTGCAACGGGCT 1200

G D D G A Y P V M G A T Y Q P R A G I A -

1201 CCAAGCAGCAGCAGCGACCGGTCATGGGGCCGGCAGGAAACGGAATGGGTATGGTGGACA 1260

K H D H V A W A F A R K A N E M G V D I -

1261 TCATCGGATCGAATCACCCTGGCCTGTGGCAAGGACGAGATCTGCAAGGACGAGATCTGCAACGGGCT 1320

I Q N C E V T G F L K D G E K V T G V K -

1321 AAACACGCGCAGCACCACATCCACAGCGCGCCAGGCAATGGGTCACTGGGCCGGCGGGCGGCCTCCT 1380

T T R G T I H A G K V A L A G A G H S -

1381 CCGTGAGGCAGCAGCAGCGACCGGATCTGCAAGGACGAGATCTGCAAGGACGAGATCTGCAACGGGCT 1440

V L A E L A G F L P I Q S H P L Q A L -

1441 TGATCGGATCGAATCACCCTGGCCTGTGGCAAGGACGAGATCTGCAAGGACGAGATCTGCAACGGGCT 1500

V S L E F P V H P T V V M S N H I H V -

1501 TCATCGGATCGAATCACCCTGGCCTGTGGCAAGGACGAGATCTGCACTGGGCCGGCGGGCAATCGACACTCAAC 1560

Y V S Q A H K G E L V M G A G I D T Y N -

1561 AACCGTACGGCGCAGCGCGGCGGTTCACGATCTGAGGAGCAAAATGGGCCGGCGGGCGGTGG 1620

G Y Q R G A F H V I E B Q M A A A V E -

1621 AGCTGGTCCCGGATCTTCCGGCAGGCGGCTGGCACTGGGTGAGGAGGAGAATGGGATCGGAGACCACCTGTGG 1680

L F P I F A R A H V L R T W G G I V D T -

1681 CCACATGGGAGCCTTGGCAGTATCTCAGGAAACGCGGATCCACAGGAAACCTCTACGTCACACT 1740
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

1741 GCGGCTGGGGCACCAGCGGCTTCAAGGGGACCCCTGGGCCGCTTCACCCTGGGCAAC 1800
G W G T G G F K G T P G A G F T L A H T -
1801 CCATGCGAATGCGAAGCCCACGCTGAACGCCGCTTCGATGAGGGAAATCTTTCGAA 1860
I A N D E A H A L N A P F S L E R F -
1861 CGGGCCACCTTGATCGAGAACACCGGCACGCCGGCGTGGCCCACATTAGAGCGCCACCAT 1920
G H L I D E H G A A A V A H -
1921 GATGCTGTATTGGAATGCGCCCCAATCCTGCGCGAGCGGGACGAAATTCTCTCCGCTGCGG 1980
M L I E C R N P C R N E T E F S Y G G -
1981 CCAGGGCCACGTCGCTACCCGAGGACCCGGAACACCGCGAAGAAATTCTTCAAGCGGG 2040
Q A H V A Y P E D P N T L S D K E W S R -
2041 CTACCTGGTCTACCCGAGGAACTCCATGCGAGAACCGGGATCTTCGAGAACGCTGGGTCCACTCCGG 2100
Y L F Y R G N S K G I F A R E R W V H S G -
2101 CGGGTGGCGGAGTGTTTCCAGCGCCTGCGCGACACCGGAGACCGGACATTGATATTACGCGGG 2160
G C R K W F N A L R D T A T Y E F K A V -
2161 CTACCGGCGGCGGAGACCCCGAGCCGAGCCGATGAGCCAGCCCGATCGGCTGCGG 2220
Y R A G E P R P F E L N T Q G G S R * M S O -
2221 AACAAGTGCCGTACGGCAGGCGAGCACATTCCCGCGCCCGCCGATCGAGCCGCGGAA 2280
N K S Y R L P A E Q S P A A R I D R G E -
2281 CGCGCTGGTCTGAGGCTGAGCAGCCGAAGACGATGCGATCGGCTTCGCCGCCCGACAGCCCGG 2340
A L V L S V D G K Q L D A F R G D T V A -
2341 AGCCGGCATGCTGCGGCAACCCGAGGCGCTTCGCGGCATCTCCATGATCGGCGGAACCGGGC 2400
S A M L A N G Q R S C G N S M Y L D R P -
2401 CGCCGCATCTCTTCCGCCGGGCTGAGGAGGACCCCTGGTTGAGCCGCTGCCGCCGGGC 2460
R G I F S A G V E E P N A L V T V A A R -
2461 CACAGAGAGGACATCAACGAGGCTCCTCGCGCGCACTCCATGATCCTGCAGCCGGAAC 2520
H E E D I N E S M L A A T T V P V T A N -
2521 CTACAGCGCCACGCTGCTGCGGCTCGCTGGATGCGTGGCTGAGCCCGAGCAGCGATCGGCTAC 2580
L S A T L L R G L V G V L D P S T D P A Y -
2581 TACGCCATGCTGCACTCCACACGGCGACGCTGCTGCTGCGGCGCCGACCGCGCGGCTG 2640
Y D H V H V H T D V L V V G A P A G L -
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2641 GCCGCCGCCGCCGAGGATCCCGCTTCCCGGGGCCCCCCGGCTGCTGCTGCTGGATGAGCGCGCC 2700
            AAAAREASRSRSGARVLLLDERA

2761 GCGTGAGTCGAGGCCGCCGCGAATCTGTCGCCGCCGCCGCCAACCCCACTGTGAGCGGACC 2720
            A W IDATAAELASAASAE T THLQ

2821 GCACCCACCTGCTGGTCTATAGACCCCACTACGTGATCGCCGCCGCAACCGGACACC 2800
            RTTTLGSYDANYVIAAAQRRT

2941 GCCGACCTGCGCCGCCCTCCTGGCGGCTGGCTGACGGCTGAGCGCATCTGGGACATCCGC 2900
            VHLDAPSAGVSREIRIWHIR

3001 GACCGCGCGCGCACTGCTGCCGCCGCCGCGGAGCTACCTGAAACCCACCTCGCGGACC 3000
            ANQVVLATGAHERPIVFQNN

3121 GACCTGCTGCGCCGCCCGTGGTCGCCGCCCTGCTCGCCGCCATCGTGTTCCAGAACAAC 3180
            DRPGLMAGAVRSYLNTRYGV

3301 GCCGCCGCCGCCAGGCCGAGCGGCCGCGGGCGCTGCTGCTGCTGGATGAGCGCCCG 3360
             RELGAQPRFEADVLAVAGGF

3481 CTGTTCGACCGGAGCGGCCGCGGCTGCTGCTGCTGCTGGATGAGCGCCCG 3540
             LFDTASALTGAAATGAAAT

3601 GCCGCCGCCGGCTGCAGCCAGGCATCCCGCCAGGGATGAGCGGACC 3660

85
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AAGFERIAQVPQALAVPAGE

3601 GCCCGTCGCTCGCTGCTGCTGCCCTCTCGCTGACAGCGACCCAGGCCGGAATACACCAC
3660
ARPVWLVPSLNGDQAANYTT

3661 CACTTCGTGCAGCAGCGACACAGCCGTCTCCAGCTGCTGCGCGCACCACCGGCC
3720
HFVDLQRDTVSVDVRATGA

3721 GGCCTGGAATCGGTGAGACATCAAGCGGTACACCTGATCTCACCGCAACCACGACAC
3780
GLESVEHIKRTSTANDQ

3781 GGCAAGACCTCGGCGCCATCGCGCGTGACATCGCGCGGCCGCGCTGTAAAAGAC
3840
GKTSGVAAIGVIAAVLGIEN

3841 CCGCCGAGATCGCGACCACACACTTCGCGCCCGGTACACCCGGTGTCCTCGCGGCC
3900
PAEIGHTTPFRAPYTPVSFAA

3901 CTGCGCGCAGGGCAACCGCGGCGCGCTGCGACCAGCGCCGATGACCCCGATC
3960
LAGRTRGALDPARITPMHS

3961 TGGCACCTGCCCACGCGCGGCGGCGGCGGCGGAGTGGAGAGCAGCGCGCTGAC
4020
WHLAHGAPEDVGQWKRAWY

4021 TTCCCGCAGGACGGGAGACATGGGAGCGCCGCTGCTACGGCCAATCCAAGGCGGTG
4080
FPQDGEDMAAVYRESKAHR

4081 GACTCGGTGCAGCGCATGCTGCGCAACACCCGCAACCTGCGAGAGATACGAGATCA
4140
DSVGMALDATTLGKIEIRGBK

4141 GCGGCCGAGTTCTGAAACCGCATCTACACAAACCGGGTACACACCAGTGAAAGGTGC
4200
AAEFLNRTTYNTGKTALKVG

4201 GCCCGCTACGCGGTATGTGCAAGCGGCAACGGGATCAGCTCTCGAGACGCGGCGGAC
4260
ARYGVMCADGMPFDDGVTL

4261 CGCCTGCGGACGGAGCCGGTCTCTCGGCAACCCGGGAGGGGCGGGCGGGCGGTGTC
4320
RLADRFMHTTGGAAAGVL

4321 GACTCGGCTGGAGGAATGGCTGCAGACCGAATGGCCGGAGCTGGACGTCACCTGCACCTG
4380
DWLEEWLQTEWPEDLVCTS

4381 GTCAACCCGCCAAGCTGCGCCATCGGCGGTGCGCTCGCGCGCGGACGCTGGAGCC
4440
VTEQLATVAVGGPSRSDVVA

4441 AAGCTGGCCAGGGCTGAGCTGCTCCAAACGAGCGGCTCAGGTACGTTCCTCCGGGAC
4500
KLASGLDVSNENAFKFMFSRD

4501 GTGACCCCTGGATCGGGCATCGAGCGCACTCAGCCGCACTCTCCTCCGGAACTG
4560
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

V T L D S G I E A R I S R I S F S G E L -

4561 GCCTACGAGATCGCGATCCCGTCCCTGGGATGGCGTCTGGAAAGACGTGTTCGCC 4620
A Y E I A I P S W H G L R V W K D V F A -

4621 GCCGGCCAGGAAATTCACATCACCCCGTACCGGACAGGAAACCATGCACTGCTGCCGC 4680
A G Q E F N I T P Y G T E ' T M H V L R A -

4681 GAAAAGGGCTTTCACTATGCGGCGAGACCCGAGGAGGACCTGCTGCAACCAGGCC 4740
E K G F I I V G Q D T D G T V T P Q D A -

4741 GCATGGGAATGGGTGGTTTCAAGCTCAAGGACTCTGCTGGCAAGGCGCTACGGC 4800
G M E W V V S L K D F V G K R S F A R -

4801 GCCGAACACCTGCGGGAGGACCCGAGGACCTGCTGCTGCCGGTGGACACCACG 4860
A D N L R E D R K H L V S V L P V D T T -

4861 CTGGCGTGTCGGCGAGGCGCGGCCTGCGGGAAATGCCGATTGCTACCCGAGTGGAAACCCG 4920
L R L A E G A A L V A D G A V E T G C -

4921 ACCCCGATGGAAGGCTGGGCTACCCGAGGTACCTCTACCACCTCGGCGCTGCACCCTTC 4980
T P M E G W V T S S Y N S A L G R T F -

4981 GCCTGGCAGCTAGTCAAGAACCAGGGCGGACCGCACATCGGAAAGTCTCAAGACC CGGCT 5040
G L A L I K N G R S R I G E V L K T P V -

5041 AACGGGCAGCTGGTGATGGCTCGGTTCCCTGGGTATCGCTACTCTGTCCCGAAAGGGGAC 5100
N G Q L V D V L V S D L V L P D P E G S -

5101 CGTCGGCATGCGCTACCGAACCCTCCTGTGGGCCGCGCTCGCCCGGCGCA 5160
R R D G * M A S N T L I E S T S L R R S P A E-

5161 GCACCTGGCGCGAGGCAATGCCGACGCGGCTCCACCCCGTGCCGGTGTTGCTGCGG 5220
H L A E A M A Q G S T A G A V V L R E I-

5221 GCCTTCCGCACCCACAGGCTGCGGCGCGGCTCCCGGAAGGCCGCACTGCGGCCTG 5280
A P A T Q V G R A V P G S A C H A A L-

5281 GCCGGCGCGACTGCGAACCAGGGCTGCGCAGCGAGTTGGCGAGGTCGCTGGGGCGCA 5340
A A A L G T G L P Q Q V G E V A G A A E-

5341 GCCACCGGGCTGGCTCCTGGGGGACCCGATGGCTCCTGCTGGCCCTCCGCAAGGG 5400
G T A V L W L G P D E F L A I A P E G S-

5401 CGGGCTGGCGCGAGAACCCTGGCTGCGGCGCGGAGGAGCCACCGCCAGGTCACCTC 5460
G L A G E L V A A L G G E P G Q V I D L -
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

5461 GTGCGCCCAACCGCAGGTGTCCGAAACTGTTCCGACCGGCCACCGCGCCACGGCGCTTGGCTGCGCAA 5520
     S A N R S V L E L S G P A A P L V L R K -
5521 GAGCTGCCGCGAGCCGACCTGCAACCGCGCCGCGCTCAGCTGCCACCGGCCAGATCGCCACCACAC 5580
     S C P A D L H P R A F G V N R A I A T T -
5581 GCTGGCCGAATATCCCGGTGTGCTGTGCTGCGACCACCGGCCACGCGATGTCCTGTACGTCTGCC 5640
     L A N I P V L L W R T G E Q S W Y V L P -
5641 GCGGTCTTCCTCCACCGAGCCACACCGTGACTGGCTGATCGACGCGATGACGTTCCACCGCAGCAC 5700
     R V F P T E H T V H W L I D A M T E F A -
5701 CTCCCCGAGGTTCGCTAGGCGAGAAAATTCCTCCATCCCCGGCCCCCGGGCAGTTCCCGAG 5760
     S P T V A -
5761 GCCACCGGCTTCCCCGACCTCCAGTGCCCGGGAACCCGTCCGCGGTCGCGAAGTTGCGCC 5820
5781 CAGCAGGGATAGAAGACGCGCCCGCCGCGGATCCGGAGGTCCGCGGCGCCGAGCTTGAG 5880
5881 CGGCCCTGGCCGGCGGGTGATGCTGTCGCGGGCGCTCGGGGCTCGGCGCGCAGCC 5940
5941 CGCCGGGCGGAGTACGGCTCAAGAAACACAAGGGCGGAGAAACACCAAAACAAAACTTTTCC 6000
6001 GCCCGACCCGCCCCCTCCAGGGCGCTTCGCCCGCCCAGACATCTGAATGGAAGTGAACCAGTA 6060
     M S -
6061 GCGAGCGGTTCCGCAAGTGCTGGAACCGGCGGATCCGCGGATCGCGGCTATG 6120
     E Q V R H V L T L Q C P E G G I G I V H A -
6121 CGGTGACGGCAAGATTCGCTTCCGACCGGACGCGACCACCTCGTGGAGGTTCGAGTAGACAGCAG 6180
     V T G F L V R Q Q R T I V E L K Q Y D D -
6181 ACATGACCGGGGGCGGTGGTCTGGCTGAGGCAGATTTGCGCCGGACGCCGCCCTCGAT 6240
     M S A G R L F L R V D P A G D G A P D L -
6241 TGCTCGCTACGATGGCGAGCCAGGGAGGTTCTCCGAGGCCGCAGATCGCCATGGGAATGCGC 6300
     L A T M R S E F S E V A E Q F D M E W Q -
6301 AGCTGCCGAGGCGCCGCGCGAGAAAGACCAGAGCGTCTGATCAGGCTCAAGTTGGCAACTCTGAC 6360
     L R E R G E K T K V L I M V S K F D H C -
6361 GCCGTCCAGGCAGCTGCTTTGCTCCGAGCGCAGGCGCCGACCTGCAGGCGCGACATGGGCTGGG 6420
     L Q D L L F R M H S G D L P I E V V G V -
6421 TCGGCTCCAACGACCTCGGCGAGCCACCGTGCTCCTGGTGAATGCTACGGAGTTGCAGATTCCAC 6480
     A S N H P D H R S L M U L T I P L E C L O N I N G S I T E -
6481 CAACATCCGAGTCAGGGACGCCACCCAAAGCCTC - 6515
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3.2.2 Sequence Analysis

The nucleotide sequence and deduced amino acid sequence of the four sox genes (sox B,D,A,G) is shown in Figure 3.2. Regions upstream and downstream of this sequence have also been sequenced and indicate the presence of a gene encoding a serine hydroxymethyl transferase (glyA; Miyata et al., 1993; Chlumsky et al., 1995) upstream of the four sox genes. Downstream of the last sox gene (soxG) there is the start of an open reading frame which shows similarity with an E. coli gene encoding a protein important for the regulation of folate metabolism and purine biosynthesis (purU) (Nagy et al., 1993; Chlumsky et al., 1995). The sox genes are arranged in the order sox BDAG and code for the β, δ, α, and γ subunits of TSOX, respectively (Figure 3.1).

The serine-hydroxymethyltransferase gene (SHMT) from H. methyllovorum (glyA; Miyata et al., 1993) showed homology with the Arthrobacter genomic sequence upstream of the sox genes. Only about 30% of the full SHMT gene has been sequenced, but the full gene should be present in the 5 kb region upstream of the sox genes. An amino acid sequence alignment of the Arthrobacter gene with a serine hydroxymethyl transferase from H. methyllovorum shows 60% identity (Figure 3.3.b.). This protein is involved in the interconversion of 5,10-methylenetetrahydrofolate (active formaldehyde) to serine to regulate levels of folate and reducing equivalents in the cell. Other enzymes involved in folate and choline metabolism may also be found upstream of this gene.

Downstream of the four sox genes is an open reading frame (ORF) virtually identical to the one identified downstream of the Corynebacterial sox operon (Chlumsky et al., 1995). Only 50% of this gene is present in the Arthrobacter genomic fragment but it is located the same distance from the sox genes after a region of ~350 bases which shows very little sequence identity to the Corynebacterial intergenic region. This partial Arthrobacterial ORF showed 96% sequence identity with the N-terminal half of the purU gene from Corynebacterium (Figure 3.3b; Chlumsky et al., 1995) the corresponding Arthrobacterial ORF was assigned as encoding a 10-formyltetrahydrofolate hydroxylase involved in purine biosynthesis.
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Figure 3.3. Multiple sequence alignments for the deduced amino acid sequence of partial genes encoding (a) Formyltetrahydrofolate deformylase (b) Serine hydroxymethyltransferase. A.sp. 1-IN, Arthrobacter sp. 1-IN; C.sp. P-1, Corynebacterium sp. P-1; P. aerugi, Pseudomonas aeruginosa; S. coelic, Streptomyces coelicolor; M. tuberc, Mycobacterium tuberculosis; H. methyl, Hyphomicrobium methyllovorum.
The two genes encoding the smallest TSOX subunits are soxD and soxG. SoxG encodes a protein of 203 residues and its molecular weight estimated from SDS PAGE is 24 kDa. This compares well with the molecular weight estimated from the deduced amino acid sequence of 21.6 kDa. SoxD codes for a smaller subunit of only 98 residues and its molecular weight estimated by SDS PAGE is 14 kDa. The molecular weight estimated from the deduced amino acid sequence is 12.6 kDa. A BLAST search with these subunits showed no homology with any other proteins in the sequence databases except for the previously sequenced TSOX subunits from Corynebacterium (Chlumsky et al., 1995; Figure 3.4).

The soxB gene (Figure 3.5b) encodes a protein with 405 residues. Its molecular weight estimated by SDS PAGE is 43 kDa. This compares with the molecular weight deduced from the amino acid sequence of 46 kDa. The β subunit exhibits an ADP-binding motif near the N-terminus (Figure 3.5a) which satisfies all of the 11 consensus sequence requirements described by Wierenga et al. (1986). A BLAST search showed that the Arthrobacter TSOX β subunit has sequence homology with a range of proteins including the β subunit of the previously sequenced Corynebacterial enzyme (99%), and several other βTSOX enzymes from different sources (Figure 3.5b). Other homologies included four bacterial MSOX enzymes, an E. coli enzyme N-methyltryptophan oxidase (MTOX) and a mammalian pipecolate oxidase (PIPOX). Modest sequence homology is also seen between βTSOX and the N-terminal half of rat liver dimethylglycine dehydrogenase (DMGDH), sarcosine dehydrogenase (SDH) as well as the recently sequenced dimethylglycine oxidase (DMGO) gene from Arthrobacter globiformis. All these enzymes belong to the same protein family, each has a covalently bound flavin and all catalyse oxidation reactions of secondary or tertiary amino acids. Each enzyme also contains an ADP-binding motif near the N-terminus. Pairwise alignments using the GAP program indicate 22-25% identity and 45-47% similarity between the β subunit of TSOX and the MSOX enzymes. The degree of homology between the MSOX enzymes varies from 37-86% identity (58-91% similarity). A sequence alignment of the β subunit of TSOX and MSOX enzymes reveals 42 conserved residues. The most highly conserved regions are in the N-terminal ADP-binding motif and in a 60 amino acid region near the C-terminus.
Figure 3.4. GAP alignment of deduced amino acid sequence of soxD and soxG from Arthrobacter sp. 1-IN (Arthro) with the genes for the previously published Corynebacterium P-1 (Coryne) enzyme (Chlumsky et al., 1995).
Figure 3.5. (a) Alignment of the ADP-binding motif of the β subunit from *Arthrobacter* sp 1-IN with the βTSOX subunit from *Corynebacterium* sp. P-1, MSOX from *Arthrobacter* sp. TE-1826 (ArthMSOX), *Bacillus* sp. B-0618 (0618MSOX) & NS-129 (s129MSOX), *Streptomyces* sp. KB210-8SY (streMSOX), plus MTOX from *E. coli*, PIPOX from rabbit, DMGDH & SDH from human and rat, pyruvate dehydrogenase phosphatase (PYRDHP) from *E. coli* and DMGO from *Arthrobacter* sp. The 11 positions that define the ADP binding motif are marked with asterisks (*); conserved residues are shaded. An arrow shows the flavin attachment site in DMGDH (†).
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(b)

--- ADP Binding domain

| A.PTSOX | ADP.Binding domain | MADLLPEH | PEF..LWANP | EP.. | KKSYDV | VIVGGGGL | ATAYLAKNH |
| A.PTSOX | A.PTSOX | ADP.Binding domain | MADLLPEH | PEF..LWANP | EP.. | KKSYDV | VIVGGGGL | ATAYLAKNH |
| ArthMSOX | 0618MSOX | ADP.Binding domain | MADLLPEH | PEF..LWANP | EP.. | KKSYDV | VIVGGGGL | ATAYLAKNH |
| streMSOX | PIPOX | ADP.Binding domain | MADLLPEH | PEF..LWANP | EP.. | KKSYDV | VIVGGGGL | ATAYLAKNH |

| A.PTSOX | A.PTSOX | ArthMSOX | 0618MSOX | S129MSOX | M TOX | streMSOX | PIPOX | PIPOX |
| A.PTSOX | A.PTSOX | ArthMSOX | 0618MSOX | S129MSOX | M TOX | streMSOX | PIPOX | PIPOX |

--- MTOX ---

| A.PTSOX | ArthMSOX | 0618MSOX | S129MSOX | M TOX | streMSOX | PIPOX | PIPOX |
| A.PTSOX | ArthMSOX | 0618MSOX | S129MSOX | M TOX | streMSOX | PIPOX | PIPOX |

--- MTOX ---

| A.PTSOX | ArthMSOX | 0618MSOX | S129MSOX | M TOX | streMSOX | PIPOX | PIPOX |
| A.PTSOX | ArthMSOX | 0618MSOX | S129MSOX | M TOX | streMSOX | PIPOX | PIPOX |
Figure 3.5. (b) Alignment of the deduced amino acid sequence of the β subunit of TSOX from Arthrobacter sp I-1N with TSOXs, MSOXs, MTOX and PIPOX (same abbreviations as for Figure 3.5.a). The histidine that forms the covalent link to FMN in TSOX is marked by a down arrow (\(\downarrow\)), the cysteine that forms the covalent link to FAD in MSOX is marked by an up arrow (\(\uparrow\)). Putative catalytic residues identified in Bacillus sp. B-0618 MSOX are marked by asterisk (*). The shaded region represents a region of highly conserved residues (60 in total).
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The soxA gene codes for a 967 residue protein and its molecular weight estimated by SDS PAGE is 106 kDa, which compares with the molecular weight estimated from the deduced amino acid sequence of 99.6 kDa. An ADP-binding motif is also found in this protein near the N-terminus and again meets the 11 consensus sequence requirements (Wierenga et al., 1986). A BLAST analysis using the deduced Arthrobacterial αTSOX sequence identified the previously sequenced α subunit from the Corynebacterial enzyme (Figure 3.6), as well as two separate groups of sequences that showed homologies in the N- or C-terminal halves of αTSOX.

Bacterial T-proteins were found to exhibit some sequence similarity (28% identity, 50% similarity) with 40% of the C-terminal end of αTSOX. T-proteins are one of a series of enzymes involved in glycine cleavage and in the synthesis of 5,10-methylene tetrahydrofolate. The C-terminal half of a group of bacterial enzymes, whose N-terminal half showed similarity with the β subunit of TSOX, also showed some similarity with the C-terminal region of the α-subunit. These are DMGDH and SDH (~32% identity, 56% similarity), and DMGO (Figure 3.6b). These enzymes are also involved in glycine cleavage and in the synthesis of 5,10-methyltetrahydrofolate.

The N-terminal half of the α-subunit of TSOX showed similarity with octopine and nopaline oxidases, which are heterodimeric enzymes that catalyse oxidative cleavage reactions with N-substituted arginine derivatives analogous to the sarcosine oxidase reaction (Zanker et al., 1994). Subunit A from octopine and nopaline oxidases showed sequence similarity (30-32% identity, 49-52% similarity) with 30% of the N-terminal region of αTSOX, which includes the ADP binding motif (Figure 3.6.a). Two recently sequenced enzymes also showed sequence similarity to the α subunit in this region, i.e. hydrogen cyanide synthase (HcnB) from Pseudomonas fluorescens, and coenzyme F₄₂₀-quinone oxidoreductase (FqoF) from Archaeoglobus fulgidus. Hydrogen cyanide synthase is a flavoenzyme, which produces HCN and CO₂ from glycine using the cofactors NAD⁺ and FAD, and consists of 3 different subunits. The N-terminal half of subunit B shows ~32% identity with the N-terminal half of αTSOX (170 residues). HcnB contains an ADP binding domain and binds either NAD or FAD. The similarities seen between HcnB and αTSOX may indicate a downstream processing of glycine, a product of sarcosine oxidation. F₄₂₀-quinone oxidoreductase is less well understood, but is a multi-subunit protein which catalyses the oxidation of the F₄₂₀ cofactor and reduction of a
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(a)

| A. αTSOX | MSQNKSYRLP ABQSPAARID RBEALVLSVD GKLQDAFRGD TVASAMLANG |
| C. αTSOX | MSQNTSYRLP ABQSPAARID RBEALVLTVD GKLQLEAFRGD TVASAMLANG |
| F420 | MVRTFPGRLQ BHIVPDF..K RGERVTYIYN GPFLVAYEGD TVAALAYAG |
| NOPOX | ----MSTVRN BPHILDPEKX RKKKTVYYK GQPIEAYEGD TIAALHAAAG |
| HCNSYNTH | ----------------- ----------------- ----------------- ----------------- ----------------- |

| A. αTSOX | QRSQNSMYRL DRPRGIFSAG VEEPNALVTV AARH.......EEDINE |
| C. αTSOX | QRCQNSMYRL DRPRGIFSAG VEEPNALVTV EARH.......EQDINE |
| F420 | VRVFSRSFWR HRRPGFFCPI GKCSACMMEV DG......IPN VRTCKVYRD |
| NOPOX | IRVLNYSRV KRPRGLPCAI GKCSSCMTV NG......IPN VRTCITLVED |
| HCNSYNTH | ----------------- ----------------- ----------------- ----------------- ----------------- |

| A. αTSOX | SMLAATT... . VPVTANLSA |
| C. αTSOX | SMLAATT... . VPVTANLSA |
| F420 | GM QVEQTGQP DPAEKDVFSI LDDVIDKVFP HGSHYTKFTT SKKAREFM.V |
| NOPOX | GMKI...EENK P......ILPANINK......K DKKRRAKI.V |
| HCNSYNTH | ----------------- ----------------- ----------------- ----------------- ----------------- |

1- | ----------------- ----------------- ----------------- ----------------- ----------------- |

| A. αTSOX | TLLR... GLG VLDPSTDPAY YDHVVHHTDV LUVGGAPQLG AAREREASRG |
| C. αTSOX | TLLR... GLG VLDPSTDPAY YDHVVHHTDV LUVGGAPQLG AAREREASRG |
| F420 | KRMRKFTGFG NPPKAVFQGR AELEEIETDV LVIGGGPGGM SAAINAGKYG |
| NOPOX | K......GDI IVVGGGPACG MAAIRASDTG |
| HCNSYNTH | ----------------- ----------------- ----------------- ----------------- ----------------- |

| A. αTSOX | E.TTHLQRTT VLGSYDANYV IAQQRTVHL DAPSGAQVSR ERWHIRANQ |
| C. αTSOX | E.TTHLQRTT VLGSYDANYV VAVQRTVHL DAPSGAQVSR ERWHIRANQ |
| F420 | N.VEVRRETFR VFYGI.NGRA GAYQRLNDD........G EKLLRIKAKK |
| NOPOX | N.IKAIYRTS AVGIFQDGEE KLIGVR.K........G E MELIEFGK |
| HCNSYNTH | QMIDVRLNASR VVGAERTQSL VLLDG........G EQVQQSYESQ |

| A. αTSOX | VVLATGAHER PIVFQNDRP GIMLAGAVRS YLNYGVVRA TRIVATTN |
| C. αTSOX | VVLATGAHER PIVفنDRP GIMLAGAVRS YLNYGVVRA SQIVATTN |
| F420 | IVYFGYGER TLPFENDNLG GYGGAGGQV LGTVYIKPG NAGLIVGSG |
| NOPOX | VIVAGGAMEK TLPFENDNLG GYGGAGGQV LGTVYIKPG NAGLIVGSG |
| HCNSYNTH | LILAAGCHER SVPFGNLTLGP GKVLLOGLQL QIKSGVVXKP SPVIIATGTP |

| A. αTSOX | SAYPLVADLA ASG.GVAVV D......AR TTVSA......A.AA |
| C. αTSOX | SAYPLVADLA ASG.GVAVV D......AR TTVSA......A.AA |
| F420 | VGLILTYQLM QAGVNLAAV E.....AM PRIGY......FVHAA |
| NOPOX | VGLILAYQQL QANVQVAAE E.....AM PKIGY......FVHAA |
| HCNSYNTH | LLPLVACQLH AGSRVAAGY EACALGKIAK QSLAMLNKPQ LFIDGLSMLA |
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Figure 3.6 (a). Sequence alignment of the deduced amino acid sequence for the N-terminal half of *Arthrobacter* sp. 1-IN α subunit with αTSOX from *Corynebacterium* sp. P-1 and F420H2: quinone oxidoreductase from *Archaeoglobus fulgidas* (F420), Nopaline oxidase from *Agrobacterium tumefaciens* (NOPOX) and Hydrogen cyanide synthase from *Pseudomonas fluorescens* (HCNSYNTH). The 11 positions that define the ADP binding motif are with marked asterisks (*) and the GG and ATG motifs involved in nicotinamide binding are shown in bold.
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(b)

A.aTSOX

RDSVMQDLAT TLGKIEIR... GKDAAEFLNR IQTNGYTKLK VGMARYGVMC

C.aTSOX

RESVGMQDLAT TLGKIEIR... GADAAEFLNR IQTNGYTKLK VGMARYGVMC

HDMGDH

RVGV...TVDSL PKDFKNK... KQDSTQLLDH LF.ANVP... KQGTPNISHMJ

RDGDH

RVGV...TVDSL PKDFKNK... KQDSTQLLDH LF.ANVP... KQGTPNISHMJ

HSRD R.GAAVFDMS YFGKFLY... GGDANYGQLK LGDKYHTNL LGDKYHTNL

RSDH

RTAAGLFDLS HMGEIVT... GYVYMKLIGP EYFVKLMDK EYFVKLMDK

PYSHP

KAAVQDPLDS SQFKEITST QDQAELILQY LFSNLDLV... PLYHVTGMG

DMGO

RTAVAMIRMN PLKRLKESQ G...ALKDLQK LTDLTAQK LGYAVYTTLL

B.AMT

RTAAGLFDVS HMGSEYVS... GDNFLNLFLQ TMTNDVSALT PGRQYTMCC

S.AMT

RTRAGLFQLS HMGITVTV... GPQAAELNF ALVGNIGTVK PGRQYTMCC

A.aTSOX

KADGMVFDDQ VTLRLA...... EDRFLMHTTT GWIAAGVLDW

C.aTSOX

KADGMVFDDQ VTLRLA...... EDRFLMHTTT GWIAAGVLDW

HDMGDH

TPKGRVYAVE TLVHQPSPG... FLLITQS GSNLHDLRMI

RDGDH

TPKGRVYAE... TLVHQPSPG... FLLITQS GSNLHDLRMI

HSRD NHRGTGSSDL TLSRAHSQ... ASLAPAFEG AQAQNKHLS AVAQNNKHL

RSDH

NHRGTGSSDL TLSRAHSQ... ASLAPAFEG AQAQNNKHL AVAQNNKHL

PYSHP

KQYMPDE... S...NLLLEDVTT KTYALNLIGP RTVDLSEL SYAPMTDPH

DMGO

QTSGGASA... T DWWQVRDITG GCCTGIALWP LARQLVSKG SDDFTNDGL

B.AMT

KEHA... AQ...DVQIDNQSDP QIALAVQGP KABALKNL TDADV...AL

S.AMT

REDGKSLQG LIVYKL... EAYMMKVAE SNAQQVLAD

A.aTSOX

EEQWYGQDPE LDVTCTSTVE QLATAVQVS RSRDVKQAIA SGLDVSSEAF

C.aTSOX

EEQWYGQDPE LDVTCTSTVE QLATAVQVS RSRDVKQAIA SGLDVSSEAF

HDMGDH

EEAVKQG... YVEQPNTID ELGVQCVAGP QAPKVLQK... TPELSLDDVF

RDGDH

EEAVKQG... YVEQPNTID ELGVQCVAGP QAPKVLQK... TPELSLDDVF

HSRD

TLVQVQ... .K SQOQLIDSSE DLGMSIGQGP ASRAILQEV... LDAATSSEAF

RSDH

TLVQVQ... .E FRQQLMDCSE DLGMSIGQGP ASRDILQDV... LDAATSSEAF

PYSHP

KQYMPDE... S...NLLLEDVTT KTYALNLIGP RTVDLSEL SYAPMTDPH

DMGO

QTSGGASA... T DWWQVRDITG GCCTGIALWP LARQLVSKG SDDFTNDGL

B.AMT

KEHA... AQ...DVQIDNQSDP QIALAVQGP KABALKNL TDADV...AL

S.AMT

REDGKSLQG LIVYKL... EAYMMKVAE SNAQQVLAD

A.aTSOX

KSIGFSFQWLQ DSG.IEARI S... RISFSGELAY EIAIPWSHGL RWKQVDPAAG

C.aTSOX

KSIGFSFQWLQ DSG.IEARI S... RISFSGELAY EIAIPWSHGL RWKQVDPAAG

HDMGDH

KFLQKTSLKE .S.NPVTAT RISYTGELG LHYHREDSV LAYDAIMNAG

RDGDH

KFLQKTSLKE .S.NPVTAT RISYTGELG LHYHREDSV LAYDAIMNAG

HSRD

PFSTHKLRLA .A.GHLVRAM RLSDVGELG LHYVQFASG PVAYMAAG

RSDH

PFSTHKLRLA .A.GHLVRAM RLSDVGELG LHYVQFASG PVAYMAAG

PYSHP

PSLFCKEMVS... GYARIAVRSV SMHTGPEAL YLVIPYIAV HVLNEYSVSG

DMGO

KDFAKRNVV... G.SIPVTAM RLSYVQGELG NELTSADNQ RINDLNAQG

B.AMT

KPFAPIDEBAD IAG.RKALIS RTYUTEDQY EYICRSDDAM HIIKGDIDG

S.AMT

KSYAGTLPG OTG.VAPALIA RTGTYTEDQY ELVKFEPAHV GLQMAATQAG

A.aTSOX

QEFNITPYGT ETMHVLRAEK GIIFIVGQDAG TMTPQDPAGM EMIQVSKLDL

C.aTSOX

QEFNITPYGT ETMHVLRAEK GIIFIVGQDAG TMTPQDPAGM EMIQVSKLDL

HDMGDH

QEBQDNGFT YANMLAREK ARFAGMEMN CDTNPLAEAG EYFVKLMDK

RDGDH

QEBQDNGFT YANMLAREK ARFAGMEMN CDTNPLAEAG EYFVKLMDK

HSRD

AKHGLINAGY RAIDSLSIEK GYRHWHADLR PDQSPLEAGL AFTCKLKTSP

RSDH

AKHGLIVAGY RAIDSLSIEK GYRHWHALDR SDDPSLEAGL AFTCKLATSV

PYSHP

CQKQIGIRN... YARLSRRIEK PFAWQCPQLT TLLTPLECSS ESVRKLDKGV

DMGO

QFQGVIAAGR AAAFSLRLR GYRSWKTDT TEHPFSAGL GAPXVMAKE

B.AMT

DAYGLIPDLQG QARLTLFAPE TVPHAQSLR TDTIPBAGI GAPXVHBBES

S.AMT

EAAAGLIPDLQG SCRDLRELA GMFLHGNELS TLATPDAGL GRVVKFKEG
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A. αTSOX. VGKRSFAR ADNLREDKHK .LVSVLPVD TRLRLAEGAA LVADGAVETG
B. αTSOX. VGKRSFAR EDNLREDKHK .LVSVLPVD TALRLAEGAA LVADGAVETE

Table 3.6 (b) Sequence alignment of the deduced amino acid sequence for the C-terminal half of Arthrobacter sp. 1-IN α subunit with αTSOX from Corynebacterium sp. P-1 and the C-terminal half of rat and human dimethylglycine dehydrogenase (DMGDH), and sarcosine dehydrogenase (SDH), also pyruvate dehydrogenase phosphatase (PYDHP) and dimethylglycine oxidase (DMGO) from Arthrobacter sp. and two T-proteins, amino methyl transferases (AMT) from Bacillus sp. and Streptomyces sp.
menaquinone (Brugemann et al., 2000). The FgoF subunit is the catalytic subunit forming the input device of the protein complex and contains 2 [4Fe-4S] clusters and 1 mol of FAD. Its catalytic mechanism involves the transfer of a formyl group and this may also prove to be a functional role of the N-terminal portion of αTSOX.

3.2.3 Protein Purification of Native and Recombinant TSOX Enzymes

3.2.3.1 Recombinant TSOX Expression Using the pXOR9 Clone

The pXOR9 clone, supplied to the laboratory by collaborators in Lithuania, used the high copy number plasmid pTZ18R, and had been transformed into *E. coli* strain TG1. It consisted of a 12.5 kb genomic DNA fragment from *Arthrobacter* sp. 1-IN ligated into the polylinker of the parent plasmid. Restriction analysis previously demonstrated that only 7.5 kb of this fragment contained the *sox* genes (Meskys et al., 1996). The fragment had been inserted into the multi-cloning site (MCS) in the lac Z gene, close to a T7 promoter. Figure 3.1 shows the orientation of the *sox* genes and additional genomic DNA in the MCS.

Restriction digests were performed on pXOR9, isolated from *E.coli* strain TG1 to identify any unique sites in the fragment, and to compare them with those published by the Lithuanian group to confirm the isolated DNA. Digestion of the construct with Bam HI, Kpn I, Hin dIII, Xba I and Nco I confirmed that the construct was the correct size and that the sites had been identified correctly. It was also deduced that Xba I, Hin dIII and Kpn I sites were not in the 7.5 kb region of the fragment that contained the *sox* genes. The 5 kb genomic region (without the *sox* genes) was removed using a Kpn I digest with the intention that this would improve the expression of the *sox* genes. The pKOR9 construct and the full pXOR9 were transformed into *E. coli* DH5α cells for sequencing, and into JM109 (DE3) cells for protein expression. These transformants were checked using the previous digests and gave the expected banding patterns confirming that they were correct.

Plasmids containing the 7.5 kb *sox* fragment in the MCS of pTZ18R were constructed as described and transformed into the *E. coli* strain JM109 (DE3). These strains were grown in 2xYT media and minimal media in the presence and absence of IPTG. No sarcosine oxidase activity could be measured in cell extracts using the peroxidase assay and no bands corresponding to the four subunits of sarcosine
oxidase could be seen on SDS PAGE of these cell extracts. The Lithuanian group have published that 10 units of enzyme activity accumulate per litre of cell culture for the \textit{Kpn} I deletion clone when the cells were grown in the presence of IPTG; this is equivalent to 1 mg of protein per litre of cell culture (Meskys \textit{et al.}, 1996). The authors did not supply this \textit{Kpn} I-digested construct, but the \textit{Kpn} I deletion clone used in this work was constructed in an identical manner. Since 1 mg of enzyme from 1 l of cell culture is not a large quantity of recombinant enzyme, it was decided that efforts should be made to express the native enzyme which can be obtained at levels of 10-15 mg enzyme/l cell culture.

3.2.3.2 Purification of Sarcosine Oxidase from \textit{Arthrobacter} sp. 1-IN

To provide a source of TSOX prior to production of recombinant enzyme, the native enzyme was purified from \textit{Arthrobacter} sp. 1-IN. \textit{Arthrobacter} sp. 1-IN was cultured, and sarcosine oxidase was purified, as described previously (Meskys \textit{et al.}, 1996; Section 2.4.1). The purified enzyme produced four bands during SDS-PAGE of the expected molecular weights for TSOX (Figure 3.7), and exhibited an absorption spectrum characteristic of a flavoprotein. Analysis of the purification procedure is given in Table 3.1.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>French press</td>
<td>221</td>
<td>18000</td>
<td>0.012</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>35-50% ((\text{NH}_4)_2\text{SO}_4)</td>
<td>239</td>
<td>4580</td>
<td>0.052</td>
<td>108</td>
<td>4.3</td>
</tr>
<tr>
<td>DE52 anion exchange</td>
<td>123.5</td>
<td>378.5</td>
<td>0.326</td>
<td>56</td>
<td>27.2</td>
</tr>
<tr>
<td>Phenyl sepharose*</td>
<td>26.0</td>
<td>30.0</td>
<td>0.867</td>
<td>12</td>
<td>72.3</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>13.53</td>
<td>14.16</td>
<td>0.956</td>
<td>6</td>
<td>79.7</td>
</tr>
</tbody>
</table>

\textbf{Table 3.1.} Purification of native TSOX grown on 10 litres of KMT media and purified as described in the Materials and Methods section. Units = \(\mu\)moles \(\text{H}_2\text{O}_2\) formed min\(^{-1}\). * Activity only measured for pooled fractions.
**Figure 3.7.** 12.5% SDS PAGE analysis of the purification of native TSOX from *Arthrobacter* sp. I-1N. Lane 1, sample of cell contents following French press; lane 2, 35-50% ammonium sulphate fraction; lane 3, pooled fractions following DE52 ion-exchange chromatography; lane 4, pooled fractions following phenyl sepharose hydrophobic interaction chromatography; lane 5, pooled fractions following hydroxyapatite ion exchange column, lane 6, protein markers: 83, 63, 45, 29 and 14 kDa.
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The purification of native TSOX produced only limited amounts of pure protein. The fractions from the phenyl sepharose column were analysed by 12.5% SDS-PAGE, and the purest fractions were pooled and applied to a hydroxyapatite column, but less pure fractions were not processed further, and consequently there was a reduced recovery. SDS PAGE showed no improvement in the purity of TSOX after the hydroxyapatite column, consistent with the finding that the protein was eluted from the column almost immediately (although some impurities may have been retained).

Attempts were made to improve the yield of TSOX by introducing additional steps into the purification procedure, but this met with limited success. It was discovered that the French press pellet, when washed with detergent (0.5% Triton X), contained a considerable amount of TSOX activity. However, despite extensive dialysis the detergent interfered with the ammonium sulphate fractionation step resulting in loss of the enzyme, and the effectiveness of the DE52 column was also reduced significantly. To obtain enough enzyme for kinetic characterisation and crystallography, a 50 l cell culture preparation was performed which yielded ~80 mg of pure protein. The purified protein was stored in 20 mM potassium phosphate buffer pH 7.3 and was stable at -70°C for 6-12 months without any loss in activity.

Several papers (e.g. Kvalnes-Krick and Jorns, 1987, and Wagner and Jorns, 1997) have reported that TSOX from Corynebacterium will interact with tetrahydrofolate. This knowledge was used to produce an affinity column (AH-4B sepharose) containing 5-formyl tetrahydrofolate ligands using the method of Wittner & Wagner (1981). The column was equilibrated with 20 mM potassium phosphate buffer, pH 7.3. Arthrobacter TSOX was loaded and the column washed with the same buffer. The enzyme remained tightly bound to the column when 1 M KCl was applied to the column, and was only eluted when the wash buffer contained 1M KCl and 5-formyl-tetrahydrofolate (20 mM). This confirmed that the Arthrobacterial TSOX also binds folate in a manner similar to the Corynebacterial form (2.19 mol H4 folate/mol enzyme; Kvalnes-Krick and Jorns, 1987). This column aided in producing pure protein for crystallography, but this purification step was not used routinely during the purification procedure, as only a few milligrams of protein could be loaded on the column at any one time and formyl-tetrahydrofolate was an expensive elutant.
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3.2.3.2 Expression and Purification of Recombinant TSOX

It was possible, with knowledge of the complete gene sequence of the sox operon and orientation of the four sox genes, to construct a clone for recombinant TSOX expression (Section 2.2.6; Figure 3.8). This construct which had been cloned into vector pET-11d was named pSOX and was transformed into BL21 (DE3) cells. The cells were originally grown at 37°C in 2xYT media (supplemented with 100 µg/ml ampicillin) for 24 h, and harvested when they had reached stationary phase (OD$_{595nm}$ \( \approx 10 \)). High expression of TSOX could be observed easily when samples of the cell extracts were run on a 12.5% SDS gel. During purification of this preparation it was found that a large proportion of the enzyme remained in the cell pellet after French pressing (observed from SDS PAGE), indicating that the protein may have been forming inclusion bodies. Protein present in the supernatant was purified further. However, spectral analysis showed that the enzyme had lost a considerable amount of flavin and had quickly denatured, indicating that it may not have been folded correctly. The conditions of expression were changed to slow down the growth of the cells and to allow more controlled expression of active TSOX. Timentin (50 µg/ml) was used instead of ampicillin to prevent removal of the antibiotic from the growth medium and reduce contamination. The temperature was also reduced to 25°C, 21°C, and 15°C to slow down the growth rate. Luria Bertani (LB) and Minimal media were also used instead of 2xYT media and cultures were induced with IPTG.

The use of timentin, instead of ampicillin, reduced the total wet cell mass at both 37°C and 25°C and the stationary phase of growth was reached 24-30 h after inoculation. The enzyme was expressed at high levels (as judged by SDS PAGE), but still formed inclusion bodies. Reducing the growth temperature to 21°C or 15°C dramatically slowed down the growth of the cells, which took two and four days respectively to reach the stationary phase. The enzyme was still expressed highly, but did not form inclusion bodies, and was stable throughout the purification procedure. The spectrum of the purified protein, and cofactor analysis demonstrated that the enzyme contained the full complement of flavin. Growth on LB media at 21°C reduced TSOX expression levels. No expression occurred with Minimal media and IPTG induction had no effect on the level of TSOX expression under any condition.
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Figure 3.8. Recombinant TSOX expression construct pSOX. (A) Diagram of pSOX: the partial Nco I digested Kpn I/Hin dIII DNA fragment cloned into the Nco I site of the pET-11d expression vector. (B) Agarose gel of the time course of an Nco I digest of the Kpn I/Hin dIII DNA fragment containing the sox genes. Lane 1, uncut 7.5 kb fragment, lanes 2-7: samples taken, 5, 10, 15, 20, 25, 30 mins after addition of Nco I, lane 8 DNA markers: 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5 kb.
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The optimal growth conditions for TSOX expression involved culture in 2xYT media supplemented with 50 μg/ml Timentin at 21°C. A growth curve was constructed in order to estimate the optimal time to harvest the cells based on cell density (OD₅₉₅) and TSOX activity of the cell lysate (Figure 3.9). Both parameters did not begin to increase very rapidly for the first 12 h and then plateaued after 36 h with small increases for a further 12 h, before there was any detectable loss of activity. Growth of BL21 (DE3) transformed with pSOX using the optimised conditions gave higher levels of protein expression (~30 mg/l), and the recombinant enzyme was simpler to purify than the native form. Analysis of the purification procedure is given in Table 3.2 and Figure 3.10.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>French Press</td>
<td>1870</td>
<td>28000</td>
<td>0.067</td>
<td>100</td>
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</tr>
<tr>
<td>30-50% (NH₄)₂SO₄</td>
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<td>10400</td>
<td>0.202</td>
<td>112</td>
<td>3.01</td>
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<tr>
<td>DE52 anion exchange</td>
<td>760</td>
<td>1830</td>
<td>0.42</td>
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<tr>
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<td>16.1</td>
</tr>
<tr>
<td>Q sepharose</td>
<td>470</td>
<td>387</td>
<td>1.21</td>
<td>25</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Table 3.2. Purification of recombinant TSOX from 10 litres of 2xYT media and purified as described in the Materials and Methods section. Units = μmoles H₂O₂ formed min⁻¹

E. coli cells were easier to break open than Arthrobacter cells using the French press and very little enzyme remained in the cell pellet. As with native enzyme the total enzyme activity increased after the ammonium sulphate fractionation step. It is likely that this effect is due to an inhibitor compound present in the cell extract of both these cell types which affects TSOX activity or interferes with the peroxidase assay. A lot of enzyme activity is lost after the DE52 column (as with the native prepreparation), but this step doubles the purification. This activity loss may be due to loss of TSOX or contaminating enzymes assayed using the peroxidase assay e.g. catalase. The phenyl sepharose column is more efficient in the recombinant purification procedure, possibly as different conditions were used to elute the enzyme (Section 2.4.1.2). A higher degree of purification can be achieved without such a high loss of enzyme. The amount of pure protein obtained from the
recombinant preparation is much higher than the native preparation, due to higher levels of activity in the cell extract and a significant reduction in the loss of enzyme during the purification procedure.

3.2.4 Co-factor Analysis of TSOX

The β subunit of TSOX was shown to contain the covalent flavin by separating the subunits by SDS-PAGE and then exposing the unstained gel to UV light (365nm). An intrinsic fluorescence was observed from the 43 kDa subunit, which was indicative of a covalently linked flavin (Figure 3.10). This result confirmed that ascertained by Chlumsky et al., (1998) for the Corynebacterial enzyme, who used mass spectrometry to locate the covalent flavin to residue His173 on the β subunit.

The non-covalently linked cofactors NAD⁺ and FAD were shown to be present in purified recombinant TSOX using HPLC (Section 2.4.3; Figure 3.11). Using a standard calibration curve for the area under the peak of the eluted FAD (348 nm) and by measuring the absorbance of the supernatant at 450 nm, the cofactor was shown to be present at ~1 mol FAD per mol of protein. NAD⁺ was also shown to be present at a concentration approximately equal to that of the protein, based on running a sample of standard NAD at the same concentration as the enzyme (30 μM).
Figure 3.9. (a) Time course for the growth of *E. coli* BL21 DE3 (open circles, Optical Density measured from a 1/10 diluted stock) transformed with pSOX at 21°C in 2xYT (supplemented with timentin 50 μg/ml) compared with SOX activity of cell free extracts (filled circles), for 500 ml cultures.
Figure 3.9. (b) 12.5% SDS PAGE analysis of the purification of recombinant TSOX from *E. coli* BL21/DE3 transformed with pSOX. Lane 1, sample of cell contents following French press; lane 2, 35-50% ammonium sulphate fraction; lane 3, pooled fractions following DE52 ion-exchange chromatography; lane 4, pooled fractions following phenyl sepharose hydrophobic interaction chromatography; lane 5, pooled fractions following Q sepharose ion-exchange chromatography; lane 6, same as for lane 5, except that the gel was exposed to U.V light to identify the subunit containing the covalently bound flavin.
Figure 3.10. HPLC cofactor analysis of TSOX. Panel A: 348 nm standards of FAD and FMN, Panel B: 348 nm supernatant from boiled TSOX, Panel C: 260 nm standards of AMP, FAD, and NAD, Panel D: 260 nm supernatant from boiled TSOX.
3.2.5 N-terminal Sequencing of Native Sarcosine Oxidase

Analysis of the DNA sequence was used to predict the initiation Met codons for the four genes of the sox operon, which compared well to those deduced for the Corynebacterial enzyme. A further analysis is described in which N-terminal sequencing was used to determine the N-terminal residues of each of the subunits of the native enzyme isolated from Arthrobacter sp. 1-IN (Table 3.3).

<table>
<thead>
<tr>
<th>sox B</th>
<th>sox D</th>
<th>sox A</th>
<th>sox G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted</td>
<td>Actual</td>
<td>Predicted</td>
<td>Actual</td>
</tr>
<tr>
<td>Met</td>
<td>Met</td>
<td>Met</td>
<td>-</td>
</tr>
<tr>
<td>Ala</td>
<td>Ala</td>
<td>Met</td>
<td>Met</td>
</tr>
<tr>
<td>Asp</td>
<td>Asp</td>
<td>Leu</td>
<td>Leu</td>
</tr>
<tr>
<td>Leu</td>
<td>Leu</td>
<td>Ile</td>
<td>Ile</td>
</tr>
<tr>
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<td>Glu</td>
<td>Glu</td>
</tr>
<tr>
<td>Pro</td>
<td>Pro</td>
<td>Cys</td>
<td>Ser</td>
</tr>
<tr>
<td>Glu</td>
<td>Glu</td>
<td>Pro</td>
<td>Tyr</td>
</tr>
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<td>Asn</td>
<td>Arg</td>
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<tr>
<td>Pro</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ser</td>
</tr>
</tbody>
</table>

Table 3.3. N-terminal sequencing results of the four subunits of TSOX from Arthrobacter sp. 1-IN, compared to the amino acid sequenced predicted from the DNA sequencing data. Two sets of results were obtained from the sox G subunit as two distinct bands of similar molecular weight were isolated from the gel.

The N-terminal sequencing data and translation of the DNA sequence have been used to identify the start and termination codons of the four sox genes. These results compared well with those mentioned by Chlumsky et al. (1995) for the Corynebacterial enzyme. The serine hydroxymethyltransferase gene terminates 2 bases before the start of soxB. The soxB and soxD genes are separated by 11 bases, but the stop codon of soxD overlaps with the start codon of the preceding gene, soxA. The start codon of soxG also overlaps with the end of the soxA gene (Figure 3.11).
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

<table>
<thead>
<tr>
<th>Junction</th>
<th>TSAX:</th>
<th>Ribosome Binding Sites</th>
<th>Translated Sequence Confirmed by N-terminal Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. soxB-soxD</td>
<td>GTGCCTAACACTACAGAGCCACCACATGCTGATTGAA</td>
<td>VAH End MMLIE</td>
<td></td>
</tr>
<tr>
<td>2. soxD-soxA</td>
<td>CAGGCAGGATCCCATGAGGCCAGAAAGCTAC</td>
<td>QGGSREnd MSQNKSY</td>
<td></td>
</tr>
<tr>
<td>3. soxA-soxG</td>
<td>GAAGGGACCCCGCGGGCTAGCAACCCCTGATTGAA</td>
<td>EGSSRRDG End MASNTLIE</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.11. Junctions between continuous sox genes, where translational-coupling occurs. Ribosome binding sites are underlined and the translated sequence that was confirmed by N-terminal sequencing is also shown.
This overlapping of genes, or genes separated by short intergenic regions, is associated with a phenomenon known as translational coupling (Normark et al., 1983). This idea proposes that the same ribosome or a component of it will translate two continuous genes, without dissociating from the mRNA. Potential ribosome-binding sites for the sox genes have been identified all of which lie within the preceding gene. This means that translation of an upstream gene, will terminate within the ribosome-binding site of the corresponding downstream gene, a feature required for the coupling effect.

In translational coupling the ribosome 'slides' back along the mRNA after termination to locate the start codon of the next gene without dissociating from it. This leads to the efficient translation of all the genes in the operon to the same level, and such activity is proposed to occur in G-C rich operons of Corynebacterium and related species (Normark et al., 1983). The model proposed for translational coupling, where the mRNA codon in the second site of the ribosome can 'slide' back 1-2 bases, allows the start and stop codon to overlap, as is the case between soxD and soxA. However, this model does not fit with the 8 base overlap seen between soxA and soxG. The N-terminal sequencing of the sox G subunit resulted in two sets of sequence, as there were two bands of similar molecular weight isolated together from the gel. The larger protein began at the predicted start site, showing that the protein is translated from within the previous gene. However, the smaller protein began with a protein sequence corresponding to a DNA sequence, downstream of the termination codon for the previous gene. There is no possible start codon for this protein, so it is likely that it is a proteolytic product of the larger protein.

3.2.6 Crystallography of TSOX

The native protein was the first to be purified and hence attempts were made to crystallise this protein. A small sample (8 mg/ml) was dialysed against water at pH 7. Sitting drop crystallisation screens were then set up as described in Section 2.7. Of the 100 different conditions screened, only a few produced any crystals, and only one condition gave uniform 'rod like' protein crystals, which could clearly be seen under the microscope. The crystallisation reagent used was 0.2 M magnesium chloride, 0.1 M Tris-HCl (pH 8.5) and 30% w/v PEG 4000. These conditions were explored further using the native protein, and the hanging drop method to try to produce larger crystals of a better quality. Increasing the concentration of the enzyme to 15-20
mg/ml caused it to precipitate when dialysed in water. Using larger volumes of the
diluted enzyme (6-8 mg/ml), against a more concentrated crystallisation solution in a
smaller volume (to produce the same final conditions) also resulted in precipitation of
the enzyme after a few hours. The crystallisation conditions were varied by reducing
the concentration and size of the precipitant PEG, or by eliminating it completely.
The salt and buffer conditions were also varied extensively, but no further crystals
were obtained with the native enzyme.

Purification of the native enzyme to a level where the folate affinity column
could be used effectively, became increasingly difficult to achieve and it was not
until pure recombinant enzyme was obtained that crystallisation of the protein
proceeded any further. Pure recombinant enzyme was stable when dialysed against
water pH 7.0 up to concentrations of 10-15 mg/ml. The sitting drop crystallisation
screens were set up as they were for the native protein and again, of the 100
conditions screened only one produced crystals. These conditions were not the same
as those for the native enzyme. The conditions identified were: 0.2 M sodium acetate
trihydrate, 0.1 M cacodylate pH 6.5 and 30% PEG 8000. As with the native enzyme
these conditions were reproduced in hanging-drop plates to try to improve the size
and quality of the crystals produced. These conditions were varied by increasing and
decreasing the concentration of PEG, and by reducing the concentration of salt and
buffer. Unfortunately, no crystals were formed or precipitation of the enzyme occured
in these trials, and varying the enzyme concentration did not improve crystallisation.

A further area that was explored in light of the crystallisation of MSOX from Bacillus
sp. (Trickey et al., 1999) was the use of inhibitor analogues such as pyrrole-2-
carboxylic acid (PCA) at saturating concentrations. The intention was to produce a
stable and uniform protein conformation that would aid the formation of a crystal
lattice. Saturating levels of NADH and FAD, were also used to aid crystal formation,
as it was thought that these non-covalent cofactors may dissociate under the
crystallisation conditions used leading to precipitation of the protein. However, none
of these techniques produced viable crystals from which a provisional data set could
be obtained. Structural data of this complex flavoprotein would be of great benefit to
any future work on the enzyme mechanism, and mutagenesis studies, and there are
plans to carry out a more exhaustive crystallisation trial based on this provisional
work.
3.2.7 Steady-state Kinetics

The horse-radish peroxidase assay was used to obtain kinetic constants for native and recombinant enzymes reacting with sarcosine and similar substrates. However, no detectable reaction rates could be measured with either creatinine or N,N-dimethylglycine, even at high concentrations of enzyme or substrate. Creatine hydrochloride salt was not very soluble in assay buffer, preventing analysis with this substrate. Data obtained for the natural substrate sarcosine is shown in Figure 3.12 and the kinetic constants are summarised in Table 3.4.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Native TSOX</th>
<th>Recombinant TSOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (M)</td>
<td>$1.07 \times 10^2 \pm 7.70 \times 10^4$</td>
<td>$9.84 \times 10^3 \pm 5.81 \times 10^4$</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$3.32 \pm 0.06$</td>
<td>$4.32 \pm 0.06$</td>
</tr>
<tr>
<td>$k_{cat} / K_m$</td>
<td>$3.1 \times 10^2 \pm 27.9$</td>
<td>$4.4 \times 10^2 \pm 32.1$</td>
</tr>
</tbody>
</table>

Table 3.4. Kinetic constants for native and recombinant TSOX, derived from steady-state data using the Michaelis-Menten Equation.

The $K_m$ of ~10 mM sarcosine obtained for native and recombinant enzyme is relatively high for a natural substrate. However, this value is comparable to $K_m$ values for sarcosine obtained for other heterotetrameric sarcosine oxidases: 5.4, 6.4 and 12.2 mM (Ogushi et al., 1987; Suzuki, 1981; Jorns, 1985). There is a modest difference in the $k_{cat}$ values between native and recombinant enzyme: 200 and 260 min$^{-1}$, respectively (reflected in their specific activities). These compare to a turnover rates of 348 and 408 min$^{-1}$ (Suzuki, 1981; Ogushi et al., 1987) obtained for other TSOX enzymes under different conditions (pH 8.3 and 37 °C).
Figure 3.12. Steady-state data produced using the horse-radish peroxidase coupled assay in 20 mM potassium phosphate buffer, pH 7.3 at 25°C. Native TSOX open circles, recombinant TSOX filled circles.
3.3 Discussion

3.3.1 Sequence Similarities

Although the soxD and soxG genes showed no sequence homologies with previously published sequences, the N-terminal region of the soxD gene contains a pair of cysteine residues separated by two other residues. This CxxC motif has been implicated in active site disulphides, which assist electron flow in the redox reactions of the thiol oxidoreductases (Chivers et al., 1997). Work on the recombinant TSOX from Corynebacterium concluded that a flavin-thiolate adduct is involved in inactivation of the enzyme, and stabilisation of the flavin prior to reaction with sarcosine, although no specific residue has been assigned to this role (Chlumsky et al., 1993). Studies of the Corynebacterium TSOX by a different group showed that two cysteines are modified by iodoacetamide resulting in loss of enzyme activity (Hayashi et al., 1983). Also the extent of enzyme inhibition was proportional to the square of Hg²⁺ concentration, indicating again that at least two cysteines are involved in catalytic activity.

The sequence identity of sox B with the MSOX enzymes (22-25% identity, 45-47% similarity) is particularly significant. This is because the crystal structure of MSOX from Bacillus sp. B-0618 in the presence of various inhibitors has been solved and catalytically important residues have been identified (Trickey et al., 1999). These residues, at or near the active site are conserved in the monomeric and heterotetrameric sarcosine oxidases, and their substitution in other enzymes of the family may account for changes in substrate specificity. Although the β subunit of TSOX shows only modest sequence identity (23% identity) with MSOX from Bacillus sp. B-0618, all the residues implicated in catalysis are conserved, including two residues which bind the substrate carboxylate. The conservation of putative catalytic residues in βTSOX strongly suggests that this subunit binds non-covalent FAD. This conclusion is consistent with the presence of a single binding site for sarcosine near FAD that serves for the input of electrons in TSOX (Zeller et al., 1989). The covalent FMN attachment site in βTSOX aligns with Ser149 in MSOX; the hydroxyl group of this serine is located on the protein surface on the si face of the FAD ring, about 8 Å from the flavin N(5) atom. This places Ser149 on the side of the protein opposite to the channel, leading to the substrate-binding cavity on the re face of the FAD ring.
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The homologies between the β subunit of TSOX, MSOX and the N-terminal half of DMGDH, SDH and DMGO, indicate that they may all have evolved from a common ancestral flavoprotein that contained a covalent prosthetic group. A covalent flavin linkage was shown to be present in the Arthrobacterial β subunit of TSOX using a fluorescence technique (Section 3.5). As the protein sequence for the β subunit of *Arthrobacter* sp. TSOX was virtually identical to that of the Corynebacterial enzyme (99% identity), it is likely that His 173, shown to be the site of covalent FMN attachment in this enzyme (Chlumsky *et al.*, 1998), is the same in the Arthrobacterial enzyme. The covalent attachment sites in DMGDH (His 84) and SDH (His 108) is a histidine residue located much closer to the ADP binding site and aligns with histidines in MSOXs (His 45), MTOX (His 44), PIPOX (His 49) and DMGO (His 48) but not in βTSOX where an alanine (Ala 63) is found instead (Chlumsky *et al.*, 1995). In the MSOX structure, however, the flavin is linked covalently to Cys315, but this residue is spatially near the conserved histidine, which may play a role in covalent flavinylation (Trickey *et al.*, 1999). It is likely that MSOX, MTOX and PIPOX bind FAD covalently via their conserved cysteine, whilst SDH, DMGDH and DMGO bind flavin at the histidine near the ADP site. SDH, DMGDH and DMGO lack this conserved MSOX cysteine residue and the covalent flavin attachment site has been shown to be His84 in DMGDH which shows closer sequence identity to SDH and DMGO.

The presence of two ADP binding sites in TSOX as well as a site for covalent flavinylation (His 173) lead to the original conclusion that the covalent flavin was bound to the β subunit and that the non-covalent FAD was associated with the ADP binding domain of the α subunit. However, in the light of unpublished results by Jorns and co-workers which show that the covalent FMN bound to the β-subunit binds at the interface with another subunit and that FAD must bind in close proximity to sarcosine (Zeller *et al.*, 1989), it is likely that both flavins bind to the β subunit. Further support for this proposal can be seen in the sequences of the two ADP binding domains. The last residue of the ADP binding motif can be an indicator as to whether the motif binds FAD or NADH. In the β subunit, the ADP binding domain ends with a glutamate residue, common to flavoproteins that bind flavin non-covalently (asparagine in MSOX that binds FAD covalently). In the α subunit the ADP binding domain ends with an asparagine residue common to NADH binding.
proteins. Recent work on sequence motifs in flavoproteins based on sequence and structural information (Vallon, 2000), has identified conserved motifs present in further strands and loops of the Rossmann fold, downstream of the GxGxxG motif. These include a GG doublet located 5-8 residues after the ADP binding domain and an ATG motif found in FAD and NADH binding domains forming the fourth strand of the Rossman fold. The GG doublet is found after both ATP binding domains but the ATG motif is only found in the α subunit, which is thought to bind NAD⁺. The GG motif is mainly associated with non-covalently bound FAD and is found in βTSOX but not in the other proteins identified from the BLAST search, which only bind covalent flavin.

The BLAST searches of the β & α subunits of TSOX have identified sequence similarities in this expanding family of flavoenzymes which catalyse analogous oxidation reactions with secondary and tertiary amino acids (Figure 3.13). The homologies have also helped to identify differences between the monomeric and tetrameric forms of sarcosine oxidase as the presence of the large α subunit in the heterotetrameric form confers some extra function to the enzyme complex. Both forms catalyse the oxidative demethylation of sarcosine to produce formaldehyde, hydrogen peroxide and glycine. However, the TSOX enzymes are also able to utilise tetrahydrofolate as a co-substrate, generating 5-10-methylenetetrahydrofolate instead of formaldehyde (Kalvenes-Krick & Jorns, 1987). This activity is due to the presence of two folate binding domains in the α subunit identified by homologies with T-proteins and the mammalian SDH and DMGDH all of which are known to catalyse the synthesis of 5-10-methylenetetrahydrofolate, indicating they may have evolved from a common tetrahydrofolate-binding protein. A folate binding motif has not been formally identified, even for those enzymes that are folate dependent, but it is likely that it will consist of residues conserved in this set of proteins. A 52-residue region of the α subunit, shows a region of highly conserved residues that could be implicated in folate binding. An ancestral 10-formyltetrahydrofolate binding protein has been proposed as an evolutionary precursor for 10-formyltetrahydrofolate dehydrogenase and 5’phospho-ribosylglycinamide (Cook et al., 1994). The presence of identities from the N and C terminal regions of DMGDH and SDH may indicate that these enzymes evolved from a fusion of βTSOX, or a similar amino acid oxidising flavoprotein and the C terminal half of αTSOX, or a similar folate binding protein.
Chapter 3: Sequencing, Expression and Characterisation of Sarcosine Oxidase

Figure 3.13. Summary diagram of the sequence homologies identified by BLAST searches with: (A) β subunit of TSOX and (B) α subunit of TSOX. The yellow region labelled (ABM) represents the high level of sequence identity observed in the ADP binding motif. The blue region indicates an area thought to contain residues important for folate binding and the hashed area indicates low sequence homology.
The N-terminal half of αTSOX which contains the ADP binding region shows homology with octopine and nopaline oxidases which are known to catalyse oxidative cleavage reactions with N-substituted arginine derivatives similar to the sarcosine oxidation reaction. It has been shown previously, however, that TSOX is not reduced by anaerobic incubation with either compound (Chlumsky et al., 1995). Sequence comparisons of N-terminal αTSOX with the hydrogen cyanide synthase genes may indicate a common structure or function for these two multi-subunit enzymes. The N-terminal half of soxA shows similarity to HcnB (32% identity for a stretch of 170 residues) and soxB with Hcn C (24% identity). A mechanism for glycine dehydrogenation has been proposed (Wissing, 1974) in which glycine is oxidised to iminoacetic acid [H-C(NH)-COOH], followed by glycine cleavage in a second dehydrogenation reaction involving the breakage of the C-C bond. There appears to be no evidence in the literature for measurement of glycine production by the TSOX enzymes and this unusual glycine cleavage maybe linked to the use of 5,10-methylenetetrahydrofolate by these enzymes.

The sox operon codes for a series of genes which code for proteins involved in regulating levels of tetrahydrofolate C-1 pool. Based on the recently sequenced Arthrobacter sp. DNA fragment containing the DMGO gene (Meskys, Harris, Basran, and Scrutton, submitted for publication), it is likely that a series of genes lie in close proximity in the Arthrobacter genome and may be co-expressed by a translational coupling mechanism. Figure 3.14. shows a possible metabolic scheme illustrating possible fates of the methyl group of sarcosine, involving both serine hydroxymethyltransferase (glyA) and formyl tetrahydrofolate hydrolase (purU), found in the sox operon. It is likely that genes for other folate dependent enzymes will be found surrounding the sox operon. Evidence to support this is found at the start of the sequenced DNA fragment containing the DMGO gene which has the purU gene located upstream of the DMGO gene, the purU gene is found downstream of the sox genes.
Figure 3.14. Diagram of metabolic reactions in *Arthrobacter* sp. involving dimethylglycine dehydrogenase (DMGO), sarcosine oxidase (TSOX), serine hydroxymethyl transferase (SHMT), 10-formyltetrahydrofolate hydrolase (FTHH) and formaldehyde dehydrogenase (FDH), showing the possible reaction pathways of the methyl group of sarcosine.
3.3.2 Heterotetrameric Sarcosine Oxidase from *Arthrobacter* sp. I-1N

Heterotetrameric sarcosine oxidase from *Arthrobacter* sp. I-1N has a molecular weight of 180,000, similar to the molecular weight of the *Corynebacterium* sp. P-1 (178,700; Chlumsky *et al.*, 1995) determined from the amino acid sequence. The subunits are also of similar size to the *Corynebacterium* sp., (α: 100 kDa, β: 46 kDa, δ: 13 kDa, γ: 22 kDa). These compare well to those estimated by SDS PAGE but should be confirmed by mass spectrometry to check for degradation, premature termination and the presence of other co-factors.

Recombinant expression of TSOX has been achieved using the pET expression system and the enzyme can be easily purified for kinetic and structural studies. The steady state assays indicated that the purified enzyme was active with similar rates to those observed in other sarcosine oxidases (Suzuki, 1981; Ogushi *et al.*, 1987). The production of crystals of TSOX suitable for structural determination has been unsuccessful, but conditions have been identified for crystal formation, which may prove useful to any further crystallisation studies.

The presence of 1 mol FAD and NAD per mol of flavin has been shown, confirming results that identified these co-factors in the Corynebacterial enzyme. Using this information and the extinction coefficient determined previously for TSOX, it was possible to demonstrate that the enzyme was fully flavinylated. Complete non-covalent flavinylation is important for determining enzyme activity, particularly in the following chapter where reduction of the non-covalent FAD by sarcosine is monitored in stopped-flow experiments.
Chapter 4

Mechanistic Studies of Carbon-Hydrogen Bond Breakage in Sarcosine Oxidase
Chapter 4: Carbon-Hydrogen Bond Breakage in Sarcosine Oxidase

4.1 Introduction

4.1.1 Mechanistic Studies of Flavoproteins

Amine oxidation reactions catalysed by flavoproteins involve the cleavage of at least one C-H bond and the transfer of two electrons to flavin. An oxidising agent (e.g. a second substrate, a protein or O₂) is then used to reoxidise the reduced flavin and complete the catalytic cycle. Thus, catalysis always involves a reductive half-reaction, where the enzyme-bound flavin is reduced, and an oxidative half-reaction where the reduced flavin is reoxidised. As the spectrum of oxidised flavin is different to that of reduced flavin, transitions between the two forms can be monitored easily using stopped-flow techniques. The kinetic mechanisms of a number of amine oxidising flavoproteins [e.g. D-amino acid oxidase (Pollegioni et al., 1997) and monoamine oxidase (Ramsay, 1991)] have been studied in detail.

Exploring the catalytic mechanism of a complex flavoprotein like TSOX involves using a variety of steady-state and pre-steady-state kinetic techniques. Previous steady-state experiments carried out on TSOX led to the elucidation of a general kinetic mechanism (Section 1.2.3; Ali et al., 1991). This, and the fact that difficulties are encountered with the accuracy of the steady-state assay when varying physical parameters such as temperature and pH, meant that the separate steps of the enzyme mechanism were investigated using stopped-flow methods. This chapter reports on the results obtained from a stopped-flow analysis of the first stage of the reaction catalysed by TSOX, i.e. the reductive half-reaction. The majority of flavoproteins contain a single flavin whose rate of reduction by the substrate can be measured under anaerobic conditions using rapid mixing techniques. However, TSOX contains two flavin molecules, which presents difficulties when studying the rate of flavin reduction since the spectral properties of the flavins overlap and cannot be separated easily. Even under anaerobic conditions, the transfer of electrons between flavins produces multi-phasic reaction transients making the identification of individual reaction rates difficult. Fortunately, in the case of TSOX the two flavin molecules have different properties, since one flavin is non-covalently bound (FAD) whereas the FMN is covalently linked to the protein. The covalent FMN reacts with sulphite to form a covalent adduct which results in a bleaching of its visible spectrum, without affecting the spectrum of the non-covalent flavin (Jorns, 1985). By forming the TSOX-sulphite complex, the rate of reduction of non-covalent FAD can be
monitored under aerobic conditions, since formation of the FMN-sulphite-adduct also prevents the transfer of electrons to oxygen (Ali et al., 1991).

### 4.1.2 Use of pH and Temperature Effects as Probes for Studying Enzyme Mechanism.

The pH dependence of an enzyme catalysed reaction can be of particular value since it may reveal protonation state(s) that are important for enzyme activity. A simple measurement of the pH dependence of a rate at a constant substrate concentration is of little value, as this contains all the pKₐ values of a kinetic model for free enzyme and free substrate, and any complexes or intermediates of the reaction. Therefore, in order to obtain useful information, it is necessary to investigate the effects of pH across a range of substrate concentrations, and obtain kinetic parameters for the reaction whose pH dependence can then be analysed.

Using a simplified model for an enzymatic reaction, it is possible to demonstrate how the pH dependence of kinetic parameters can be used to separate ionisations, into those involved in substrate binding and those important in the catalytic complex (E.S) (Tipton & Dixon, 1979). Consider the following system:

\[
\begin{align*}
\text{EH}_2 & \rightleftharpoons k_{+1}^{E} k_{-1}^{E} \text{EH}_2 S \\
\left[ K_E \right] & \left[ K^{ES} \right] \\
\text{EH}^- & \rightleftharpoons k_{+1}^{E} k_{-1}^{E} \text{EHS}^- \rightarrow k_+ k_2 \text{EH}^- + P \\
\left[ K_E \right] & \left[ K_{ES} \right] \\
\text{E}^2- & \rightleftharpoons k_{+1}^{E} k_{-1}^{E} \text{ES}^2- \\
\end{align*}
\]

where \( K^E \) represents dissociation constants of the free enzyme and \( K^{ES} \) represents dissociation constants of the enzyme-substrate complex. The whole system is in a quasi-equilibrium both horizontally and vertically; the substrate can bind to all three ionisation states of the enzyme. The overall rate of a pre-steady state reaction for the above mechanism produces the following equation (Alberty & Massey, 1955):
Chapter 4: Carbon-Hydrogen Bond Breakage in Sarcosine Oxidase

\[ k_{\text{obs}} = \frac{k_{\text{lim(max)}}}{1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_{i}^{\text{ES}}} (1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_i^{\text{ES}}})} \]

Equation 4.1

Where \( k_{\text{lim(max)}} = k_{s2} \) and \( K_{d(\text{max})} = (\Sigma k_{s1} + k_{s2})/\Sigma k_{s1} \); these parameters are corrected for pH and represent the limits to which the observed \( k_{\text{lim}} \) and \( K_d \) values vary with pH between the relevant \( pK_i \) and \( pK_{H} \) values. By comparison of Equation 4.1 with the Strickland equation:

\[ k_{\text{obs}} = \frac{k_{\text{lim}}[S]}{K_d + [S]} \]

Equation 4.2

it can be seen that the \([H^+]\) will affect both the observed \( K_d \) and \( k_{\text{lim}} \) values. The observed \( k_{\text{lim}} \) value can therefore be described by the following equation:

\[ k_{\text{lim}} = \frac{k_{\text{lim(max)}}}{1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_i^{\text{ES}}} (1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_i^{\text{ES}}})} = \frac{k_{\text{lim(max)}}}{1 + 10^{(pK_i^\text{ES}-pH)} + 10^{(pH-pK_i^\text{ES})}} \]

Equation 4.3

The \( k_{\text{lim}} \) value will thus be dependent on ionisations of the enzyme-substrate complex.

The observed \( K_d \) value can be given by the equation below:

\[ K_d = K_{d(\text{max})} \frac{1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_i^{\text{ES}}} (1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_i^{\text{ES}}})}{1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_i^{\text{ES}}} (1 + \frac{[H^+]}{K_i^{\text{ES}}} + \frac{K_i^{\text{ES}}}{K_i^{\text{ES}}})} = K_{d(\text{max})} + 10^{(pK_i^\text{ES}-pH)} + 10^{(pH-pK_i^\text{ES})} \]

Equation 4.4

Therefore \( K_d \) will be affected by ionisations of the enzyme-substrate complex and free enzyme and free substrate. Combining Equations 4.3 and 4.4 gives:

\[ \frac{k_{\text{lim}}}{K_d} = \frac{k_{\text{lim(max)}}/K_{d(\text{max})}}{1 + 10^{(pK_i^\text{ES}-pH)} + 10^{(pH-pK_i^\text{ES})}} \]

Equation 4.5

Hence the \( k_{\text{lim}}/K_d \) value depends only on the ionisations of the free enzyme and the free substrate. The pH dependence of kinetic parameters (\( k_{\text{lim}} \) and \( k_{\text{lim}}/K_d \)) can therefore be used to determine which ionisations are important in binding and those important in the catalytic complex. Reaction rates are measured over the desired pH range and the kinetic parameters can be obtained by fitting the data to the above
equations. The resulting \( pK_a \) values can then be assigned to specific groups on the protein and/or the substrate. For the reductive half-reaction of flavoenzymes, the pH dependence of the limiting rate of flavin reduction, \( k_{\text{lim}} \), indicates ionisations important in the enzyme-substrate complex, whereas the pH dependence of the kinetic parameter, \( k_{\text{lim}}/K_d \), reveals ionisations important in the free enzyme and/or the free substrate.

An increase in temperature causes a corresponding increase in the rate of an enzyme-catalysed reaction, within the range of the stability of the enzyme. The temperature dependence of a reaction can provide important information about its thermodynamic properties. The rate constant for a reaction increases with temperature according to the Arrhenius equation:

\[
k = Ae^{(-\Delta E_a/RT)}
\]  

Equation 4.6

where \( k \) is the rate constant \( (s^{-1}) \), \( A \) is the pre-exponential factor, \( T \) the absolute temperature \( (K) \), \( \Delta E_a \) is the activation energy for the reaction \( (kJ \text{ mol}^{-1}) \) and \( R \) is the gas constant. Temperature dependence data is usually presented in the form of an Arrhenius plot (\( \ln k \) versus \( 1000/T \)), where the activation energy can be determined from the slope of the plot \( (-\Delta E_a/RT) \) and the pre-exponential factor, \( A \), from the intercept. With knowledge of the activation energy, \( \Delta E_a \), other thermodynamic parameters can be determined, such as the enthalpy, entropy and free energy of formation of the transition state.

In a reaction where C-H bond cleavage is rate limiting, an Arrhenius plot can be used to calculate the activation energy for C-H bond breakage. In systems where tunnelling has been detected the difference in activation energies can be much greater than this as protium will tunnel more effectively than deuterium. Deviations from classical behaviour observed by curvature of an Arrhenius plot are explored using the Kinetic Isotope Effect (KIE). This is used to gain a greater understanding of the mechanism of H-transfer by observing the effect deuterated substrate has on the temperature dependence of the limiting rate of reduction \( (k_{\text{lim}}) \). KIEs have been used to explore non-classical behaviour of H-transfer where KIEs > 7 have indicated quantum tunnelling of hydrogen (Bahnson & Klinman, 1995). Also large differences in the activation energies for protium and deuterium \( (\Delta \Delta E_a > 4.8 \text{ kJ mol}^{-1}) \) and values for the ratio of the pre-exponential factors not equal to 1 can indicate non-classical behaviour. These criteria have been used to demonstrate H-tunnelling in several
enzyme systems. A prediction from a static barrier plot is that tunnelling becomes more prominent as the apparent activation energy decreases and that temperature independent tunnelling of both protium and deuterium should be observed. However, recent work of Basran et al. (1999) on MADH, has shown temperature dependent tunnelling of protium and deuterium (Section 1.3.2.10). The data was modelled using a fluctuating potential energy barrier, which takes into account the dynamic motion of the protein. Since this was a novel mechanism for the breakage of a C-H bond it was of interest to see if TSOX exhibited similar non-classical behaviour during H-transfer.

4.2 Results

4.2.1 Titration of TSOX with sodium sulphite

Formation of a reversible covalent complex involving nucleophilic attack of sulphite at the N5-position of the enzyme bound flavin is a property characteristically observed with flavoprotein oxidases (Massey et al., 1969). The formation of this adduct can be followed spectrally and dissociation constants are usually in the micromolar range. The binding of sulphite is affected by the accessibility of the flavin N5 atom and by the presence of a positively charged group (e.g. the protonated side chain of a histidine or arginine residue), which stabilises the anion developed at the N1 portion of the flavin.

Reactivity of TSOX with sulphite has been shown for the Corynebacterial enzyme (Jorns, 1985). Herein it is shown that the Arthrobacterial enzyme also forms an adduct by titration of the enzyme with sulphite (Figure 4.1). A progressive loss in absorbance is observed at 450 nm corresponding to the formation of the sulphite complex; this reaction was shown to be reversible, since the addition of formaldehyde (a sulphite scavenger) resulted in a return to the original unbleached spectrum (Figure 4.1).
Figure 4.1. Effect of sarcosine on sulphite treated TSOX 20mM phosphate buffer pH 7, 25°C. Spectrum 1, oxidised enzyme (12μM); spectrum 2, TSOX in the presence of excess sulphite (20mM); spectrum 3a, reduced spectrum recorded after the addition of 4 mM sarcosine (spectrum stable for up to 3 h). Spectrum 3b, Addition of 5mM formaldehyde removes the sulphite from TSOX to yield oxidised enzyme.
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Figure 4.2. Titration of sarcosine oxidase with sulphite Spectrum 1, oxidised TSOX (8 μM); spectra 2-8 inclusive, addition of sulphite to give a final concentration of 50 μM, 100 μM, 200 μM, 500 μM, 1 mM, 6 mM, and 10 mM, respectively. Inset: Plot of $1/\Delta A_{450}$ versus $1/[SO_3^{2-}]$, $K_d = 333$ μM. Conditions: 20 mM sodium pyrophosphate buffer pH 8.5, 200 mM KCl at 25°C. Titrations were performed at different pH values and adduct dissociation constants were determined: (Table 4.1)

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_d$ sulphite (mM)</th>
<th>20 mM Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>0.071</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>7.0</td>
<td>0.095</td>
<td>Potassium phosphate</td>
</tr>
<tr>
<td>8.0</td>
<td>0.23</td>
<td>Sodium pyrophosphate</td>
</tr>
<tr>
<td>8.5</td>
<td>0.33</td>
<td>Sodium pyrophosphate</td>
</tr>
<tr>
<td>9.0</td>
<td>4.60</td>
<td>Sodium borate</td>
</tr>
<tr>
<td>10.0</td>
<td>10.0</td>
<td>Sodium borate</td>
</tr>
</tbody>
</table>

Table 4.1. TSOX sulphite adduct dissociation constants determined at different values of pH.
Chapter 4: Carbon-Hydrogen Bond Breakage in Sarcosine Oxidase

As discussed in the introduction (Section 1.2.2.2) only one of the two flavins (FMN) in TSOX reacts with sulphite as only 47% of the flavin absorbance at 450 nm is bleached upon addition of sulphite, with no further change after subsequent additions (Figure 4.2). The TSOX-sulphite complex is catalytically inactive since no activity was detected under steady-state assay conditions in the presence of excess sulphite. However, activity could be restored when a sample of sulphite-treated enzyme was diluted, hence dissociating the complex under assay conditions. Jorns (1985) had previously shown that it is the FMN involved in oxidase activity. The FMN reacts with sulphite and thus its modification accounts for loss of enzyme activity. For the Arthrobacterial enzyme, the addition of sarcosine to the sulphite-treated enzyme resulted in the rapid reduction of the sulphite unreactive flavin (Figure 4.1). Sulphite inhibition of TSOX oxidase activity was further demonstrated when low levels of sarcosine (4 mM) were added to the sulphite-treated enzyme under aerobic conditions. Unlike with non-treated enzyme, there was no reoxidation of the flavin in the TSOX-sulphite treated protein (Figure 4.1).

The transfer of electrons through the two flavins in Corynebacterial TSOX has been investigated using anaerobic stopped-flow techniques and shown to proceed by the following mechanism:

\[
\begin{align*}
E_{OX} + S & \rightleftharpoons E_{OX}\cdot S \rightarrow E_{INT} \rightleftharpoons E_{INT} + S \rightarrow E_{RED}
\end{align*}
\]

Scheme 4.2

Scheme 4.2. Kinetic scheme for TSOX reduction with sarcosine. An initial complex is formed with the fully oxidised enzyme \(E_{OX}\), which reacts to form an intermediate \(E_{INT}\). This intermediate represents the 2-electron reduced enzyme which reacts with a further molecule of sarcosine to yield the fully reduced species \(E_{RED}\) (Ali et al., 1991).

Studying the mechanism of substrate reduction is complicated by the presence of two flavins, the binding of two molecules of sarcosine and internal electron transfer between the flavins, all of which give rise to complex spectral changes (Ali et al., 1991). This can be simplified by studying reduction of the non-covalent FAD in sulphite-treated TSOX. In this case, the enzyme can only accept two electrons from the substrate since internal electron transfer is prevented by the
formation of the sulphite adduct FMN. Another advantage of this approach is that it is not necessary to work under anaerobic conditions.

The method of using sulphite-treated TSOX in the stopped-flow has been shown to work for FAD reduction in the Corynebacterial enzyme where a single reduction rate was determined, but the technique had not been exploited further (Ali et al., 1991). To use this procedure in stopped-flow studies of the reductive half-reaction of Arthrobacterial TSOX, the reactivity with sulphite was investigated in detail. Sulphite titrations were performed over a pH range from 6.5 –10.5 and dissociation constants were calculated at each pH (Table 4.1). Additionally, these dissociation constants were shown not to be significantly affected by temperature (data not shown). It was found that observed dissociation constants were tighter at lower pH values, but that a concentration of 20 mM sulphite was sufficient at all pH values to maintain inhibition of oxidase activity.

4.2.2 Analysis of FAD Reduction

4.2.2.1 Multiple Wavelength Photo Diode Array Analysis

Before commencing a detailed study of FAD reduction in sulphite-treated TSOX using single wavelength stopped-flow methods, enzyme reduction was followed using photodiode array spectroscopy to confirm the effectiveness of excess sulphite treatment on inhibiting oxidase activity. Experiments were performed aerobically by mixing equal molar concentrations of sarcosine and sulphite-treated TSOX. The data were collected over 200 s and fitted globally by numerical integration methods using Prokin software supplied by Applied Photophysics. The data were best fitted to a two step model:

\[ A \rightarrow B \rightarrow C \]

Where A represents sulphite-treated enzyme in its oxidised form, B is the 2-electron reduced form of sulphite-treated TSOX (large reduction in absorbance at 450 nm) and C represents an alternate form of reduced sulphite treated TSOX, where the absorbance at 450 nm is reduced further. This slow phase might reflect further reduction of the FAD or may be the result of product dissociation or a conformational change due to trapping the enzyme in the EH₂ state.

When the experiment was repeated and the data collected over a longer time scale (>1000 s) it was possible to detect a slight increase in absorbance at 450 nm (Figure 4.3). This occurs after 600 s and is the result of the reversible binding of
sulphite to the covalent FMN. During prolonged incubation with substrate, molecular oxygen is able to remove electrons from the enzyme via transfer to the FMN resulting in reoxidation of FAD. However, these results clearly show that the reductive phase of the reaction can be resolved from the oxidative phase in sulphite-treated TSOX.

### 4.2.2.2 Single Wavelength Stopped-flow Studies of TSOX

The rate of flavin reduction in TSOX was also analysed at a single wavelength of 450 nm [i.e the peak of the flavin absorbance spectrum for TSOX (Figure 4.3 & 4.4)]. These reductive transients were biphasic in nature with the fast phase contributing ~70% of the total absorbance change. When transients were collected over a longer time course, there was a slight increase in absorbance at 450 nm owing to TSOX reoxidation. Further studies of the concentration dependence of the fast rate showed that it was hyperbolically dependent on sarcosine concentration, which is consistent with the formation of a Michaelis (E.S) complex prior to flavin reduction. Reaction rates for the fast phase were analysed using the Strickland equation (Figure 4.5). The slow phase of the reaction was shown to be independent of sarcosine concentration (~6 s⁻¹), over the range studied (2.5 –140 mM). The slow phase was observed at pH values of 6.5–8.5, and was more prominent at lower concentrations of sarcosine. At pH values greater than 9, the reductive transients were monophasic due to the loss of the slow phase. The significance of the slow phase was not investigated further, but may be the result of product dissociation.
Figure 4.3 Photo diode array analysis of the reaction of sulphite-treated TSOX (16.5 μM) with stoichiometric sarcosine (16.5 μM). Conditions: 20 mM sodium pyrophosphate buffer, pH 8.5, 20 mM sodium sulphite, 180 mM KCl, 25°C. Panel A: Time dependent spectral changes on mixing sulphite-treated TSOX with sarcosine recorded over 260 s, first spectrum recorded after 1.3 s (only selected subsequent spectra are shown). Panel B: Deconvoluted spectra from the reaction shown in panel A; spectrum 1: enzyme species A, sulphite-treated TSOX; spectrum 2: enzyme species B following the fast phase of the reductive half-reaction; spectrum 3: enzyme species C following the slow phase of the reductive half-reaction.
Figure 4.4. Single wavelength stopped-flow spectroscopy of sulphite-treated TSOX with sarcosine. Conditions are as for Figure 4.3. Panel A: Transient obtained at 450 nm showing the biphasic nature of the reductive half-reaction. Panel B: shows the same experiment over a longer timescale to illustrate the slight reoxidation of 2-electron reduced, sulphite-treated TSOX by molecular oxygen.
Figure 4.5 Substrate dependence in potassium phosphate buffer pH 8.0 of $k_{\text{fast}}$ (open circles) and $k_{\text{slow}}$ (closed circles), for TSOX, 25°C. $k_{\text{fast}}$ was fitted to the Strickland equation (Equation 2.4) to obtain kinetic parameters.
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4.2.3 pH Dependence of the Reaction Catalysed by TSOX

The pH dependence of flavin reduction in TSOX by sarcosine was studied over the pH range 6.5 - 10.5, the range over which the enzyme is stable. The data at each pH value was fitted to the Strickland equation to determine the limiting rate of flavin reduction, \( k_{\text{lim}} \), and the dissociation constant for the E.S complex, \( K_d \). These values are shown in Table 4.2. The pH dependence of the kinetic parameters was then explored to identify ionisations important in the enzyme-substrate complex or free enzyme and free substrate (Figure 4.6).

<table>
<thead>
<tr>
<th>pH</th>
<th>( k_{\text{lim}} ) (s(^{-1}))</th>
<th>( K_d ) (mM)</th>
<th>( k_{\text{lim}}/K_d ) (mM(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>244 ± 92</td>
<td>940 ± 400</td>
<td>0.26 ± 0.21</td>
</tr>
<tr>
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<td>246 ± 45</td>
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<td>0.87 ± 0.39</td>
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<tr>
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<td>103 ± 6</td>
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<td>4.9 ± 1.2</td>
</tr>
<tr>
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</tr>
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<td>13 ± 2</td>
<td>11 ± 2.0</td>
</tr>
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<td>9.0</td>
<td>115 ± 5</td>
<td>10 ± 2</td>
<td>12 ± 2.8</td>
</tr>
<tr>
<td>9.5</td>
<td>128 ± 3</td>
<td>14 ± 1</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>10</td>
<td>127 ± 2</td>
<td>16 ± 1</td>
<td>7.9 ± 0.6</td>
</tr>
<tr>
<td>10.5</td>
<td>141 ± 3</td>
<td>23 ± 2</td>
<td>6.1 ± 0.7</td>
</tr>
</tbody>
</table>

Table 4.2. Limiting rate constants and dissociation constants for flavin reduction of sulphite-treated TSOX by sarcosine.

From Figure 4.6B, it can be seen that the \( k_{\text{lim}} \) values are independent of pH over the range studied indicating that there are no kinetically influential ionisations in the enzyme-substrate complex. The relatively high errors associated with the \( k_{\text{lim}} \) values determined below pH 7.0 are due to the high values for \( K_d \) which produce poorly defined hyperbolic plots. The pH profile for \( k_{\text{lim}}/K_d \) identifies kinetically influential ionizations in the free enzyme and/or free substrate, and in this case shows a bell-shaped dependence. These data were fitted to Equation 2.7 to determine pK\(_a\) values of 7.4 ± 0.1 and 10.4 ± 0.2. Sarcosine is zwitterionic at almost all pH values in the range studied, and has pK\(_a\) values of 2.23 and 10.01 (Budavari, 1989). It is
therefore possible that the pK$_a$ value of 10.4 represents the ionisation of the secondary amine group in free sarcosine, although it is also possible that it may represent the ionisation of a protein residue. The pK$_a$ value of 7.4 is attributed to a protein ionisation, since this value is too high to be the pK$_a$ for the carboxylate group of sarcosine (pK$_a$ = 2.23).
Figure 4.6. pH dependence of FAD reduction in sulphite treated TSOX. Panel A: Plot of $k_{lim}/K_d$ versus pH. The data were fitted to the equation for a double ionisation (equation 2.) to calculate the macroscopic p$K_a$ values of 7.4 ± 0.1 and 10.4 ± 0.2. Panel B: Plot of $k_{lim}$ versus pH showing no apparent dependence of $k_{lim}$ on pH across the range studied.
Chapter 4: Carbon-Hydrogen Bond Breakage in Sarcosine Oxidase

4.2.4 Kinetic Isotope Effect

The mechanism of C-H bond breakage by TSOX was investigated by studying the kinetic isotope effect (KIE). The KIE was determined by measuring the substrate dependence of the rate of FAD reduction at 25°C and pH 8.5 with both sarcosine and \(N\)-methyl-\(d_3\)-glycine (deuterated sarcosine) in the range 2.5 - 150 mM (Figure 4.7). As expected, the dependence on substrate concentration was hyperbolic and were fitted to the Strickland equation. Deuteration had no affect on binding of sarcosine, but did affect the limiting rate of flavin reduction (Equation 4.2). The ratio of \(k_{\text{lim}}\) values for protonated versus deuterated substrate (the kinetic isotope effect) was calculated to be 7.3. This shows that the rate of the flavin reduction step in TSOX is limited by C-H bond cleavage.

The temperature dependence of the maximum rate (\(k_{\text{lim}}\)) for flavin reduction was investigated using substrate concentrations of 150 mM (> 10 x \(K_d\)) at pH 8.5 from 5°C to 40°C. The temperature dependence of a unimolecular rate constant is given by Equation 4.7:

\[
\ln \left( \frac{k}{T} \right) = \ln \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT}
\]

where \(k_B\) and \(h\) are the Boltzmann and Planck constants respectively. A plot of \(\ln(k/T)\) versus 1000/T is linear and can be used to calculate \(\Delta H^\ddagger\) and \(\ln A^H\) or \(A^D\) (Figure 4.7). This method of plotting the temperature dependence of a unimolecular reaction using Equation 4.7 is preferred instead of the classical Arrhenius plot (i.e. \(\ln(k)\) versus 1/T) since the latter is not linear over large temperature ranges. The activation energy, \(\Delta H^\ddagger\), is calculated from the slope of the plot and \(\Delta S^\ddagger\) is calculated by extrapolation back to the ordinate-axis. The data indicate that the KIE is independent of temperature and that the difference in activation energies for protium versus deuterium transfer (0.6 ± 2.1 kJ mol\(^{-1}\)) are essentially zero. The ratio of \(A^H:A^D\) (5.8) calculated from the intercepts of the plots with the ordinate axis is similar to that of the KIE (7.3). These data have shown ground state tunnelling in TSOX, for both protium and deuterium. However, the data indicate tunnelling through a fluctuating potential energy barrier, driven by the thermal motion of the protein scaffold (Harris et al., 2000; Basran et al., 1999).
Figure 4.7. Kinetic isotope effect (KIE) data of sulphite-treated TSOX with sarcosine and deuterated sarcosine. Panel A: Concentration dependence of observed rate for FAD reduction. Conditions: 20 mM sodium pyrophosphate buffer, pH 8.5, 20 mM sodium sulphite. The total ionic strength at different sarcosine concentrations was balanced to 200 mM using KCl. Filled circles, reactions with sarcosine ($k_{lim}$ 141 s$^{-1}$, $K_d$ 13.2 mM); open circles, reactions with deuterated sarcosine ($k_{lim}$ 19.5 s$^{-1}$, $K_d$ 14.4 mM), Panel B: Temperature dependence and KIE data for sulphite treated TSOX. Filled circles, sarcosine; open circles, deuterated sarcosine. $ln A^H = 14.9 \pm 0.4$, $ln A^D = 13.2 \pm 0.5$, $\Delta H^H = 39.4 \pm 0.9$ kJ mol$^{-1}$, $\Delta H^D = 40.0 \pm 1.3$ kJ mol$^{-1}$. Panel B inset: Plot of $ln$ KIE versus 1000/T.
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4.3 Discussion

4.3.1 pH Dependence Studies

This chapter has dealt with a detailed analysis of the reductive half-reaction of TSOX. Exploiting the reactivity of the covalent FMN of TSOX with sulphite has enabled the reduction of the non-covalent FAD by sarcosine to be studied. The pH dependence of $k_{\text{lim}}$ uncovered the lack of any kinetically influential ionisations in the enzyme substrate complex in the pH range 6.5-10.5. The bell-shaped dependence of $k_{\text{lim}}/K_d$ with pH indicates two ionisations present in either the free enzyme and/or the free substrate; these ionisations are responsible for changes in the dissociation constant, $K_d$. The purpose of this study is to assign these ionisations to functional groups on the enzyme or the substrate. However, there is no structural information available for TSOX, nevertheless, a comparison between the $\beta$ subunit of TSOX and MSOX of Bacillus sp. B-0618 [whose structure was solved recently to 2.0Å (Trickey et al., 1999)] can be made. Although the MSOX sequence shows only limited identity (23%) to the $\beta$ subunit of TSOX, all the residues important for binding and catalysis in MSOX are conserved in $\beta$TSOX (Figure 4.8). The structure of MSOX was solved in the presence of two inhibitors, methylthio-acetic acid (MTA) and pyrrole-2-carboxylic acid (PCA); the binding of the carboxyl groups of these compounds gives us some understanding of how sarcosine may bind to MSOX. Analysis of the structure of the active site of MSOX indicates that binding of sarcosine is stabilised by hydrogen bonds between the substrate carboxylate and Arg-52 and Lys-348 (see Figure 4.8; Trickey et al., 1999). The p$K_a$ value of 10.4 seen in the $k_{\text{lim}}/K_d$ plot could therefore be the ionisation of Lys-348 (counterpart in TSOX is Lys-359) or, as mentioned previously, this ionisation may represent the methylated amine group of the substrate sarcosine. The remaining ionisation in the pH dependence of the $k_{\text{lim}}/K_d$ plot (p$K_a$ 7.4) must be an ionisation of the enzyme since the p$K_a$ of the carboxylate group of sarcosine is much lower than this (p$K_a$ 2.23). The active site structure of MSOX reveals that His-269 is located close to the potential binding site for the amine group of sarcosine (based on the PCA and MTA structures). This histidine residue is conserved in $\beta$TSOX (His-300) and protonation of this residue would produce two opposing positive charges. The roles of these residues in substrate binding is yet to be confirmed by site-directed mutagenesis.
Figure 4.8 Panel A: Diagram of the active site environment of MSOX of *Bacillus sp* B-0618 showing those residues important in binding the inhibitors PCA and MTA close to the isoalloxaline ring of the flavin. Panel B: Homology of MSOX sequence with sequence of βTSOX highlighting conserved residues implicated in MSOX catalysis (open boxes) including two residues which bind the substrate carboxylate. βTSOX covalent Histidine site (open circle) MSOX covalent cysteine site (star).
The results of the pH dependence study can also be examined in reference to proposed mechanisms of amine oxidation catalysed by flavoenzymes. The reaction involving the cleavage of a stable C-H bond can occur by a number of different mechanisms (Figure 4.9). Direct H-abstraction from the substrate methyl group (mechanism F), involving an amino acid radical as a H• acceptor, has been ruled out in TSOX as EPR studies do not indicate the presence of a radical species in the resting enzyme (Zeller et al., 1989). There are five other possible mechanisms by which C-H bond breakage could occur in TSOX. The first involves hydride transfer from the methyl group to the flavin N5 atom (mechanism A). The second is the so-called carbanion mechanism, which involves proton abstraction by an active site base (mechanism B). The third mechanism is the single electron transfer (SET) mechanism as proposed for monoamine oxidase [Silverman et al., 1995; mechanism C]. The fourth involves Direct nucleophilic attack of the substrate nitrogen at the flavin C4a atom, also proposed for monoamine oxidase [Kim et al., 1993; mechanism D]. Finally the fifth mechanism, involves the lone pair of electrons on the substrate amine, which attack the C4 atom of the flavin forming a covalent intermediate. This adduct pushes electrons towards the N5 atom which in turn abstracts a proton from the substrate (mechanism E). This mechanism was recently proposed for TMADH (Trickey et al., 2000). As no kinetically influential ionisations were observed in the enzyme-substrate complex in the pH range 6.5-10.5, this could potentially eliminate the carbanion mechanism and nucleophilic attack mechanism (mechanisms C and D, respectively), which both require a base. However it is quite possible that this base could have a pKₐ below pH 6.5, so neither mechanism can be ruled out based solely on this evidence. However, all of the proposed mechanisms shown in Figure 4.9 require the amine group of sarcosine to be unprotonated, but at physiological pH values sarcosine is protonated (pKₐ 10.01). Therefore, if any of these mechanisms were to account for sarcosine oxidation by TSOX there would need to be a deprotonation of the amine group in the enzyme-substrate complex. Consequently, at this stage it is not possible to determine the precise mechanism of C-H bond cleavage in TSOX. A favoured mechanism is one that was recently proposed for TMADH where the exposed flavin N5 atom acts as an internal base (Trickey et al., 2000). It is predicted that this group has a pKₐ value of ~20, which is clearly out of the range of any pH dependence study.
Chapter 4: Carbon-Hydrogen Bond Breakage in Sarcosine Oxidase

4.3.2 Temperature Dependence Studies

Flavin reduction in TSOX is associated with a KIE of 7.3, which is toward the upper limit for semi-classical behaviour (i.e. if one assumes the absence of tunnelling). However, analysis of the rate of flavin reduction as a function of temperature indicates the reaction is non-classical. The temperature independent KIE for TSOX and the large enthalpies of activation for protium and deuterium transfer are similar to the results obtained for MADH (Basran et al., 1999) and thermophilic alcohol dehydrogenase (Klinman et al., 1999). Although H-tunnelling has been recognised in several enzyme systems (Section 1.3.2.7) it is only recently that experimental evidence for a link between protein dynamics and H-tunnelling has been shown (Section 1.3.2.10). Studies carried out with MADH and thermophilic alcohol dehydrogenase have both been interpreted as indicating ground state tunnelling of hydrogen through a fluctuating potential energy barrier, produced by the intrinsic low frequency thermal motions of the protein (Basran et al., 1999; Kohen et al., 1999). A dynamic barrier model for enzymatic hydrogen tunnelling was proposed by Bruno and Bialeck (1992) that can accommodate KIEs of <7 (i.e. within semi-classical limits for the static barrier model). However, ground state tunnelling through a fluctuating barrier can accommodate KIEs<7. The work described in this chapter is thus consistent with H-tunnelling in TSOX from the substrate ground state, through a fluctuating potential energy surface. The use of this fluctuating barrier model of enzymatic H-tunnelling has now been demonstrated experimentally in a number of enzyme systems and reviewed recently (Scrutton et al., 1999, Sutcliffe and Scrutton, 2000).

There are potential complications arising from the isotope studies with TSOX, namely the issue of kinetic complexity and the magnitude of secondary isotope effects arising from perdeuteration of the substrate methyl group. Firstly regarding kinetic complexity, it is important to note that flavin reduction in TSOX was monitored by stopped flow technique to measure the rate of C-H bond cleavage. Since the precise mechanism of amine oxidation by TSOX is as yet unresolved, the question of whether the rate of flavin reduction is also a true indicator of the rate of C-H bond cleavage remains to be established. In this situation, it is possible, with decreasing temperature, that the expected increase in KIE (for semiclassical behaviour) is offset by a decrease in kinetic commitment, leading to an observed KIE.
that is apparently independent of temperature. This can be described by the following equation:

\[ \text{KIE}_{\text{obs}} = \frac{\text{KIE}_{\text{int}} + C}{1 + C} \]

Equation 4.8

where \( C \) is the ratio of rate constants reflecting the degree by which C-H bond breakage is rate-determining, \( \text{KIE}_{\text{obs}} \) is the observed KIE and \( \text{KIE}_{\text{int}} \) is the intrinsic KIE (Schimerlik et al., 1977). This kind of argument is commonplace in steady-state kinetic analyses, where passage over multiple potential energy barriers takes place. In single turnover stopped flow studies, which focus on 'one step' of the reaction, the rate of substrate oxidation/flavin reduction may in fact reflect more than one kinetic step, giving rise to internal commitments. However, the following three factors combine to argue against internal commitments in stopped flow studies with TSOX, although unequivocal demonstration will require further work. (i) the analogous temperature-dependent kinetic behaviour of TSOX, MADH (Basran et al., 1999) and TMADH (Basran, unpublished data); (ii) the fact that MADH, TMADH and TSOX catalyse similar reactions (i.e. amine oxidation via C-H bond breakage); and (iii) the improbability of a perfectly matched compensatory change in intrinsic KIE and internal commitment yielding an observed KIE that is independent of temperature for both TSOX and TMADH. Second, an upper limit for secondary KIEs of 1.36 (Klinman, 1978) for the perdeuterated methyl group of sarcosine may complicate the analysis if the full secondary KIE is realised. The stopped-flow data shows strong evidence for the VEGST mechanism in TSOX catalysed oxidation of sarcosine, but further studies are required to prove the role of VEGST of hydrogen during C-H bond cleavage. Further study of the mechanism will involve a lot more time and effort, particularly as measurement of secondary isotope effects are not practical or easily achievable with this enzyme-substrate system.
Chapter 5

Redox Potentials and Internal Electron Transfer


5.1 Introduction

5.1.1 Introduction

The work presented in this chapter explores intraprotein electron transfer. TSOX has previously been shown to stabilise the biradical state at pH 7, but less so at pH 8 (Jorns, 1985; Ali et al., 1991). Two-electron reduced TSOX exists as an equilibrium between a flavin dihydroquinone/flavin oxidised state and a state where both flavins are in the semiquinone form (flavin biradical). At pH 7, the equilibrium is shifted towards the biradical state and at pH 8 the equilibrium is more in favour of the dihydroquinone/oxidised state. The rate of intraprotein electron transfer between the two flavins can thus be measured by reducing the enzyme to the two-electron level at pH 7 and monitoring the loss of the semiquinone signal at 550 nm, when the pH is jumped from pH 7 to pH 8. The work described in this chapter explores the intraprotein electron transfer process further by investigating the pH dependence of the stability of the semiquinone forms and the role of the redox potentials of the two flavins on the electron transfer rate. The electron transfer rate between the redox centres was measured using a pH jump method, monitoring the formation or loss of the semiquinone biradical species at 550 nm.

5.1.2 Intramolecular Electron Transfer and pH-Jump Experiments.

Intramolecular electron transfer rates between redox centres can often be measured using the pH jump technique (Porras & Palmer, 1982; Rohlfs & Hille, 1991). In pH-jump experiments with TSOX the enzyme is reduced to the two-electron level by dithionite or L-proline in a weakly buffered (10 mM) solution at a known initial pH, and then mixed rapidly with a strongly buffered (100 mM) solution at a different final pH. The initial equilibrium distribution of reducing equivalents within the enzyme (determined by the initial pH) is thus perturbed, allowing the kinetics of the approach of the system to the new equilibrium position (determined by the final pH) to be followed spectrophotometrically. Corynebacterial TSOX forms a biradical species upon reduction with L-proline at pH 7 (Zeller et al., 1989). In kinetic studies of electron transfer the pH of the solution was then rapidly jumped to pH 8 at 25°C. This pH jump resulted in a rapid disappearance of 55% of the biradical signal at 550 nm (Ali et al., 1991). The reaction exhibited first order kinetics with $k_{obs}$...
= 750 s⁻¹ (25°C). It was determined that 58% of the total spectral change occurred during mixing, based on the observed rate constant and the dead time of the instrument. Biradical disappearance was accompanied by an increase in the absorbance at 450 nm consistent with a disproportionation reaction, Scheme 5.1:

\[
\text{FlH}^+ + \text{Fl}^\bullet \xleftrightarrow{k_r} \text{Fl} + \text{FlH}^-
\]

Scheme 5.1

In Scheme 5.1 \(k_{\text{obs}} = k_r + k_r\) for the relaxation to the new equilibrium position when the pH is changed. The fully reduced flavin has an unknown protonation state and is shown as an anion, FlH⁻. This chapter is concerned with similar pH-jump studies of internal electron transfer in the highly related Arthrobacterial enzyme, and extension of the work reported for the Corynebacterial enzyme. As part of this study, the redox potentials of the individual flavin couples were determined (Section 5.1.3), in addition to the direction of electron transfer during the pH jump experiment. A similar pH jump study has also been used to investigate intramolecular electron transfer between the flavin and iron-sulphur centre of TMADH (Rohlfs & Hille, 1991; Rohlfs et al., 1995), and has helped in the elucidation of a complex mechanism of electron and hydrogen transfer in this enzyme system.

5.1.3 Redox Potentiometry

An enzyme redox couple, \(A_{\text{red}}/A_{\text{ox}}\) can be represented as undergoing the following reaction:

\[
A_{\text{red}} \xleftrightarrow{n\text{e}^-} A_{\text{ox}}
\]

Scheme 5.2

Where \(n\) is the number of electrons released as the reduced component is oxidised or the number of electrons taken up as the oxidised component is reduced. This scheme is a half reaction as electrons are associated with a reduced compound and not as free entities. The complete reaction would be:
Where the amounts of A and B are such that the number of electrons transferred between the compounds is balanced with the number of electrons that can be accepted. If compound A can accept two electrons but compound B can only accept one electron there must be twice as much of compound B to balance the reaction. The equilibrium constant, $K_{eq} = [A_{red}][B_{ox}]/[A_{ox}][B_{red}]$, will depend on the individual affinities of the couples for electrons. The Gibbs free energy for the reaction can therefore be expressed according to the following equation (Dutton, 1978):

$$
\Delta G = \Delta G^\circ + RT \ln\left(\frac{[A_{red}][B_{ox}]}{[A_{ox}][B_{red}]}\right)
$$

Equation 5.1

The free energy, $\Delta G$, required to transfer $n$ moles of electrons is related to the potential difference, $\Delta E_h$, through which the electrons are transferred (Equation 5.2):

$$
\Delta G = -nF\Delta E_h
$$

Equation 5.2

In Equation 5.2 $F$ is the Faraday constant, a conversion factor of chemical potential to electrical potential, equal to 96,493 J>V. In equation 5.2 a positive redox potential, $\Delta E_h$, results in a negative $\Delta G$ and hence indicates an exergonic reaction. Equations 5.1 and 5.2 can be combined to yield the Nernst equation, by making an analogous substitution of $\Delta G^\circ$:

$$
\Delta E_h = \Delta E^\circ - \frac{RT}{nF} \ln\left(\frac{[A_{red}][B_{ox}]}{[A_{ox}][B_{red}]}\right)
$$

Equation 5.3

In Equation 5.3 $\Delta E_h$ is the redox potential for the reaction and $\Delta E^\circ$ is the redox potential when all components are in their standard states.

The redox potential difference of a solution can be measured by potentiometry where the system is adjusted so that zero flux is maintained by balancing the voltage difference by applying a potential of opposite charge and by adjusting the current, measured with a sensitive galvanometer, to zero. The voltage measured shows the
driving force, which is the difference in free energy of the electrons in equilibrium with the redox couple. As the redox potential difference is measured under conditions of zero flux, it is a direct measure of the free energy change of the reaction. The redox potential difference is measured with respect to a standard electrode, the hydrogen half-cell. However, the hydrogen electrode is not a convenient device to use and instead a calomel electrode was used and the resulting potentials standardised to the hydrogen electrode.

The measurement of redox potentials for enzyme cofactors is not as easy as measurements for inorganic chemical reactions. This is because the redox centres in enzymes are often shielded by the surrounding protein and cannot make direct contact with the electrode surface to transfer electrons. Consequently, redox mediators are added at low concentrations to the protein solution to act as 'go-betweens', between the electrode and enzyme redox centre. This method has been used to measure redox potentials effectively in many biological redox molecules (Daff et al., 1997; Noble et al., 1998). The method of using absorbance changes to determine $K_{eq}$ and midpoint potentials is detailed in Section 2.3.7.

5.2 Results

5.2.1 Anaerobic Reductive Titrations at Various pH Values.

When TSOX is reduced anaerobically with dithionite spectral changes are observed which reflect the distribution of electrons and consequently indicate the oxidation states of the two flavins. Titration of TSOX with dithionite under anaerobic conditions at pH 7 results in a decrease in absorbance at 450 nm, which is accompanied by a small and transient increase in absorbance at wavelengths between 510 nm and 650 nm (Figure 5.1A). The end point of the titration produces a value of 4 electron equivalents of dithionite required for complete reduction of TSOX. This result indicates that four electrons have been added to the system to achieve complete reduction and that no other redox component reacts with the dithionite (i.e. the non-covalent NAD or a redox active disulphide).
Figure 5.1. Anaerobic reduction of TSOX (7 μM) with dithionite at various pH values at 25°C (Open circles 450 nm, filled circles 550 nm). Panel A: potassium phosphate buffer, pH 7, Panel B: potassium phosphate, pH 8, Panel C: sodium borate buffer, pH 9, Panel D: sodium borate buffer, pH 10.
Chapter 5: Redox Potentials and Internal Electron Transfer

It is likely that the broad absorbance increase observed between 510 nm and 650 nm is due to a blue neutral flavin radical since it shares similar spectral properties with those observed for other neutral radicals (Massey & Palmer, 1969). Also, this absorbance increase has been identified in the reduction of Corynebacterial TSOX by dithionite (Jorns, 1985; Zeller et al., 1989), and attributed to a blue neutral flavin radical. Further evidence is provided by a plot of absorbance at 550 nm versus electron equivalents of dithionite, which indicates that the absorbance increase is maximal when two electron equivalents have entered the system and the spectral change is lost with the addition of the subsequent electrons. The nature of biradical formation was explored further by reduction at higher pH values of pH 8, 9 and 10. These titrations were all followed at 450 nm and again all indicated that 4 electron equivalents were added to the system to obtain full reduction. Formation of the biradical spectral form was observed at pH 8, but its yield was reduced by 50%. No biradical spectral signal was observed in titration experiments carried out at pH 9 and 10 when only two electron equivalents were supplied to the system, indicating lack of biradical formation at these pH values.

Previous work by Jorns and co-workers identified several heterocyclic carboxylic acids which bound to Corynebacterial TSOX producing perturbations in the absorbance spectra (Zeller et al., 1989). In particular, L-proline was shown to act as a substrate for the enzyme forming a strong biradical signal at pH 7, but only reducing the enzyme to the two-electron level. This experiment was repeated with Arthrobacter TSOX which was anaerobically reduced with L-proline at pH 7 and the absorbance changes recorded after addition of 10, 30, 50 and 100 μM L-proline after which no further change in absorbance was observed (Figure 5.2A). Titration of TSOX with L-proline was also performed at pH 9 (Figure 5.2B), the absorbance changes were recorded after the addition of 2, 5, 10, 20 and 30 μM L-proline. Further absorbance changes where not observed for 90min, after this time a slow absorbance decreased occurred at 450 nm. During reduction with L-proline at pH 9 no long wavelength intermediate was observed.
Figure 5.2. Anaerobic reduction of TSOX (30 μM) with L-proline. (A) TSOX in 20 mM potassium phosphate buffer, pH 7 at 25°C. Spectrum 1, oxidised TSOX, spectrum 2, 10 μM L-proline, spectrum 3, 30 μM L-proline, spectrum 4, 50 μM L-proline, spectrum 5, 100 μM L-proline. (B) TSOX in 20 mM sodium borate buffer, pH 9 at 25°C. Spectrum 1, oxidised enzyme, spectra 2-6 after addition of 2, 5, 10, 20, and 30 μM L-proline, respectively. Spectrum 7 after 30 minutes in the presence of 100 μM L-proline.
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5.2.2 Redox Potential Measurements

It is possible that the changes in the electron distribution in the half-reduced enzyme may be the result of a pH dependent change in the redox properties of the two flavins. The difference in redox potential between the two flavins can also provide information about the driving force of interflavin electron transfer. This hypothesis was explored by measurement of the redox potentials of the two flavins at pH 7 and pH 9 using the method of Dutton (Dutton, 1978). TSOX was artificially reduced with dithionite and oxidised with ferricyanide and changes in the absorbance spectrum and redox potential were recorded, as described in Section 2.6.

TSOX was artificially reduced and oxidised under anaerobic conditions in 20 mM potassium phosphate buffer pH 7; the spectrum and redox potential was recorded after each addition. These spectra were then adjusted according to Equations 2.8 and 2.9 to allow for evaporation and precipitation of the protein during the time course of the experiment. Figure 5.3A shows the spectral changes monitored between 300 nm and 750 nm. Figure 5.3B shows a plot of the values for absorbance at 454 nm versus the potential of the enzyme solution. The absorbance at 454 nm versus potential data were fitted to the four electron equation (Equation 2.14) assuming four sequential one-electron transfer steps occur. Absorbance changes were observed at 550 nm, but the biradical (semiquinone) signal change was too small to give useful data for determining the redox potentials of the semiquinone. However, from the Nemst fit of the 454 nm data, the midpoint potentials for the oxidised/semiquinone and semiquinone/hydroquinone states of both flavins could be determined at pH 7 (Table 5.1).

TSOX was also reduced anaerobically by dithionite in 20 mM potassium borate buffer, pH 9, and the spectrum and redox potential was recorded after each addition. Figure 5.4A shows the spectral changes observed between 300 nm and 700 nm. Figure 5.4B plots the values for absorbance at 454 nm versus potential of the enzyme solution; no absorbance change was observed at 550 nm as the biradical signal is not stable at pH 9 (Section 5.2.1). The absorbance at 454 nm versus potential data were similar to that obtained at pH 7 and the data were fitted to the four-electron equation (Equation 2.14). From this fit it was possible to determine the midpoint potentials for the oxidation states of the two flavins as separate sigmoidal steps could be observed for the majority of the one electron steps.
Chapter 5: Redox Potentials and Internal Electron Transfer

Figure 5.3. Redox titration of fully flavinylated TSOX in 20 mM phosphate buffer, pH 7 at 25°C. Panel A: Spectral reduction of TSOX, values on selected maxima (454 nm) indicate the potential of the enzyme solution when the spectrum was recorded. Panel B: plot of absorbance at 454 nm versus standard hydrogen electrode potential. Midpoint potentials were determined using Nernst equation for four one-electron steps.
Figure 5.4. Redox titration of fully flavinyalted TSOX in 20 mM borate buffer, pH 9 at 25°C. Panel A: Spectral reduction of TSOX, values on selected maxima (454 nm) indicate the potential of the enzyme solution when the spectrum was recorded. Panel B: plot of absorbance at 454 nm versus standard hydrogen electrode potential. Midpoint potentials were determined using Nernst equation for four one-electron steps.
Figure 5.5. Redox titration of the covalent FMN of TSOX in 20 mM borate buffer, pH9, at 25°C. Panel A: spectral reduction of FMN, values on selected maxima (454 nm) indicate the potential of the enzyme solution when the spectrum was recorded. Panel B: plot of absorbance at 454 nm versus standard hydrogen electrode potential. Midpoint potentials were determined using a Nernst equation for a two-electron step.
The non-covalent cofactor FAD was removed from TSOX by exhaustive dialysis of the fully flavinylated enzyme in 20 mM potassium phosphate buffer, pH 7, containing 3 M potassium bromide for >24 h. The enzyme was subsequently dialysed exhaustively for a further 24 h in 20 mM potassium phosphate buffer, pH 7, to remove free flavin and potassium bromide. The absorbance of the resulting semi-apoenzyme was recorded and this demonstrated that it had lost 48% of its absorbance at 450 nm. The 450 nm absorbance of the semi-apoenzyme could not be reduced aerobically by sarcosine (4 mM), but could still be reduced by sulphite. This confirmed that sulphite reacts with the covalent flavin, and sarcosine reacts only with the non-covalent FAD. The semi-apoenzyme was then dialysed against 20 mM potassium borate buffer, pH 9, and reduced anaerobically with dithionite. The spectrum and redox potential were recorded after each addition. Figure 5.5A shows the spectral changes observed between 350 nm and 700 nm. Figure 5.5B plots the values for absorbance at 454 nm versus potential of the semi-apoenzyme. The absorbance at 454 nm versus potential data were fitted to the two-electron equation (Equation 2.12), to obtain data for the midpoint potentials of the covalent FMN. All the midpoint potentials for the fully flavinylated TSOX at pH 7 and pH 9 are given in Table 5.1, along with the midpoint potentials for covalent FMN in the semi-apoenzyme.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Flavin midpoint potentials (mV)</th>
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<tr>
<td></td>
<td>FAD</td>
</tr>
<tr>
<td>pH / Absorbance</td>
<td></td>
</tr>
<tr>
<td>pH7 A&lt;sub&gt;454 nm&lt;/sub&gt;</td>
<td>E&lt;sub&gt;01(ox/sq)&lt;/sub&gt; = +148 ± 2.8</td>
</tr>
<tr>
<td>pH9 A&lt;sub&gt;454 nm&lt;/sub&gt;</td>
<td>E&lt;sub&gt;02(sq/hq)&lt;/sub&gt; = -49.2 ± 5.4</td>
</tr>
<tr>
<td>pH9 A&lt;sub&gt;454 nm (FMN)&lt;/sub&gt;</td>
<td>E&lt;sub&gt;02(sq/hq)&lt;/sub&gt; = -82.1 ± 9.5</td>
</tr>
</tbody>
</table>

**Table 5.1.** Midpoint potentials vs. standard hydrogen electrode (25°C, pH 7.1) for the flavins of TSOX at pH 7 and pH 9.

From a knowledge of the midpoint potentials obtained for FMN in the semi-apoenzyme it is possible to assign the midpoint potential of FMN in the diflavinylated TSOX (Table 5.1). At pH 7, FAD has higher transfer potentials \((E_{01(ox/sq)} = 148 ± 2.8 \text{ mV}; E_{02(sq/hq)} = -49.2 ± 5.4 \text{ mV})\) than the FMN \((E_{03(ox/sq)} = -165 ± 6.3 \text{ mV}; E_{04(sq/hq)} = -200 ± 5.3 \text{ mV})\) implying that exergonic electron transfer will
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occur from FMN to FAD. These results are surprising in light of information about
the catalytic cycle of TSOX in which sarcosine reduces the non-covalent FAD and
electrons are transferred to covalent FMN from FAD, and finally from FMN to
reduction of each flavin can be determined at each pH value from which the redox
potential difference, $\Delta E_h$, is obtained. This value can be used to determine the Gibbs
free energy accompanying the transfer of electrons between FAD and FMN using
Equation 5.2. The results are summarised in Table 5.2:

<table>
<thead>
<tr>
<th>pH 7.0</th>
<th>FAD $E_{02(\text{sq}/\text{hq})}$</th>
<th>FMN $E_{02(\text{sq}/\text{hq})}$</th>
<th>$\Delta E_h$ (mV)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-49.2</td>
<td>-165</td>
<td>-115.8</td>
<td>+11.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2. The midpoint potentials of the semiquinone states of FAD and FMN and
the redox potential difference between them, across which one electron is transferred,
with a positive Gibbs free energy change.

These results indicate that electron transfer from FAD to FMN is
substantially endergonic. Consequently, electron transfer is predicted to be very slow
in the FAD to FMN direction and the pH jump technique was used to measure the
kinetics of electron transfer in both directions (Section 5.2.3).

5.2.3 Interflavin Electron Transfer Rates

Intramolecular electron transfer was investigated with two electron reduced
TSOX (reduced by dithionite or L-proline) using pH jump stopped-flow techniques.
This technique allows the kinetics of electron distribution between the two flavins to
be followed spectrophotometrically. Based on the results of the anaerobic reduction
titrations where the biradical was formed at pH 7 but not at pH 9, the two-electron
distribution was monitored by jumping the pH from pH 7 to pH 9. TSOX in 10 mM
potassium phosphate buffer, pH 7, with 100 mM KCl was reduced to the two-electron
level with dithionite. The two electron reduced TSOX was then mixed rapidly with
100 mM potassium borate buffer, pH 9, at 5°C; loss of the biradical was monitored at
550 nm. However in this experiment the majority of the absorbance change occurred
within the dead time of the stopped-flow apparatus and no electron transfer rate could
be measured. Two electron reduced TSOX was also mixed with 100 mM potassium
phosphate pH 7 to show that no absorbance change occurred when the pH was not
altered.
TSOX was also reduced to the two-electron level with L-proline in 10 mM potassium phosphate buffer pH 7, and then rapidly mixed with 100 mM potassium borate buffer pH 9 at 5°C. In this experiment, a rapid absorbance decrease was observed at 550 nm consistent with loss of the biradical (Figure 5.5B). This transient was monophasic and fitted to a single exponential expression to obtain an interflavin electron transfer rate of 500 s\(^{-1}\) (Figure 5.6). However, it was determined that 47% of the absorbance change was not observed since this had occurred during mixing; this is expected based on the measured rate constant and the instruments dead time of 1.1 ms. This result indicates that electrons equilibrate between the two flavins at a rate faster than the rate of substrate reduction of non-covalent FAD (130 s\(^{-1}\) at 25°C or 36 s\(^{-1}\) at 5°C) and the overall reaction rate \(k_{cat}\) (4.3 s\(^{-1}\) at 25°C). The pH jump experiment was repeated by reducing TSOX to the two electron level with 0.1 mM L-proline in 10 mM potassium borate buffer, pH 9. At this concentration of L-proline, formation of the two-electron reduced enzyme is slow (30 min) and further reduction is much slower (90 min). The two-electron reduced TSOX was quickly transferred to the anaerobic stopped-flow apparatus and rapidly mixed with 100 mM potassium phosphate buffer pH 7 at 5°C. Formation of the biradical was observed as an absorbance increase at 550 nm; the transient produced was fitted to a single exponential expression to obtain a rate of 3 s\(^{-1}\). This figure is similar to the measured \(k_{cat}\) value for the enzyme of 4.3 s\(^{-1}\) measured under different conditions: 25°C using the steady state assay. The pH jump from 9 to 7 is likely to represent the physiological electron transfer reaction and this may be limiting the overall rate of reaction. The result is also consistent with an uphill electron transfer process predicted from the redox potentials where FAD transfers electrons to the FMN, which has a lower potential.
Figure 5.6. Transients observed for pH jump of two-electron reduced TSOX by L-proline. Panel A: pH 7 to pH 9 jump, TSOX (10 μM in 10mM potassium phosphate buffer, 100mM potassium chloride, pH 7.0) was reduced by L-proline (0.5 mM) to the two-electron level and mixed with 100 mM sodium borate, pH 9, at 5°C. Panel B: pH 9 to pH 7 jump, TSOX (10 μM in 10 mM sodium borate buffer, 100mM Potassium chloride, pH 9.0) was reduced by L-proline (0.1 mM) to the two electron level and mixed with 100 mM potassium phosphate, pH 7 at 25°C
5.3 Discussion

5.3.1 Endergonic electron tunnelling

Electrons can be transferred up to a distance 14 Å between redox centres and the intramolecular electron tunnelling is often much faster than the substrate redox reactions, due to the close proximity of redox centres. A simple expression has been developed from 'Fermi's Golden Rule' (Section 1.3.2), which relates the exponential decay of the rate of electron tunnelling, $k_{et}$ (s$^{-1}$), to the edge to edge distance, $R$ (Å), between redox centres and also $\Delta G$ and $\lambda$ (Moser et al., 1992). This relationship is as follows:

$$\log_{10} k_{et} = 15 - 0.6 R - \frac{3.1(\Delta G + \lambda)^2}{\lambda}$$

Equation 5.1

where 0.6 is the log$_{10}$ of a $\beta$ value of 1.4 Å$^{-1}$, which is the weighted intermediate value of the extreme values $\beta$ can take [~0.9 Å$^{-1}$ for covalently linked redox centres, or 3.5 Å$^{-1}$ for tunnelling through a vacuum; (Moser et al., 1992)]. The coefficient value of 3.1 is a collection of the constants at room temperature for the quantised nuclear term. This expression gives useful approximations of electron transfer distance or electron transfer rate. The expression can be expanded to include a term for packing density, $\rho$, of protein atoms between redox centres to account for $\beta$ variations in an exergonic electron tunnelling regime (Page et al., 1999):

$$\log_{10} k_{et}^{ex} = 13 - (1.2 - 0.8 \rho)(R - 3.6) - \frac{3.1(\Delta G + \lambda)^2}{\lambda}$$

Equation 5.2

Where the packing density, $\rho$, is the fraction of volume between redox centres which are within van der Waals radius of intervening atoms, and ranges from 1 for fully packed medium ($\beta = 0.9$ Å$^{-1}$), to 0 corresponding to a space within the protein structure outside van der Waals radii ($\beta = 2.8$ Å$^{-1}$). The $\rho$ weighting of these extreme $\beta$ values, $\beta = (\rho)0.9$ Å$^{-1} + (1-\rho)2.8$ Å$^{-1}$, generates the $(2 - 0.8\rho)$ coefficient. The log of the optimal rate at 3.6 Å van der Waals contact is 13 (Moser et al., 1992). Dutton and co-workers performed a survey of electron transfer proteins, which have multiple cofactors for intramolecular electron transfer and for which structural data was available (Page et al., 1999). They showed that the packing fraction, $\rho$, was almost
identical for the volume between redox centres whether the redox centres took part in
physiologically productive reactions or not. This indicated that the heterogeneity of
the protein structure is not used to physiological benefit, but that the majority of
physiological redox centres are within a distance of 14 Å. Within this distance the
calculated tunnelling rates from Equation 5.2, when ΔG is optimised, are very high
(10^{13}-10^7 \text{s}^{-1}) and remain higher than $k_{\text{cat}}$ at physiological values of ΔG (0.0 to -0.1
eV). Importantly, they also showed that at shorter distances, uphill electron tunnelling
could occur at functionally relevant rates for example at a separation of 6 Å, rates of
10^2 \text{s}^{-1} were predicted for a +0.5 eV endergonic step. Equation 5.2 can be modified to
estimate rates of endergonic tunnelling by dividing by the temperature-dependent
Boltzmann factor (10^{ΔG/0.06}) to give the following equation:

$$\log_{10} k_{\text{et}}^\alpha = 13 - (1.2 - 0.8\rho)(R - 3.6) - \frac{3.1(ΔG + \lambda)^2}{\lambda - \frac{ΔG}{0.06}}$$

Equation 5.3

This equation has been used to calculate electron transfer rates of 10^5-10^7 \text{s}^{-1} for
endergonic steps of 0.21 eV and 0.40 eV, between iron sulphur clusters in several
cytochrome chains. Further support for this equation comes from agreement of
calculated rates with those measured in several other cytochrome systems which
show that endergonic tunnelling can be part of physiological electron transfer chains.

### 5.3.2 Uphill electron transfer in TSOX.

The pH jump experiment performed following reduction by L-proline is
summarised in Figure 5.8. In the jump from pH 7 to 9, a single electron is transferred
from the FMN semiquinone to the FAD semiquinone, producing reduced FAD and
oxidised FMN. The rate of transfer of the electron is fast because the FMN has a
lower redox potential than FAD. However, a jump from pH 9 to 7 involves the
transfer of the electron from reduced FAD to oxidised FMN and this is the
physiological direction of electron transfer. The rate of electron transfer is slow in
this direction because the FAD hq/sq couple has a higher redox potential than the
FMN ox/sq couple and the electron is transferred across a negative potential
difference. The Gibbs free energy change for the transfer of an electron from the
FAD hydroquinone to oxidised FMN can be determined from the potential energy
difference of the midpoint potentials for FAD(sq/hq) and FMN(ox/sq) couples. At pH
Chapter 5: Redox Potentials and Internal Electron Transfer

This difference is -116 mV equivalent to a Gibbs free energy change of +11 kJ mol\(^{-1}\). This indicates that the single electron transfer is an endergonic step requiring a considerable input of energy.

The redox potentials were measured by reduction with dithionite so it is difficult to compare these results with the electron transfer rates measured by L-proline reduction. However, a prediction of the redox potentials of L-proline can be made based on a comparison of the anaerobic reduction experiments. L-proline stabilises the neutral blue semiquinone to a greater extent than dithionite and exhibits a strong biphasic reduction pattern. It is therefore likely that the midpoint potentials for the FAD are likely to be lower in the presence of L-proline or the substrate sarcosine, with the potentials for FMN remaining constant. This is summarised in Figure 5.8, which compares the individual redox potentials for FAD and FMN at pH 7 and pH 9 and those predicted for FAD in the presence of L-proline.
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**Figure 5.7.** Diagram summarising the proposed redox states of the two flavins at pH 7 and pH 9. A shift in the equilibrium position of electrons occurs when the pH is jumped between pH 7 and pH 9.
Figure 5.10. Comparison of the individual midpoint potentials for FAD and FMN at pH 7 and pH 9. Potentials were assigned based on the results of the FMN reduction at pH 9. Estimated potentials for TSOX in the presence of L-proline are shown based on the assumption that the potential difference between FAD and FMN would be reduced as the semiquinone state is more pronounced and stable in the presence of L-proline.
5.3.3 Comparison with Redox Potentials of Other Flavoenzymes

Although redox potentials have not been determined for the flavins of any of the other members of the SOX family, comparisons can be made with flavoenzymes, which have both FMN and FAD bound non-covalently. Examples of these types of flavoenzymes whose redox potentials have been measured include cytochrome P450 reductases, NADPH-sulphite reductase and nNOS. In all these cases, the FMN has the higher potential and is the exit port for electrons. However, the FAD, which is reduced by NADPH, has a lower potential that is still higher than the potential for NADPH. The extremely high midpoint potential for the non-covalent FAD in TSOX ($E_{01(ox/sq)} = +148 \text{ mV}$) is at the higher end for any recorded flavoenzyme flavin potential. However, in contrast all the examples of high flavin midpoint potentials have been observed in covalent flavoenzymes. Examples include succinate dehydrogenase ($E_m = -3 \text{ mV}$; Gutman et al., 1980), trimethylamine dehydrogenase ($E_m = +40 \text{ mV}$; Barber et al., 1988), thiamine oxidase ($E_m = +55 \text{ mV}$; Gomez-Monero et al., 1979), and vanillyl-alcohol oxidase ($E_m = +55 \text{ mV}$; Fraaije et al., 1999). In the case of vanillyl-alcohol oxidase the covalent histidyl linkage was mutated producing enzyme that had non-covalently bound FAD and exhibited a much lower $E_m = -65 \text{ mV}$ (Fraaije et al., 1999). The change in redox potential was attributed to the loss of the covalent link as structural data indicated very little change in the active site structure, which would affect the redox potential. By contrast, TSOX is a complex flavoprotein with the unique property of containing both covalent and non-covalent flavin. The heterotetrameric enzyme may have evolved from the combining of the MSOX enzyme, which contains covalent FAD and domains from other flavoenzymes. With respect to MSOX, for which structural data is available, the environment of the covalent flavin is said to be unique amongst flavoenzyme structures determined to date (Trickey et al., 1999). There are only neutral or basic residues in the region surrounding the flavin isoalloxazine ring. This sea of positive charges and the positive end of a $\alpha$ helix dipole may produce a positive shift in the flavin dipole. No redox potential has been measured for MSOX, but further evidence to support a high redox potential for the flavin includes the fact that it is reducible with thiols, a feature observed with high potential flavins in model studies (Raibekas et al., 1993) and the presence of a covalent link. The $\beta$ subunit of TSOX shows sequence identity with MSOX and its non-covalent FAD is reduced by substrate. It is
therefore possible that the non-covalent nature of this link in TSOX may result in a lower midpoint potential for FAD than the covalent form found in MSOX. However, this midpoint potential could still be unusually high due to the similar environment of the flavin. The determination of redox potentials for MSOX and structural information for TSOX will help in the understanding of these unusually high redox potentials.
Chapter 6

Discussion & Future work
Chapter 6: Discussion and Future work

6.1 Discussion

The work presented in this thesis has looked at several different aspects of the complex flavoenzyme TSOX from *Arthrobacter* sp. 1-IN. The genes encoding the four subunits have been successfully sequenced, cloned, and expressed at high levels in a host strain of *E.coli*. Work has begun on gaining a better understanding of the kinetic mechanism and developing techniques to use TSOX as a model system for investigating the transfer of hydrogen and electrons during enzyme catalysis. TSOX is an interesting flavoenzyme with many unique properties and functions, which still remain to be investigated.

The work presented in Chapter 3 involved a preliminary characterisation of wild-type and recombinant TSOX, and will provide a background to any future studies of the enzyme. The sequencing information is a valuable resource not only for comparison with similar sequences in other enzymes but will also be crucial for future structural work. The sequence information indicates that TSOX may have evolved as the result of a fusion of domains from a number of different flavoenzymes and that its function may not simply be the oxidation of sarcosine. The β subunit shows sequence similarity to the MSOX enzymes, which only require a single subunit with a single covalent flavin to perform the same reaction. The question remains as to what the roles of the additional subunits are. It is possible that these subunits perform a completely separate function as yet unknown. Based on sequence similarities of the α subunit it can be proposed that this subunit performs an additional function involving glycine cleavage and the transfer of a formyl group to tetrahydrofolate. The presence of a putative folate binding region in the α subunit confers the ability to generate 5,10-methylenetetrahydrofolate from tetrahydrofolate. This may be of physiological significance as 5,10-methylene tetrahydrofolate is used as a co-substrate in many metabolic pathways involved in purine biosynthesis, and the synthesis of precursors for various amino acids. In particular 5,10-methylene tetrahydrofolate is used by serinehydroxymethyl transferase (SHMT) in the production of serine from glycine; the gene for SHMT is located upstream of the *sox* genes but may form part of the same operon. The presence of folate dependent genes surrounding the DMGO gene in *Arthrobacter* sp. indicates that it is likely that there are a series of enzymes whose genes will be located in close proximity in the Arthrobacter genome and catalyse consecutive reactions.
The high level expression and purification of active recombinant TSOX containing all the relevant cofactors is also of great benefit for future work. Stopped-flow experiments often require large quantities of enzyme (200-500 mg), which must be spectrally pure, and not contain contaminating redox proteins, which could affect observed rates. Pure protein is also a key requirement for any crystallography trials.

The use of sulphite to inactivate the covalent FMN has been an important technique as it has allowed the study of the non-covalent flavin in isolation without the need for anaerobic conditions. The pH dependence of the reductive half reaction has proved useful in the elucidation of possible kinetic mechanisms for TSOX. The lack of any structural data prevents a definitive assignment of residues important in the mechanism of TSOX, but conserved residue with the MSOX enzymes provide possible candidates.

The mechanism of C-H bond cleavage was further investigated by kinetic isotope effect studies, which indicated that hydrogen was transferred by a ground state quantum tunnelling mechanism, aided by the low frequency thermal motions of the protein. The results provide another example of a recent model for H-tunnelling, driven by protein dynamics (VEGST), which has now been identified in various enzyme systems (MADH, AADH, TMADH, TSOX). This extreme tunnelling is an attractive mechanism for the cleavage of the stable C-H bond, which has a large energy of activation, making it energetically unfavourable for a classical over-the-barrier mechanism. H-tunnelling driven by protein dynamics may be a common mechanism for C-H bond breakage by enzymes and may extend to other types of H-transfer mechanism. This model of H-transfer using a dynamic barrier has serious implications for the use of transition state theory for H-transfer and other enzyme catalysed reactions.

The results of Chapter 5 are particularly interesting and they suggest that the transfer of electrons between the two flavins occur endergonically. The requirement to transfer electrons over a long distance or in a certain direction may outweigh the energy requirement of the step. Endergonic steps have been revealed in the iron sulphur clusters of the cytochrome c3 chain of nickel-iron hydrogenase (Volbeda et al., 1995) and in the haem chain of the photosynthetic reaction centre of Rhodopseudomonas viridis (Knaff et al., 1991). These examples support the theory that the use of redox centres within enzymes are not solely governed by favourable midpoint potentials, and that the proximity of redox centres and thermal activation
control the viability of a transfer chain. In the case of TSOX, little is known about the overall mechanism and the requirement for two flavins with different properties. The redox potentials may be modulated by the presence of substrate allowing reduction to the two-electron level. This would prevent the harmful build up of reaction products like formaldehyde and hydrogen peroxide.

6.2 Future work

The results described in this thesis have been a preliminary investigation exploring some of the mechanistic aspects of TSOX, and developing a system for its high level expression and purification. This work will form the basis for future experimental analysis and will be of benefit in understanding the mechanisms involved in the transfer of electrons and hydrogen. Only a limited number of groups work on this unusual flavoenzyme and a lot of the results presented in this thesis have raised questions and opened new avenues for research in the future. In this final section a number of possible future investigations are proposed.

In the long term, if TSOX is to be exploited fully, both in understanding its mechanistic function and developing its use in the biosensor industry, structural data on the enzyme will be a key accomplishment. Structural determination was not a major focus of this thesis, although the preliminary crystallisation trials should form a good basis for obtaining TSOX crystals. The sequencing information will also be of benefit when a structural data set has been obtained. Future work should definitely be focused on obtaining structural information as this will aid in understanding many facets of enzyme function. In particular, data on the active site architecture will provide information on residues involved in reaction mechanism. Structural similarities with other flavoenzymes whose functions are understood will also help in investigating a TSOX mechanism. Knowledge of the environment of the two flavins, and distance between them will help in understanding the redox potentials and rates of interflavin electron transfer. The overall structure may also provide important clues to the role of the δ and γ subunits of TSOX, and any structural benefit of the NAD+ in the α subunit.

Site directed mutagenesis (SDM) is a powerful tool for the enzymologist, whether structural information is available or not. In the case of TSOX, SDM can be used to investigate the importance of catalytic residues identified in the β subunit.
from comparison of those identified in MSOX. Additionally, the technique can be used to mutate the histidine involved in covalent flavinylation, producing an enzyme containing two non-covalent flavins. This enzyme can then be used to explore the role of the covalent linkage in TSOX with particular reference to the unusual redox properties it exhibits.

This thesis has explored the kinetics of some of the steps of the catalytic cycle of TSOX using stopped-flow techniques. A natural progression of this work would involve studying the oxidative half-reaction of TSOX under pre-steady state conditions. This can be achieved by artificially reducing the enzyme anaerobically and studying flavin oxidation by rapidly mixing the reduced enzyme with buffer containing different percentages of oxygen. TSOX has the advantage of allowing the study of the kinetics of covalent FMN oxidation alone, achieved by removing the FAD using the ‘KBr technique’. The covalent FMN is known to be the exit port for electrons to oxygen and spectral changes upon oxidation will be easier to resolve in the absence of the second flavin.

A lot of interesting and unresolved issues have been produced from the preliminary investigation of inter flavin electron transfer. Further work is needed in this area to get a better idea of the processes involved. A more in depth study of pH jump experiments with dithionite, L-proline, and sarcosine at ambient and low temperatures is required, with rates measured in the high to low as well as low to high direction. The rates of semiquinone loss and formation were studied in this thesis using pH-jump techniques measuring absorbance changes at 550 nm. Examination of absorbance changes at 450 nm and 365 nm in conjunction with 550 nm may provide a fuller picture of the oxidation states involved in the electron transfer event.

The redox potentials measured for TSOX have posed some interesting questions as to why TSOX exhibits an endergonic electron transfer event. It may be that sarcosine oxidation is not the physiological role of the enzyme, or that it has evolved an additional unknown function for which a slow rate of electron transfer is the cost involved. As the kinetics of intraflavin electron transfer were measured in the presence of L-proline it would be beneficial to determine the redox potentials of the two flavins with L-proline and sarcosine as well. This may indicate that the binding of L-proline effects the non-covalent FAD midpoint potential and consequently the rate of electron transfer.
Isotopic substitution methods can be further exploited in investigating electron and hydrogen transfer during flavin reduction in TSOX. Solvent isotope effect experiments can provide information on the number of hydrogens transferred during flavin reduction by substrate and therefore provide further support for particular mechanisms. A further long-term aim is the redesign of TSOX substrate specificity using ‘forced-evolution’ techniques. Sarcosine oxidase is currently used as part of a multi enzyme system for assaying creatinine levels in the blood of patients, to diagnose heart attacks or kidney failure. The current assay is labour intensive and could be improved and simplified if sarcosine oxidase could use creatinine directly as a substrate.

In conclusion, future investigations into TSOX structure and function could prove to be very interesting. The potential value of this unique flavoprotein as a model for exploring electron and proton transfer has not been fully realised and it has received very little attention in the past.
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