KINETIC AND SPECTROSCOPIC CHARACTERIZATION OF MEMBERS OF THE SULFITE OXIDASE FAMILY OF MONONUCLEAR MOLYBDENUM ENZYMES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

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Enzymes that possess mononuclear molybdenum can be classified into three
distinct families based on their active site geometry, molybdenum coordination and the
number of equivalents of a unique pyranopterin cofactor. In the present work, several
members of the sulfite oxidase family of mononuclear molybdenum enzymes have been
spectroscopically and kinetically investigated to provide a deeper understanding of the
nature of catalysis and electron transfer between the various redox-active centers these
enzymes possess.

A novel sulfite oxidase from *Arabidopsis thaliana* has been characterized and
shown to be a true sulfite oxidase. This enzyme is a 43-kDa monomer that contains a
single equivalent of the molybdopterin cofactor and no other redox-active centers. This
is in contrast to other members in the sulfite oxidase family that typically contain heme
domains. The plant enzyme is shown to catalyze the oxidation of sulfite to sulfate with
similar kinetics as enzymes from mammalian sources, however does so with a
significantly faster reductive half-reaction. In addition, the *A. thaliana* sulfite oxidase
exhibits similar EPR features to other sulfite oxidases, and resonance Raman reveals
peaks representative of an LMoO$_2$(S-Cys) active site with a single pyranopterin
cofactor.
Catalytic turnover experiments with mouse sulfite oxidase in $^{18}$O-labeled water have established that the source of oxygen incorporated into product is derived from solvent and not dioxygen. This observation supports the findings seen for members of the other mononuclear molybdenum enzyme families. The crystal structure for chicken sulfite oxidase revealed that the heme domain is approximately 32 Å from the molybdenum center, and not opposite the pyranopterin cofactor as would be expected. This distance does not correlate to the observed rate of electron transfer between the two domains, hence it is likely that the heme domain may be significantly mobile during catalytic turnover. Initial experiments using NMR spectroscopy have revealed conditions that will allow for the determination of the dynamic nature of the heme domain under catalytic conditions.

Investigation of several constructs of spinach assimilatory nitrate reductase mutants in the flavin domain of spinach assimilatory nitrate reductase has yielded information on the nature of electron transfer. Steady-state and rapid-reaction kinetics of these substrate-binding pocket mutants has revealed catalytic roles for each. Additionally, formation of the long-wavelength charge-transfer complex between reduced flavin and NAD$^+$ has been shown for all mutants. A possible new function for this complex in electron transfer between the flavin and heme domains is suggested.

Lastly, cDNAs encoding the human and chicken xanthine dehydrogenase enzymes have been cloned, expression systems have been developed and a number of active site mutants have been generated to investigate their roles in catalytic turnover. These systems will allow investigation of the various redox-active centers that these
enzymes possess for a more complete understanding of the detailed mechanism of
electron transfer between them.
ACKNOWLEDGMENTS

In lieu of a dedication, my acknowledgements will be many as I try to thank all those who supported and encouraged me through the long journey that is only partially described in this dissertation. I thank you ahead of time for your patience. Here goes.

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cytochrome $b_5$ cDNA construct. I would also like to thank Dr. Mark Foster and Craig McElroy (The Ohio State University) for their assistance with the mouse sulfite oxidase NMR experiments.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Abstract</strong></td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td><strong>Acknowledgments</strong></td>
<td>v</td>
</tr>
<tr>
<td></td>
<td><strong>Vita</strong></td>
<td>viii</td>
</tr>
<tr>
<td></td>
<td><strong>List of Tables</strong></td>
<td>xi</td>
</tr>
<tr>
<td></td>
<td><strong>List of Figures</strong></td>
<td>xii</td>
</tr>
<tr>
<td></td>
<td><strong>List of Abbreviations</strong></td>
<td>xv</td>
</tr>
<tr>
<td></td>
<td><strong>Chapters:</strong></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Kinetic and spectroscopic characterization of the sulfite oxidase</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>from <em>Arabidopsis thaliana</em></td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>2.2</td>
<td>Materials and Methods</td>
<td>14</td>
</tr>
<tr>
<td>2.3</td>
<td>Results</td>
<td>18</td>
</tr>
<tr>
<td>2.4</td>
<td>Discussion</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>Investigation of mouse sulfite oxidase chemistry: evidence for oxygen</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>transfer from solvent and dynamic motion of the heme domain</td>
<td></td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>42</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials and Methods</td>
<td>46</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
<td>50</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
<td>53</td>
</tr>
<tr>
<td>4.</td>
<td>Spectroscopic and kinetic investigation of the flavin-binding domain of spinach assimilatory nitrate reductase</td>
<td>75</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>75</td>
</tr>
<tr>
<td>4.2</td>
<td>Materials and Methods</td>
<td>81</td>
</tr>
<tr>
<td>4.3</td>
<td>Results</td>
<td>83</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
<td>86</td>
</tr>
<tr>
<td>5.</td>
<td>Cloning and mutagenesis of the cDNAs for human and chicken xanthine dehydrogenase</td>
<td>112</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>112</td>
</tr>
<tr>
<td>5.2</td>
<td>Materials and Methods</td>
<td>118</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>125</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
<td>132</td>
</tr>
<tr>
<td>6.</td>
<td>Summary</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>List of References</td>
<td>168</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.  $g_{1,2,3}$ and $g_{av}$ for <em>A. thaliana</em> sulfite oxidase and other sulfite oxidases ..........41</td>
<td></td>
</tr>
<tr>
<td>3.1.  Mass spectrometry standards ..............................................................................74</td>
<td></td>
</tr>
<tr>
<td>4.1.  Steady-state kinetics of the wild-type and P900A flavin domains ...................110</td>
<td></td>
</tr>
<tr>
<td>4.2.  Rapid reaction kinetic parameters for the wild-type and mutant flavin domains .................................................................111</td>
<td></td>
</tr>
<tr>
<td>5.1.  hXDH and cXDH corrections and mutations .......................................................161</td>
<td></td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Biosynthesis of the pyranopterin cofactor</td>
</tr>
<tr>
<td>1.2.</td>
<td>The mononuclear molybdenum enzyme families</td>
</tr>
<tr>
<td>2.1.</td>
<td>Reaction mechanism for sulfite oxidase</td>
</tr>
<tr>
<td>2.2.</td>
<td>pH dependence of <em>A. thaliana</em> sulfite oxidase</td>
</tr>
<tr>
<td>2.3.</td>
<td>Rapid-reaction reduction of <em>A. thaliana</em> sulfite oxidase</td>
</tr>
<tr>
<td>2.4.</td>
<td>UV-visible and CD spectra of <em>A. thaliana</em> sulfite oxidase</td>
</tr>
<tr>
<td>2.5.</td>
<td>EPR spectra of <em>A. thaliana</em> sulfite oxidase</td>
</tr>
<tr>
<td>2.6.</td>
<td>Resonance Raman spectra of <em>A. thaliana</em> sulfite oxidase</td>
</tr>
<tr>
<td>2.7.</td>
<td>Depolarization resonance Raman spectra of <em>A. thaliana</em> sulfite oxidase</td>
</tr>
<tr>
<td>3.1.</td>
<td>Chicken sulfite oxidase active site</td>
</tr>
<tr>
<td>3.2.</td>
<td>Reaction mechanism for sulfite oxidase</td>
</tr>
<tr>
<td>3.3.</td>
<td>Tris-acetate standard mass spectra</td>
</tr>
</tbody>
</table>
3.4. Sodium sulfate and sodium sulfite standard mass spectra..............................61
3.5. Sodium sulfate standard (in H2O) mass spectra..............................................63
3.6. Sodium sulfite standard (in H2O) mass spectra ..............................................65
3.7. Sample #1 mass spectra ..............................................................................67
3.8. Sample #2 mass spectra ..............................................................................68
3.9. NMR spectra of mouse sulfite oxidase at pD 7.0 ..............................................71
3.10. NMR spectra of rat outer mitochondrial membrane cytochrome b5 ............72
3.11. NMR spectra of mouse sulfite oxidase at pD 8.0 ..............................................73
4.1. Assimilatory nitrate reductase redox-active centers ........................................93
4.2. Nitrate reductase flavin domain crystal structures........................................94
4.3. Formation of the wild-type sNR-FAD charge-transfer complex with NADH and NADPH.................................................................96
4.4. Formation of the C898 mutant sNR-FAD charge-transfer complexes ..........98
4.5. Formation of the G899 mutant sNR-FAD charge-transfer complexes ..........100
4.6. Formation of the P900A mutant sNR-FAD charge-transfer complex with NADH and NADPH.................................................................102
4.7. Formation of the charge-transfer complex of wild-type and C898S mutant sNR-FAD with NADH ..............................104

4.8. Single wavelength rapid reaction kinetics of C898A mutant flavin reduction by NADH ........................................106

4.9. Full spectrum rapid reaction kinetics of flavin reduction by NADH .................................................................107

4.10. Synthetic spinach assimilatory nitrate reductase heme domain .................................................................108

5.1. Xanthine oxidase redox centers ......................................................................................................................138

5.2. UV-visible spectra and proposed reaction mechanism of xanthine oxidase ......................................................139

5.3. XDH cloning PCR methods ..........................................................................................................................141

5.4. hXDH cloning plan .............................................................................................................................................143

5.5. Expression of hXDH and cXDH ......................................................................................................................146

5.6. hXDH sequence alignment ............................................................................................................................148

5.7. cXDH cloning plan .............................................................................................................................................151

5.8. cXDH sequence alignment ............................................................................................................................154

5.9. Alignment of select XDH protein sequences .................................................................................................157
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2xYT</td>
<td>2x yeast tryptone</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>cNR</td>
<td>corn (maize) assimilatory nitrate reductase</td>
</tr>
<tr>
<td>CbR</td>
<td>cytochrome b reductase</td>
</tr>
<tr>
<td>CcR</td>
<td>cytochrome c reductase</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CODH</td>
<td>carbon monoxide dehydrogenase</td>
</tr>
<tr>
<td>cXDH</td>
<td>chicken xanthine dehydrogenase</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethylsulfide</td>
</tr>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
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<td>DMSOR</td>
<td>dimethylsulfoxide reductase</td>
</tr>
<tr>
<td>E_red</td>
<td>reduced enzyme</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>ENDOR</td>
<td>electron nuclear double resonance</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ESEEM</td>
<td>electron spin-echo envelope modulation</td>
</tr>
<tr>
<td>EXAFS</td>
<td>extended X-ray absorption fine structure</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>hpH</td>
<td>high pH</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>hXDH</td>
<td>human xanthine dehydrogenase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-thiogalactoside</td>
</tr>
<tr>
<td>lpH</td>
<td>low pH</td>
</tr>
<tr>
<td>Mo</td>
<td>molybdenum</td>
</tr>
<tr>
<td>Moco</td>
<td>molybdenum cofactor</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-([N-morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>MoR</td>
<td>molybdenum reductase</td>
</tr>
<tr>
<td>MPT</td>
<td>molybdopterin</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide (oxidized form)</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>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NR</td>
<td>nitrate reductase</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PDR</td>
<td>phthalate dioxygenase reductase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>sNR-FAD</td>
<td>spinach assimilatory nitrate reductase flavin-binding domain</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SO</td>
<td>sulfite oxidase</td>
</tr>
<tr>
<td>SOR</td>
<td>sulfite:cytochrome c oxidoreductase</td>
</tr>
<tr>
<td>Tricine</td>
<td>N-[Tris-(hydroxymethyl)methyl]glycine</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>XDH</td>
<td>xanthine dehydrogenase</td>
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<td>XO</td>
<td>xanthine oxidase</td>
</tr>
<tr>
<td>XOR</td>
<td>xanthine oxidoreductase</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Introduction

Molybdenum is found in a variety of enzymes in nearly every organism, with the exception of some archaea, and is essential for life in those organisms. It is the only second row transition metal with known biological function. Enzymes possessing molybdenum in their active sites constitute a super family of enzymes that catalyze a variety of novel reduction-oxidation reactions (Hille, 1996). Molybdenum is abundant in seawater where it is found primarily as soluble molybdate (MoO$_4^{2-}$) and is thus readily available to biological systems (Henderson, 1986). Molybdenum-containing enzymes play significant roles in global sulfur, nitrogen and carbon cycles, with several molybdoenzymes represented in each cycle (Stiefel, 2002). In humans, deficiencies in these enzymes result in the disruption of numerous metabolic processes and severe defects which can ultimately be fatal in some instances (Johnson and Duran, 2001, Raivio et al., 2001).

With the exception of nitrogenase, which contains a unique molybdenum-iron-sulfur cluster, all known mononuclear molybdenum enzymes are found coordinated by
a unique pterin cofactor originally designated molybdopterin, but now also called pyranopterin in the literature (as it also found in tungsten-containing enzymes). The cofactor consists of a pterin moiety fused to a pyran ring, which contains a side chain with a pair of dithiolate groups that coordinate the molybdenum (Romão et al., 1995). In addition, the phosphate side chain has been found in bacteria as the conjugate of adenosine, cytosine, or guanosine resulting in a dinucleotide (Hille, 1996). Pyranopterin is the product of a dedicated biosynthetic pathway (Figure 1.1), beginning with either GTP or a guanosine derivative whose biosynthesis requires the action of several different gene products (Hille, 1996). The initial moiety is converted to a precursor cofactor, called precursor Z, which does not contain the sulfur atoms required for molybdenum coordination. Addition of the dithiolene moiety creates MPT, which is followed by incorporation of molybdenum to generate the complete molybdenum cofactor, designated Moco (Mendel et al., 2002). It is prior to this last step that the nucleotide conjugation in bacteria occurs, a reaction catalyzed by the mobAB gene products. The development of a bacterial strain with a deletion in this gene (TP1000) has allowed for the expression of eukaryotic molybdoenzymes with the MPT form of the pyranopterin (Temple et al., 2000).

The pyranopterin cofactor has been proposed to play a variety of significant roles in molybdenum chemistry. It generally forms extensive hydrogen-bonding interactions with the protein backbone, thereby allowing retention of the metal center, especially in those enzymes that do not supply a protein ligand to the molybdenum (see below). It has also been proposed that the pyranopterin modulates the electronic properties of the molybdenum, fine-tunes the oxidation-reduction potential of the metal,
and acts as a conduit for electron transfer out of or into the molybdenum center (Helton et al., 2000a, Helton et al., 2000b, Joshi et al., 2003). In addition, several enzymes have two equivalents of the cofactor, each of which may play distinct roles in those systems.

The mononuclear molybdenum enzymes consist minimally of three distinct families (Figure 1.2) based on their active site composition and the general chemistry they catalyze and are described below (Hille, 1996). Determination of the crystal structures for several different molybdenum enzymes and the results of various spectroscopic measurements have shown a wide diversity of coordination environments can exist at the molybdenum center. These enzymes can possess one or two equivalents of the pyranopterin cofactor, with oxo and/or sulfido ligands to the metal and even protein ligands in some instances. In addition, it has been shown that some of these enzymes even contain active site metals in addition to the molybdenum that are critical to catalysis (Dobbek et al., 2002). Finally, a wide variety of additional redox-active centers are found in most molybdenum enzymes that function to shuttle reducing equivalents into or out of the molybdenum center. The three molybdenum enzyme families are distinguished on the basis of their coordination spheres, the number of pyranopterin equivalents and the nature of the additional ligands to the metal. Catalysis within each family is based on oxygen atom transfer chemistry, with each having distinct properties that will be discussed below.

The Xanthine Oxidase Family

The xanthine oxidase family, more commonly known as the molybdenum hydroxylases, is the most extensively studied of the three families of mononuclear molybdenum enzymes. These enzymes catalyze hydroxylation reactions at activated
carbon centers (typically aromatic heterocycles). Their active sites are typically of the LMoOS variety, with a bound water or hydroxide that is mechanistically involved in catalytic turnover (Hille, 1996, Xia et al., 1999, Enroth et al., 2000). Crystal structures exist for a number of enzymes in this family, including aldehyde oxidoreductase from *D. gigas* (Romão et al., 1995, Rebelo et al., 2001), xanthine dehydrogenase/oxidase from *B. taurus* (Enroth et al., 2000) and *R. capsulatas* (Truglio et al., 2002), and carbon monoxide dehydrogenase (CODH) from *O. carboxydivorans* (Dobbek et al., 1999, Dobbek et al., 2002). These enzymes have similar architectures, with an N-terminal iron-sulfur domain and a C-terminal molybdenum-binding domain common to all three. Xanthine oxidoreductase and CODH also have a central FAD-binding domain which is absent in the aldehyde oxidoreductase. Interestingly, CODH has an LMoO$_2$ active site coordination, similar to that seen in the sulfite oxidase family of enzymes, and has been shown to contain a catalytic copper-sulfur center (coordinated by a cysteine residue) in addition to the molybdenum (Dobbek et al., 2002).

Xanthine oxidoreductase is the eponymous enzyme in this family, although recombinant technology has provided a means for significant insights into other family members. The enzyme catalyzes the hydroxylation of hypoxanthine to xanthine and further on to uric acid in the final steps of purine catabolism. It has the distinction of being the focus of the first tandem-drug design therapy involving administration of a chemotherapeutic agent, the inventors of which won the Nobel Prize in Physiology or Medicine in 1988. The reaction at the molybdenum center is proposed to be initiated through base-assisted catalysis via proton abstraction from the Mo-OH group by an active site glutamate residue, followed by nucleophilic attack by substrate to form an
intermediate with concomitant transfer of a hydride group to the molybdenum sulfido (Manikandan et al., 2001). Displacement of product by solvent water is followed by transfer of reducing equivalents out of the molybdenum center transiently through the two iron-sulfur centers and finally to the flavin center, where they pass to the oxidizing substrate, NAD$^+$ or oxygen for the dehydrogenase or oxidase forms of the enzyme respectively.

**The Sulfite Oxidase Family**

Enzymes that comprise the sulfite oxidase family, which include the plant assimilatory nitrate reductases in addition to the sulfite oxidases, possess a LMoO$_2$(S-Cys) active site structure. These enzymes, also known as the eukaryotic oxotransferases, catalyze oxygen atom transfer reactions to or from substrate with a lone pair of electrons. These reactions are somewhat simpler than the hydroxylation reaction seen for xanthine oxidoreductase. In sulfite oxidase for instance, nucleophilic attack of the substrate lone electron pair on an oxo group on the molybdenum results in a bidentate intermediate (essentially bound product), which is subsequently displaced by solvent. The opposite reaction is thought to occur for the assimilatory nitrate reductases, although the mechanism has been less studied.

Sulfite oxidase catalyzes the terminal step in the degradation of sulfur containing amino acids and lipids and is associated with the resulting pathology seen for combined molybdenum cofactor deficiency (Johnson and Duran, 2001). Dislocation of the ocular lenses, mental retardation, seizures, brain atrophy and death result from loss of sulfite oxidase function, most likely due to a build up of sulfatide-derived sulfite in the myelin sheath (Vos et al., 1994). The assimilatory nitrate reductases catalyze the
first and rate-limiting step in nitrogen assimilation in higher plants, algae, and fungi (Solomonson and Barber, 1990). The crystal structure for chicken sulfite oxidase has been reported (Kisker et al., 1997) and has revealed much about the active site architecture of these enzymes, as will be discussed later. A partial crystal structure for the FAD-binding domain of assimilatory nitrate reductase has been solved, but further structural work remains to determine the full structure (Lu et al., 1995).

**The Dimethylsulfoxide Reductase Family**

The last family of molybdenum enzymes, the dimethylsulfoxide (DMSO) reductase family, consists of the bacterial oxotransferases, which typically possess L₂MoO(X) active sites. Two spectroscopically distinct equivalents of the pyranopterin cofactor are coordinated to the molybdenum along with an oxo ligand and a protein ligand (X), either a serine, cysteine, or selenocysteine. This family, which includes the dissimilatory nitrate reductases, biotin sulfoxide reductase, arsenite oxidoreductase, and formate dehydrogenase, catalyze important reactions in nitrogen and sulfur cycles in the environment and a wide range of other oxygen atom transfer reactions. DMSO reductase, for example, catalyzes the transfer of oxygen from substrate and the resulting dimethylsulfide, which is volatile, enters the atmosphere where it is proposed to play a role in global climate conditions. Arsenite oxidoreductase, which lacks a protein ligand and instead has a bound hydroxide or water molecule in the oxidized state, plays a significant role in reducing toxic levels of arsenite, whose toxicity arises from its binding for vicinal sulfhydryl groups of proteins. In addition to the molybdenum center, these enzymes may contain one or more additional redox-active centers, which
Dissertation Research

The research presently described primarily focuses on enzymes in the sulfite oxidase family. The spectroscopic and kinetic characterization of a novel plant sulfite oxidase is presented in Chapter 2, the results of which demonstrate significant similarities to sulfite oxidases from mammalian sources. This enzyme differs from all other eukaryotic enzymes, however, in that it contains only a molybdenum-binding domain with no other redox-active centers present. This work provides a solid foundation for more detailed spectroscopic investigation of the molybdenum active site of a member of the sulfite oxidase family without confounding spectroscopic interference from other redox active centers.

Investigation of the reaction mechanism of mouse sulfite oxidase in Chapter 3 provides preliminary results addressing two significant mechanistic questions. First, turnover experiments in $^{18}$O enriched water have shown unequivocally that the source of oxygen incorporated into product is derived from solvent, following suit with other molybdenum enzymes. This finding is different from typical oxidases that utilize molecular oxygen as the catalytic source of oxygen. In addition, nuclear magnetic resonance (NMR) spectroscopic investigation of the enzyme has been undertaken to determine changes in the structural dynamics of sulfite oxidase associated with changes in oxidation state. The crystal structure of the chicken enzyme suggests that the heme domain is bound to the molybdenum domain in a location unfavorable for efficient electron transfer between the two centers. The “linker” region between the metal center
domains is thought to be very flexible in solution, functioning to tether the heme domain to the rest of the enzyme. These NMR experiments reveal useful information regarding the state of the enzyme at various catalytic points in comparison to that seen for a homologous free heme protein.

Kinetic experiments described in Chapter 4 for the flavin-binding domain of spinach assimilatory nitrate reductase and for a number of mutants in the substrate-binding site of the flavin-binding fragment provide understanding for the roles of those residues in catalysis and substrate binding. In addition, preliminary results from spectroscopic investigation of the FADH$_2$-NAD$^+$ charge-transfer complex will provide direction for future experiments to fully establish its overall role in catalysis.

Finally, protocols for the cloning of the cDNAs for human and chicken xanthine dehydrogenases are outlined in Chapter 5, along with several site-directed mutants that were generated for kinetic analysis. These systems will allow for a detailed investigation of the reaction mechanism and questions regarding substrate binding and ionization. Investigation of residues critical to the functioning of the individual redox-active centers will also be of significant value.
Figure 1.1. Biosynthesis of the pyranopterin cofactor. Synthesis of the unique pterin cofactor found in molybdenum enzymes, beginning with GTP (or guanosine derivative), forming Precursor Z, followed by addition of sulfur and molybdenum to form the molybdopterin (MPT) and molybdenum cofactor (Moco) respectively. (Adapted from Mendel et al., 2002)
Figure 1.2. The mononuclear molybdenum enzyme families. The active site coordination of the xanthine oxidase (top), sulfite oxidase (middle) and DMSO reductase (bottom) families of the mononuclear molybdenum enzymes.
CHAPTER 2

KINETIC AND SPECTROSCOPIC CHARACTERIZATION OF THE
SULFITE OXIDASE FROM Arabidopsis thaliana

2.1. Introduction

Sulfite oxidase catalyzes the oxidation of sulfite to sulfate, the final step in the oxidative degradation of the sulfur containing amino acids and lipids, the latter being prevalent in the cell membranes of the myelin sheath. The severe neurological disorders manifested by individuals with mutations in the structural gene for sulfite oxidase suggest that clinical symptoms arise principally from a dysfunction in lipid rather than protein metabolism. Sulfite oxidase belongs to the same family of mononuclear molybdenum enzymes as the plant assimilatory nitrate reductases, enzymes whose (oxidized) active sites can be formulated as LMo\textsuperscript{VI}O\textsubscript{2}(S-Cys) (Hille, 1996), with L representing the pterin cofactor common to all mononuclear molybdenum and tungsten enzymes. The enzyme is typically a 110-kDa homodimer, containing an N-terminal cytochrome \textit{b}_5-type domain, a central molybdenum-binding domain and a C-terminal dimerization domain. In vertebrates, sulfite oxidase is located in the intermembrane space of mitochondria and passes reducing equivalents on to
cytochrome c, the physiological electron acceptor (Figure 2.1). Deficiencies in this enzyme, either from mutations in the protein sequence (Garrett et al., 1998) or from combined molybdenum cofactor deficiency (Johnson and Duran, 2001), lead to the severe neurological abnormalities referred to above, which presumably arise from accumulation of the strongly nucleophilic sulfite.

The active site structures of sulfite oxidases from mammalian and avian sources have been studied by electron paramagnetic resonance (EPR) (Lamy et al., 1980, Bray et al., 1983, Dhawan and Enemark, 1996, Cramer et al., 1979), electron spin echo-envelope modulation (ESEEM) (Pacheco et al., 1996, Raitsimring et al., 1998, Astashkin et al., 2000), extended x-ray absorption fine-spectra (EXAFS) (Cramer et al., 1981, George et al., 1996, George et al., 1989) and resonance Raman spectroscopy (Garton et al., 1997a, Johnson et al., 1997b). In addition, the crystal structure of the reduced form of the enzyme from chicken liver has been reported at a resolution of 1.9 Å (Kisker et al., 1997). The active site sits in the bottom of a positively charged binding pocket, with a Mo-OH ligand (presumably Mo=O in the oxidized enzyme) in an equatorial position, facing into the solvent access channel. The other Mo=O is in an apical position, with the remaining apical position being blocked by the protein backbone, resulting in a distorted square pyramidal coordination geometry.

Atypical sulfite oxidases have been found which do not have the same redox-active centers as the enzyme from vertebrate sources. Sulfite:cytochrome c oxidoreductase (SOR) from *Starkeya novella* (formerly *Thiobacillus novellus*), a member of the α-2 subclass of the proteobacteria (Kelly et al., 2000), has been cloned and expressed, and the protein characterized (Kappler et al., 2000). Sulfite oxidase
from this source is a heterodimeric enzyme, possessing a 40.6-kDa molybdenum-containing protein and an associated 8.8-kDa cytochrome $c_{552}$ protein rather than a $b$-type cytochrome. Reduction of sulfite at the molybdenum center is followed by transfer of reducing equivalents to the cytochrome $c_{552}$ and further on to cytochrome $c_{550}$, with similar kinetic parameters as seen for the chicken enzyme. Interestingly, Mo$^V$ EPR studies of this bacterial enzyme reveal identical features typical of a high-pH EPR signal at low or high pH, where as most sulfite oxidases have pH dependent EPR signals, and did not exhibit a phosphate-inhibited signal (Kappler et al., 2000). Additionally, electrochemical studies have shown a pH dependence of the reduction potentials for the molybdenum and heme centers (Aguey-Zinsou et al., 2003), analogous to that seen for the chicken enzyme (Sunde et al., 1991).

The presence of sulfite oxidation in plants would seem questionable, working counteractive to the activity of the well-known sulfite reductases. In chloroplasts, sulfur is assimilated via the conversion of sulfate, through sulfite, to sulfide (Leustek and Saito, 1999). Sulfide is then used to generate cysteine, which in turn is used for the synthesis of methionine and other sulfur-containing metabolites. Nevertheless, sulfite oxidase activity has been detected in spinach and wheat chloroplasts (Jolivet et al., 1995a, Jolivet et al., 1995b), and the isolation of a functionally similar enzyme from *Malva sylvestris* (Ganai et al., 1997) has led to further investigation. Recently a sulfite oxidase from *Arabidopsis thaliana* (*A. thaliana* sulfite oxidase) has been cloned and expressed (Eilers et al., 2001). The enzyme is a 43.3-kDa protein containing only the molybdenum-binding domain and a vestigial dimerization domain seen in typical sulfite oxidases, although it exists primarily as a monomer in solution. While the plant enzyme
does not contain a heme domain, it seems likely that a $b$-type cytochrome would be its physiological electron acceptor (Eilers et al., 2001). Antibodies raised to *A. thaliana* sulfite oxidase were found to react with similarly sized proteins from a variety of higher plants, suggesting the existence of sulfite oxidases in a variety of plants (Eilers et al., 2001). Preliminary investigation of *A. thaliana* sulfite oxidase has revealed similarities to the chicken enzyme with regard to catalysis and the EPR signals seen at low- and high-pH, although the low-pH signal reported appears to be a mixture of signals.

The lack of an associated heme domain makes *A. thaliana* sulfite oxidase unique to eukaryotic molybdoproteins, which permits a detailed examination of the molybdenum center of this family of enzymes (although recombinant systems have been established for the purification of the molybdenum domains of rat (Garrett and Rajagopalan, 1996) and human sulfite oxidase (Temple et al., 2000)). Studies of *A. thaliana* sulfite oxidase, will provide significant information regarding the molybdenum center and the pyranopterin cofactor. Results from the present spectroscopic and kinetic studies demonstrate that *A. thaliana* sulfite oxidase is a true sulfite oxidase with many features in common with sulfite oxidases isolated from other sources.

### 2.2. Materials and Methods

*Protein Expression and Purification*

Sulfite oxidase from *A. thaliana* was expressed and purified according to Eilers *et al.* (Eilers et al., 2001) with the following modifications. *E. coli* TP1000 ($\Delta$mobAB) cells carrying the plasmid pQE-80-sox were grown aerobically at 30°C for 24 hours after induction with 0.1 mM isopropyl-$\beta$-thiogalactoside at low cell density ($A_{600}=0.05$). Crude lysate from cell lysis / sonication was loaded onto a Ni-NTA column (Qiagen)
pre-equilibrated with lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole). The column was washed with 2-3 times the bed volume of lysis buffer followed by wash buffer consisting of 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole, until the eluate had an OD₂₈₀ less than 0.05. *A. thaliana* sulfite oxidase was eluted with 100 mM imidazole lysis buffer. All buffers contained 0.01 mM PMSF to prevent proteolysis. Fractions containing *A. thaliana* sulfite oxidase as judged by SDS-PAGE analysis were pooled, concentrated by ultrafiltration and loaded onto a Mono-Q column pre-equilibrated with 50 mM NaH₂PO₄, pH 8.0. *A. thaliana* sulfite oxidase was eluted with the same buffer and fractions were concentrated based on purity (UV-visible spectrum, SDS-PAGE). Concentrated *A. thaliana* sulfite oxidase was exchanged into 20 mM Tris-acetate, pH 8.0, 300 mM NaCl by gel filtration on a Sephadex G-25 column and stored at 5°C for immediate use or in liquid nitrogen for long-term storage. Enzyme concentration was determined spectrophotometrically using an extinction coefficient of 69,820 M⁻¹ cm⁻¹ at 280 nm (Eilers *et al.*, 2001).

*Sulfite Oxidase Assays*

Assays for *A. thaliana* sulfite oxidase were carried out in 20 mM Tris-acetate, pH 8.0 following reduction of potassium ferricyanide (ε=1,020 M⁻¹ cm⁻¹) at 420 nm (Gardlik and Rajagopalan, 1991) or reduction of horse heart cytochrome c (ε=19,630 M⁻¹ cm⁻¹) at 550 nm (Brody and Hille, 1999). Steady-state kinetic measurements were performed aerobically at 25°C using a 1.0 cm light path cuvette and a final sample buffer volume of 1.0 ml, monitoring reduction of potassium ferricyanide. The buffers used for the pH dependence profile were Bis-Tris (pH 6.0-6.5), Bis-Tris-propane (pH 7.0-7.5), Tris (pH 7.5-8.5) and glycine (pH 9.0-10.0). All buffers (in their conjugate
base forms) were used at 20 mM concentration and adjusted to a pH of 8.0 with acetic acid rather than HCl to avoid inhibition by chloride (Kessler and Rajagopalan, 1972, Eilers et al., 2001). Sulfite concentration was varied from 5-200 µM while potassium ferricyanide was kept at a saturating concentration of 400 µM. Rat outer mitochondrial membrane cytochrome $b_5$ was prepared according to Rivera et. al. (Rivera et al., 1992) with an additional S-200 purification step (see Chapter 3, Materials and Methods). Purity was based on $A_{280}/A_{414}$ ratio of $\leq 0.3$ and enzyme concentration was determined spectrophotometrically using an extinction coefficient of 117 mM$^{-1}$ cm$^{-1}$ at 414 nm (Fukushima and Sato, 1973) and a calculated molecular weight of 10,367 Da. Horse heart cytochrome $c$ and superoxide dismutase solutions were purchased from Sigma and prepared in the appropriate buffer prior to use in steady-state assays.

Rapid reaction kinetics was performed on an Applied Photophysics Inc. stopped-flow apparatus. Single-wavelength kinetic transients were measured at 360 nm using a photomultiplier tube and full spectrum analysis (250-750 nm) using a diode array detector. Reductive half-reactions were carried out anaerobically at 5°C using varying concentrations of $A. thaliana$ sulfite oxidase (7.5–25 µM) and sulfite (180-800 µM). Samples were made anaerobic by alternately flushing with argon and evacuating every ten minutes over the course of 1.5 hours in a glass tonometer.

**Spectroscopic Methods**

UV-visible spectra and kinetic assays were recorded using a Hewlett-Packard 8452A single beam diode-array spectrophotometer. Reduction of the enzyme was performed using either sodium dithionite or sodium sulfite as circumstances dictated.
Circular dichroism spectra were recorded using an AVIV 40DS UV-VIS-IR Circular Dichroism Spectrometer. Enzyme was 125 µM in 20 mM Tris-acetate, pH 8.0. Data were recorded (4 repeats) from 350 – 600 nm in a 1 mL path length quartz cuvette with a scan step of 2 nm/s and a 4 nm bandwidth.

Electron paramagnetic resonance spectra were recorded using a Brüker ER 300 spectrometer equipped with an ER 035 M gaussmeter and a Hewlett-Packard 5352 B microwave frequency counter. Temperature was controlled at 150 K using a Brüker ER 4111 VT continuous flow liquid nitrogen cryostat. Samples were prepared by exchange into the appropriate buffer through gel filtration. Sodium sulfite was added in a stoichiometric excess to the sample in an EPR tube, which had been flushed with argon for 15 min. A half-molar equivalent of potassium ferricyanide solution was added to partially reoxidize the sample, which was then immediately frozen in a dry-ice/acetone bath and stored in liquid nitrogen.

Resonance Raman spectra were recorded using 488 nm excitation from a Coherent Innova 307 argon-ion laser. Plasma emission lines were removed using Pellin-Broca prisms and Rayleigh-scattered photons rejected using a holographic notch filter. Raman-scattered light was collected into a Chromex 500 IS single-stage spectrograph equipped with a Princeton Instruments 1024 KTB, back-thinned, charge-coupled device as detector. Band positions were calibrated using an external indene standard and are accurate to ± 2 cm⁻¹. Data were collected from the surface of a 30 µl sample maintained at 30 K using a custom-built cold finger and an APD Cryogenics closed cycle liquid helium refrigerator. Each sample was illuminated for several hours prior to data collection to reduce background fluorescence. Samples for resonance
Raman studies were prepared in 50 mM Tricine, pH 8.0, and were concentrated to 1-3 mM by Centricon ultrafiltration. Redox-cycled enzyme was prepared by reduction/re-oxidation of the enzyme with an excess of sodium sulfite and ferricyanide, followed by gel-filtration. Exchange into H$_2^{18}$O (95-97% isotopically enriched H$_2^{18}$O from Isotec, Inc.) was accomplished by two 10-fold dilution/reconcentration cycles, reduction using sodium sulfite and air reoxidation, followed by three additional 10-fold dilution/reconcentration cycles.

Depolarization ratio measurements were performed on an aqueous sample held in a standard quartz EPR tube. The sample was suspended in the excitation beam using a modified Teflon sleeve that fit into a standard NMR tube spinner. A 2” diameter polarization analyzer (Ealing), held in a 360°-rotatable mount, was included as part of the illumination stage of the resonance Raman system. A polarization scrambler (Oriel) was placed directly at the entrance slit of the Chromex 500 IS spectrograph. A 1:1 mixture of carbon tetrachloride (CCl$_4$) and benzene was used to both calibrate the Raman shift as well as to determine the proper settings of the polarization analyzer for $I_{\text{parallel}}$ and $I_{\text{perpendicular}}$. Using the 314.2 cm$^{-1}$ ($\rho \sim 0.75$) and 459 cm$^{-1}$ ($\rho \sim 0.01$) bands of CCl$_4$ and the 991.6 cm$^{-1}$ ($\rho \sim 0.05$) band of benzene it was determined that $I_{\text{parallel}}$ is measured with the polarization analyzer at 44° and the $I_{\text{perpendicular}}$ at 134°. From this the depolarization ratio ($\rho$) was determined as the ratio of $I_{\text{perpendicular}} / I_{\text{parallel}}$.

2.3. Results

Kinetics of A. thaliana sulfite oxidase

To explore the effect of pH on the kinetics of A. thaliana sulfite oxidase, a steady-state analysis was carried out in the sulfite concentration range 5-200 µM while
keeping potassium ferricyanide constant at 400 µM over the pH range of 6.0 – 10.0. A plot of $k_{\text{cat}}$ versus pH yielded a bell-shaped curve indicating a maximal activity at pH 8.5, and apparent $pK_a$s of 8.1 and 8.9 (Figure 2.2). These results are similar to those observed for chicken sulfite oxidase (Brody and Hille, 1999), although the differences in the $pK_a$ values for the chicken enzyme (7.0 and 10.2 respectively) may have interesting implications for active site chemistry as will be addressed below.

*A. thaliana* sulfite oxidase lacks the cytochrome $b_5$ domain typical of mammalian and avian sulfite oxidases, and at present the physiological electron acceptor is unknown. It has been suggested that a $b$-type cytochrome from plant peroxisomes is a likely candidate to fill this role (Eilers *et al.*, 2001). We have investigated the reactivity of *A. thaliana* sulfite oxidase with rat outer mitochondrial membrane cytochrome $b_5$ (Rivera *et al.*, 1992), and with the cytochrome $b_5$ domain of nitrate reductase (see Chapters 3 and 4 for details on each domain). No reduction of either heme group is observed when *A. thaliana* sulfite oxidase was reacted with an excess of sulfite.

Additional reactions were carried out using horse heart cytochrome $c$ as electron acceptor. Assays of *A. thaliana* sulfite oxidase with sulfite in the presence of cytochrome $c$ showed a significantly slower activity than that observed using potassium ferricyanide as electron acceptor (~1%). Addition of superoxide dismutase to the reaction mixture reduced the observed activity by almost half, suggesting that $O_2^{-}$ is largely responsible for the observed reduction of cytochrome $c$ (McCord and Fridovich, 1968, Fridovich, 1970), not electron transfer from *A. thaliana* sulfite oxidase. Reaction
of *A. thaliana* sulfite oxidase with selenite (SeO$_3^{2-}$) showed approximately 5% of the observed sulfite activity using potassium ferricyanide as electron acceptor.

An extensive rapid kinetics study of the reaction of *A. thaliana* sulfite oxidase with sulfite was prohibited by the rate of the reaction. Single-wavelength transients showed that the reduction of molybdenum at even the lowest concentrations of sulfite was essentially complete within 5 msec after mixing at 5°C in the stopped-flow apparatus. With a diode array detector, it was clear that reduction had already been completed after the first spectrum when compared with that of unreacted oxidized enzyme shot against buffer rather than a solution of sulfite (Figure 2.3). Longer reaction times did not show any appreciable re-oxidation artifacts ruling out the presence of oxygen in the tonometer.

*Spectroscopic Characterization of A. thaliana sulfite oxidase*

With the addition of the Mono-Q chromatography step to the *A. thaliana* sulfite oxidase purification, which removed the apo-enzyme lacking the molybdenum center, an improved UV-visible absorption spectrum has been obtained as compared to that reported previously (Figure 2.4.A). The absorbance maxima seen at 360 nm and 480 nm compare well with the maxima seen for the molybdenum domain obtained by proteolytic cleavage of the rat (Johnson and Rajagopalan, 1977) and recombinant human (Garrett and Rajagopalan, 1996) sulfite oxidases, as well as a truncated expression construct of human sulfite oxidase (Temple et al., 2000). The maximum seen at 360 nm, attributed to dithiolene-molybdenum charge-transfer transition(s), and the broad shoulder observed at 480 nm, proposed to arise from cysteine-to-molybdenum charge-transfer (Garrett and Rajagopalan, 1996, Garton et al., 1997a), are readily
evident in the spectrum of the plant enzyme. Upon reduction of the enzyme with either sulfite or dithionite there is a loss of the observed maxima at 360 nm and 480 nm with a slight maximum appearing at approximately 400 nm, again consistent with previous results with the molybdenum fragment of the enzyme from vertebrate sources. A circular dichroism spectrum of oxidized *A. thaliana* sulfite oxidase showed strong positive features at approximately 365 nm and 480 nm (Figure 2.4.B), which correlate with the observed UV-visible maxima at 360 nm and 480 nm.

It is well-established that vertebrate sulfite oxidases give different Mo$^V$ electron paramagnetic resonance signals depending on experimental conditions (e.g., pH and anion concentration). EPR spectra of the low-pH (lpH), high-pH (hpH) and phosphate-complexed forms of sulfite oxidase from the chicken and bovine enzymes have been well characterized (Lamy *et al.*, 1980, Cohen *et al.*, 1971, Kessler and Rajagopalan, 1972). The EPR spectra of the lpH and hpH forms of *A. thaliana* sulfite oxidase have been examined (Figure 2.5). The hyperfine splitting due to a solvent-exchangeable proton seen in the lpH spectra of bovine and chicken enzyme is not readily observed in *A. thaliana* sulfite oxidase, although the broad shoulder observed at $g = 2$ in the lpH form of *A. thaliana* sulfite oxidase is slightly sharpened upon exchange of the protein into deuterated buffer (suggesting unresolved proton hyperfine in $^1$H$_2$O). Addition of sodium chloride to the lpH sample buffer resulted in a slightly more intense low-field signal with greater evidence of proton hyperfine splitting. The estimated $g$-value for the midpoint of the lpH-NaCl low-field feature ($g_t = 2.0033$, $A_1 = 1.12$ mT) agrees well with that seen for chicken sulfite oxidase ($g_t = 2.0037$, $A(H)_1 = 0.85$ mT). Consistent with previous work with the plant enzyme, we have been unable to observe the
phosphate-inhibited signal such as has been seen with the chicken protein (signal appeared as the lPH form in up to 0.1 M NaH$_2$PO$_4$); we have also been unable to obtain an arsenate-inhibited signal (George et al., 1998). The $g$-values obtained for the various forms of *A. thaliana* sulfite oxidase as well as the corresponding parameters for the chicken and *S. novella* enzymes are presented in Table 2.1.

In the absence of the heme domain typically seen in vertebrate sulfite oxidases, the molybdenum center of *A. thaliana* sulfite oxidase can be conveniently examined using resonance Raman spectroscopy, with maximal resonance enhancement of the molybdenum coordination sphere modes at 488 nm, which falls under the 480-nm absorption envelope. The resonance Raman spectra of *A. thaliana* sulfite oxidase were obtained for the as-prepared and redox-cycled (both H$_2^{16}$O and H$_2^{18}$O cycled) enzymes (Figure 2.6). The as-prepared and H$_2^{16}$O redox-cycled samples showed typical Mo=O modes at 896 cm$^{-1}$ and 876 cm$^{-1}$, representing symmetric and antisymmetric Mo=O stretching modes, respectively, similar to those seen for wild-type recombinant human sulfite oxidase (Garton et al., 1997a) and in dioxomolybdenum (VI) complex studies (Willis and Loehr, 1987). Upon redox cycling of the enzyme in H$_2^{18}$O, the 896 cm$^{-1}$ mode shifts to 884 cm$^{-1}$ ($\Delta 12$ cm$^{-1}$) and the 876 cm$^{-1}$ mode to 855 cm$^{-1}$ ($\Delta 21$ cm$^{-1}$) these previously representing the Mo=O symmetric and asymmetric modes respectively. This is less than half of the expected shift for full $^{18}$O incorporation at both Mo=O sites ($\Delta 33$ cm$^{-1}$ versus $\Delta 88$ cm$^{-1}$), suggesting that only one of the two Mo=O groups is catalytically exchangeable in the course of redox-cycling.
The \( \pi \)-delocalized dithiolene modes, seen with other molybdenum enzymes were not strongly resonance-enhanced with 488 nm excitation in \( A. \) thaliana sulfite oxidase, and assignments here are based on previously made mode assignments for human sulfite oxidase (Garton \textit{et al.}, 1997a) and DMSO reductase (Garton \textit{et al.}, 1997b). Pyranopterin dithiolene peaks were observed at 1004 cm\(^{-1}\), 1158 cm\(^{-1}\) and 1528 cm\(^{-1}\) (attributed to C-C stretching mode, coupled C-S and C-C stretching modes and C=C stretching mode respectively). Lower intensity peaks at 1094 cm\(^{-1}\) and 1127 cm\(^{-1}\) can be assigned to the coupled C-S / C-C stretching modes reported for DMSOR (1126 cm\(^{-1}\)) and sulfite oxidase (1093 cm\(^{-1}\), Garton \textit{et al.}, 1997b). The peak seen at 864 cm\(^{-1}\), previously assumed to be due to the dithiolene C-S stretching modes (Garton \textit{et al.}, 1997a, Johnson \textit{et al.}, 1997), is lost upon \(^{18}\)O labeling of the molybdenum.

Additional peaks are observed at 290 cm\(^{-1}\), corresponding to \( S_\gamma-C_\beta-C_\alpha \) bending, and 364 cm\(^{-1}\), corresponding to Mo-S stretching, for the Cys-S-Mo bond in the chicken enzyme (Garton \textit{et al.}, 1997a). Loss of the 480-nm absorption band upon reduction of \( A. \) thaliana sulfite oxidase with sodium dithionite has prevented acquisition of data for the Mo\(^{IV}\) coordination sphere due to lack of resonance enhancement.

A resonance Raman polarization experiment has been performed to determine the depolarization ratios for the Mo=O modes attributed to the symmetric and asymmetric stretching, and to determine the amount of coupling between the two Mo=O groups at the active site (Figure 2.7). Excitation was performed at 488 nm on a room temperature sample with parallel and perpendicular polarized light. Depolarization ratios \((\rho)\) of 0.30, 0.32 and 0.35 were determined for the 864 cm\(^{-1}\), 876 cm\(^{-1}\) and 896
cm$^{-1}$ peaks (the undetermined mode and the asymmetric and symmetric Mo=O modes respectively).

2.4. Discussion

The present work provides for a more detailed characterization of the kinetic and spectroscopic properties of the sulfite oxidase from *Arabidopsis thaliana*. The pH-dependence of the steady-state kinetics of *A. thaliana* sulfite oxidase is similar to that seen previously with the chicken enzyme, and the previously reported $K_m^{\text{sulfite}}$ value is comparable (Eilers et al., 2001). However, the pK$_a$ values determined for *A. thaliana* sulfite oxidase are slightly different than those determined for the chicken enzyme, which could have significant mechanistic implications for the plant enzyme. The three arginines (Arg138, Arg190, Arg450), Trp204 and Tyr322 (numbered from the chicken sequence), shown from the crystal structure of the chicken enzyme to be involved in forming the substrate binding pocket (Kisker et al., 1997), are conserved in the plant protein, suggesting a similar arrangement for *A. thaliana* sulfite oxidase. The higher pK$_a$ seen for *A. thaliana* sulfite oxidase (pK$_a$ = 8.9) is significantly different than the observed pK$_a$ for the chicken enzyme (pK$_a$ = 10.2) for the pH dependence of $k_{\text{cat}}$ (Brody and Hille, 1999). This does not correlate well to the expected pK$_a$ of a catalytically relevant tyrosine residue, which when deprotonated would likely compromise the positively charged binding pocket (Brody and Hille, 1999), and the observed differences could instead be due to local environment perturbations. A crystal structure for the plant enzyme would yield crucial information regarding the arrangement of residues in the active site to further elucidate the role of the tyrosine residue and the source of the higher pK$_a$ seen for *A. thaliana* sulfite oxidase. In addition, a kinetic
investigation of a Tyr to Phe mutant would facilitate an assignment. The only obvious
difference in the proposed binding pocket of the plant enzyme is an Arg at the position
equivalent to Lys200 in the chicken sequence (and conserved amongst other
mammalian SOs). This residue, while not within interacting distance of the bound
sulfate in the crystal structure (Kisker et al., 1997), may still contribute to substrate
binding and thus have a more important role than previously thought. The same residue
in corn leaf assimilatory nitrate reductase is a Leu, potentially supporting an importance
in the sulfite oxidases in binding a more negatively charged sulfite molecule.

Rapid reaction kinetic experiments following the reduction of *A. thaliana* sulfite
oxidase by sulfite have demonstrated that reduction of the molybdenum center in the
plant enzyme is extremely fast. Even at 5°C the reaction is essentially complete in the
dead-time of the stopped-flow apparatus (ca. 2 msec), indicating that $k_{\text{red}}$ for the plant
enzyme is $> 1000 \text{ s}^{-1}$, significantly greater than the 190 s$^{-1}$ seen for the chicken liver
enzyme. Investigation using full-spectrum analysis confirmed that reduction of the
molybdenum was very rapid, essentially preventing accurate determination of $k_{\text{red}}$ and
$K_d$ values for the sulfite reaction.

Neither rat outer mitochondrial membrane cytochrome $b_5$, spinach nitrate
reductase cytochrome $b_5$ or horse heart cytochrome $c$ are effective oxidizing substrates
for *A. thaliana* sulfite oxidase, leaving open the question as to the physiological electron
acceptor for the enzyme. While cytochrome $b_5$ is known to exist in the peroxisome, the
location of *A. thaliana* sulfite oxidase, it is thought to be the redox partner of an
NADH:cytochrome $b_5$ reductase (López-Huertas et al., 1999), which has similar
activity to that seen by the functional flavin domain of nitrate reductase (see Chapter 4)
and therefore might have similar reduction potentials as seen for the heme domains of those enzymes. The reduction potential for the heme in chicken sulfite oxidase is ~50-90 mV (Spence et al., 1991, Elliot et al., 2002), while that of the Mo$^{IV}/V$ couple is –60 mV (Barber et al., 1988), -110 mV (Cramer et al., 1980), and -239 mV (Spence et al., 1991) for the chicken and beef liver enzymes. The reduction potentials for the molybdenum center of chicken sulfite oxidase are significantly affected by pH and anion concentration, while the reduction potential for the heme center remains relatively stable (Spence et al., 1991). If the reduction potential of the molybdenum center of A. thaliana sulfite oxidase is similar to that seen for the chicken enzyme then we would likely not see electron transfer to the individual cytochrome $b_5$ domains used in this work. The rat outer mitochondrial membrane cytochrome $b_5$ domain has a reduction potential of –102 mV (Rivera et al., 1994) while that of the synthetic heme domain from spinach nitrate reductase is -118 mV (Barber et al., 2002). If this were representative of the reduction potentials of the $b_5$–type cytochromes in the peroxisome, this would suggest an alternate redox partner. The cleaved heme domain from rat sulfite oxidase is able to transfer electrons from pig liver microsomal NADH:cytochrome $b_5$ reductase to cytochrome $c$, although at reduced efficiency. This result makes the present data, indicating that it cannot accept an electron from the plant enzyme, all the more surprising (Johnson and Rajagopalan, 1977).

Interestingly, the recombinant corn leaf assimilatory nitrate reductase heme domain has a different potential than that seen for the heme domains listed above: –50 mV (Skipper et al., 2001). Indeed, molybdenum reductase (Mo:R) constructs of the corn enzyme containing the linker region between the heme and molybdenum domains
have shown a decrease in the reduction potentials of the heme, from +20 mV to –60 mV, implicating the linker domain and/or the molybdenum domain in moderating the heme reduction potential (Mertens et al., 2000). This conclusion is further supported by the location of the phosphorylatable serine in this linker region, given its proposed role in the regulation of the enzyme due to interference with electron transfer between the two domains (Hille, 1996).

*Arabidopsis thaliana* sulfite oxidase exhibits MoV EPR signals at low and high pH with g-values very similar to those seen under comparable conditions with vertebrate sulfite oxidases. Still, significant differences are observed that indicate unique aspects of the *Arabidopsis thaliana* sulfite oxidase active site. We have been unable, for example, to detect a phosphate- or arsenate-complexed EPR signal under any of a variety of experimental conditions. In addition, the lpH signal, particularly in the low-field region, does not exhibit the obvious proton hyperfine splitting seen in the lpH signal for chicken sulfite oxidase (although the lpH samples did exhibit some sharpening of spectral features on exchange into D$_2$O, indicative of unresolved proton splitting). ESEEM experiments on chicken sulfite oxidase have suggested that the proton splitting, seen in the lpH form but not in the hpH form, is due to a rotation of the Mo-OH bond (Raitsimring et al., 1998, Astashkin et al., 2000). In the lpH form, strong coupling is seen when the proton lies in the equatorial plane of the molybdenum center, maximizing interaction with the d$_{xy}$ orbital that possesses the unpaired electron (Astashkin et al., 2000). In the hpH form, the Mo$^V$-OH bond is thought to have rotated out of plane due to an interaction with a hydroxide or water molecule, which results in the loss of the observed splitting. Taken as a whole, the data suggest that the geometry of the *Arabidopsis thaliana* sulfite oxidase active
site resembles that of the high-pH form, but with the Mo-OH proton lying out of the xy plane to a greater degree, even at low pH. This behavior may be compared with that of the sulfite oxidase from S. novella has also been shown to only have a hpH EPR signal regardless of pH and also does not exhibit the phosphate-inhibited signal (Kappler et al., 2000), which may indicate a somewhat altered active or binding site geometry.

An improved purification scheme has allowed the distinct visible absorption features characteristic of other sulfite oxidase molybdenum domains to be observed more clearly with the plant enzyme. The presence of these features, particularly the 480 nm absorption envelope, has allowed us to investigate the molybdenum center with resonance Raman spectroscopy. The resonance Raman spectra of A. thaliana sulfite oxidase provide clear support for an LMo\textsuperscript{VI}O\textsubscript{2}(S-Cys) active site structure, definitively placing the enzyme in the sulfite oxidase family of mononuclear molybdenum enzymes. Specifically, peaks at 896 cm\textsuperscript{-1} and 876 cm\textsuperscript{-1}, corresponding to symmetric and antisymmetric Mo=O stretching modes are very similar to those seen for the molybdenum domain of human sulfite oxidase (Garton et al., 1997a). Upon redox-cycling of the enzyme in H\textsubscript{2}\textsuperscript{18}O, modest shifts in these modes indicate the exchange of only a single oxygen, derived from solvent, into the molybdenum center during catalytic turnover, indicating that the two Mo=O groups are not catalytically equivalent. Peaks representative of the pyranopterin bonds are weaker than expected based on the human sulfite oxidase and DMSOR data, but this behavior has also been seen with xanthine oxidase (Maiti et al., 2003) and CODH (Hemann and Hille, personal communication).

To determine the amount of coupling between the two Mo=O groups at the active site, depolarization ratios (\(\rho\)) for the 864 cm\textsuperscript{-1}, 876 cm\textsuperscript{-1} and 896 cm\textsuperscript{-1} peaks were
determined to be 0.30, 0.32 and 0.35 respectively. Preliminary \textit{ab initio} calculations performed in this laboratory on minimal molybdenum coordination sphere models for sulfite oxidase suggest that the depolarization ratios should be closer to 0.7 and 0.1 for the symmetric and asymmetric Mo=O modes respectively (Hemann and Hille, personal communication). These results would be expected for excitation of the sample off resonance but excitation in the present data were resonance enhanced (under the cysteine-to-molybdenum charge-transfer absorption envelope). Depolarization ratio measurements made in resonance with an electronic transition provide information regarding the electronic transition to which the Raman bands are vibronically coupled (Sieler \textit{et al.}, 1999). A single wavelength depolarization ratio measurement is not sufficient to draw any specific conclusions regarding an electronic transition. For a given absorption envelope, if $\rho$ is approximately 0.33 for all excitations within that envelope, then it may be concluded that vibronic coupling is to a single non-degenerate electronic transition (Sieler \textit{et al.} 1999). The situation becomes much more complicated when the Raman band intensity is derived from the overlap of two or more electronic transitions. Suffice it to say that $\rho$ can vary from 0 to 0.75 depending on the details. It is obvious that in future work the depolarization ratio, $\rho$, needs to be measured at several more wavelengths throughout the cysteine-to-molybdenum charge-transfer electronic transition to measure any dispersion in $\rho$.

In addition, the \textit{ab initio} calculations show a decoupling of the Mo=O modes when either of the dioxo-center oxygens is labeled with $^{18}$O, resulting in two independent Mo=O stretching modes. This suggests that the shifts for the symmetric and asymmetric Mo=O modes seen for the recombinant human sulfite oxidase
molybdenum domain (Garton et al., 1997a) and for the A. thaliana sulfite oxidase are not shifts per se but instead arise from individual modes representing Mo=^{16}\text{O} and Mo=^{18}\text{O} stretching.

In summary, the present data demonstrate that the A. thaliana sulfite oxidase is a bona fide sulfite oxidase. The similarity of the UV-visible spectrum to that obtained for the molybdenum domain of human sulfite oxidase, as well as the EPR and resonance Raman data, support the placement of A. thaliana sulfite oxidase in the eukaryotic oxotransferase family of molybdenum enzymes. Investigation of the slight differences in the kinetic and spectroscopic properties of this enzyme will hopefully yield significant information regarding the active-site conformation and chemistry of this group of enzymes.
Figure 2.1. Reaction mechanism for sulfite oxidase. The proposed reaction of sulfite oxidase with sulfite showing formation of the Mo$^{IV}$-product intermediate followed by displacement of product by solvent and transfer of electrons to the physiological electron acceptor, cytochrome $c$, through the $b_5$-type heme. Transfer of one electron to the heme domain results in the Mo$^V$ oxidation state, which is conveniently investigated by EPR. (Adapted from Brody and Hille, 1999).
Figure 2.2. pH dependence of *A. thaliana* sulfite oxidase. The pH dependence plot of the steady-state reaction of *A. thaliana* sulfite oxidase with sulfite. Experiments were performed as described in Materials and Methods. A pH optimum of 8.5 and apparent pKₐ values of 8.1 and 8.9 are observed.
Figure 2.3. Rapid-reaction reduction of *A. thaliana* sulfite oxidase. Full spectrum analysis of the reaction of *A. thaliana* sulfite oxidase with sulfite at 5°C showing almost complete reduction of the molybdenum center within the dead-time of the stopped flow instrument (~ 2 ms).
Figure 2.4. UV-visible and CD spectra of *A. thaliana* sulfite oxidase. *A*, UV-visible spectrum of oxidized (solid-line) and sulfite-reduced (dashed-line) enzyme. The 360- and 480-nm absorption bands (oxidized enzyme) are attributed to enedithiolate-to-molybdenum and cysteine-to-molybdenum charge-transfer bands respectively. *B*, Circular-dichroism spectrum of oxidized enzyme.
Figure 2.4.
Figure 2.5. EPR spectra of *A. thaliana* sulfite oxidase. EPR spectra were recorded on *A. thaliana* sulfite oxidase (100-200 µM) reduced with excess sulfite (4 mM) and reoxidized with one-half equivalent of potassium ferricyanide. Buffers were 50 mM Bis-tris-propane, pH 6.0 (lpH), 50 mM Bis-tris-propane, pH 6.0, 100 mM NaCl (lpH-NaCl, full spectrum not shown), 50 mM Bis-tris-propane, pD 6.0 (lpH-D$_2$O) and 50 mM glycine, pH 10.0 (hpH). Instrument settings were 9.46 GHz microwave frequency, 2 mW microwave power, 100 kHz modulation frequency, 5.0 G modulation amplitude. Sample temperature was 150K. Inset shows the expanded $g = 2$ region for the three lpH samples.
Figure 2.5.
Figure 2.6. Resonance Raman spectra of *A. thaliana* sulfite oxidase. Spectra of as-prepared and redox-cycled *A. thaliana* sulfite oxidase. Samples were prepared as described in Materials and Methods. Insets show expanded region from 840 cm$^{-1}$ to 940 cm$^{-1}$ for the redox-cycled (top) and H$_2^{18}$O-labeled enzyme (bottom).
Figure 2.6.
Figure 2.7. Depolarization resonance Raman spectra of *A. thaliana* sulfite oxidase. Spectra were recorded in the Mo=O stretching mode energy region with the polarization analyzer set at 44° (I_{parallel}) and at 134° (I_{perpendicular}).
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<th>$g_1$</th>
<th>$g_2$</th>
<th>$g_3$</th>
<th>$g_{av}$</th>
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**Table 2.1.** $g_{1,2,3}$ and $g_{av}$ for $A.\ thaliana$ sulfite oxidase and other sulfite oxidases.
3.1. **Introduction**

As discussed in the previous chapter, sulfite oxidase is the best characterized member of the eukaryotic oxotransferase family of mononuclear molybdenum enzymes. While the plant form of this protein possesses only a molybdenum center, the mouse form (like that from all other vertebrate sources) has a cytochrome $b_5$-type heme domain associated with it, similar to the heme domain of the assimilatory nitrate reductases. This class of enzymes have an $\text{LMoO}_2(\text{S-Cys})$ active site composition (Figure 3.1), with a distorted square pyramidal coordination geometry (Kisker *et al.*, 1997). The enedithiolate ligands from the pyranopterin cofactor reside in the equatorial plane, along with the cysteine ligand from the protein and one of the oxo groups, with the other oxo group occupying the axial position (Kisker *et al.*, 1997). The axial position opposite the oxo group is blocked from coordination by the protein backbone, which also forms a positively charged binding pocket for the negatively charged
substrate. The crystal structure of chicken sulfite oxidase shows a bound product molecule in the active site close to the molybdenum center, which itself appears to be in the reduced Mo$^{IV}$ oxidation state (Kisker et al., 1997). For the sulfite oxidases, transfer of oxygen to substrate is thought to be initiated by attack of the lone pair electrons of substrate on an oxo group in the molybdenum coordination sphere (Figure 3.2, Brody and Hille, 1999). Transfer of reducing equivalents to the molybdenum center and formation of an intermediate Mo$^{IV}$-product complex is followed by displacement of product by a hydroxyl group. Reducing equivalents are transferred sequentially from the now-reduced molybdenum center to the cytochrome $b_5$ center (going from Mo$^{IV}$ to Mo$^V$ and Mo$^V$ to Mo$^VI$) and then further on to cytochrome $c$, the physiological electron acceptor.

Molybdenum enzymes typically carry out oxygen atom transfer chemistry, with the xanthine oxidase family being true hydroxylases. Biological hydroxylation reactions are important biochemical reactions and are involved in a wide variety of pathways ranging from the detoxification of aromatic compounds to purine metabolism. Most enzymes catalyzing such reactions do so by creating a highly unstable oxygen intermediate or “oxygen gun,” where the source of the oxygen incorporated into product is dioxygen and the enzyme utilizes an external source of reducing equivalents in the course of the reaction. In the flavoenzyme para-hydroxybenzoate hydroxylase for example, reaction of $E_{\text{red}}$ with molecular oxygen results in formation of a 4a-peroxy flavin intermediate (the hydroxylating intermediate) that progresses on to the 4a-hydroxy flavin (with concomitant hydroxylation of substrate), which subsequently loses the hydroxyl group as water (Entsch et al., 1976). The tetrahydrobiopterin substrates
used by the non-heme iron-containing aromatic amino acid hydroxylases appear to act in a similar manner, using molecular oxygen to form a 4a-peroxypterin species, which then generates a ferryl-oxo species that is thought to be the true hydroxylating intermediate (Dix et al., 1985). However, it is known for the molybdenum hydroxylases that in the course of turnover, the source of the oxygen incorporated into product is derived from water and not from molecular oxygen (Murray, 1966).

Model compound studies using inorganic molybdenum complexes suggest that the oxygen incorporated into product derives from a Mo=O group in the molybdenum coordination sphere (Xiao et al., 1992, Schultz et al., 1993, Pietsch and Hall, 1996, Thomson and Hall, 2001). An oxo transfer mechanism has been proposed where oxidation of substrate by the Mo=O group is followed by replacement of the Mo=O group by a solvent hydroxide molecule (Pietsch and Hall, 1996). Isotopic labeling experiments with xanthine oxidase have shown that the oxygen incorporated into product was derived from the molybdenum coordination sphere and that in the course of catalysis the oxygen is replaced from solvent (Hille and Sprecher, 1987). Investigation of DMSO reductase has also shown that similar oxo transfer chemistry is carried out at the molybdenum center of this prokaryotic oxotransferase, with transfer of $^{18}$O from oxo donor substrate (DMSO) to the molybdenum coordination sphere, which is then transferred to the oxo acceptor, a water-soluble phosphine (Schultz et al., 1995). It remains to be definitively established, however, that oxygen atom transfer from solvent occurs for members of the sulfite oxidase family. We show here that mouse sulfite oxidase does indeed use solvent as the ultimate source of the oxygen atom through the use of $H_2^{18}O$ and a combination single-multiple turnover experiment.
The crystal structure of chicken sulfite oxidase has revealed much about the enzyme, but also generated additional questions regarding active site chemistry and structure, as well as electron transfer between the two domains. The ligand coordination at the molybdenum center in the crystal structure suggests that the enzyme is in the reduced form with one oxo ligand to the metal. Although the enzyme was crystallized in the oxidized form, reduction most likely occurred from prolonged exposure to the ionizing radiation in the synchrotron beam (George et al., 1999). Indeed, a water molecule is found in the position which otherwise is presumably occupied by an equatorial oxygen (Figure 3.1).

In the crystal structure, the heme domains are located in different positions in relation to their respective molybdenum domains, and their iron centers are approximately 32 Å from the molybdenum centers and not opposite the pyranopterin ring (Kisker et al., 1997). This seems counter to the idea that the pterin ring acts as a conduit or pathway for reducing equivalent egress out of the molybdenum center to the heme. It is also an unexpectedly large distance for electron transfer to occur between the two domains (Gray and Winkler, 1996). It appears likely that the heme domain is not static in the course of turnover but can assume different positions by virtue of the flexible linker region that tethers it to the larger molybdenum domain. By using proton NMR spectroscopy, the dynamics of the heme domain have been investigated in the oxidized and reduced states of the enzyme and the effect of oxidation state on relaxation times. Additionally, similar states have been examined for the rat outer mitochondrial membrane cytochrome b<sub>5</sub>, which has relatively high homology to the cytochrome b<sub>5</sub> domain of mouse sulfite oxidase, as a standard for heme domain motions. Initial
experiments have yielded conditions that will allow the accurate determination of the relaxation rates for both proteins, which will help determine the dynamic nature of the heme domain.

3.2. Materials and Methods

Mouse Sulfite Oxidase Expression and Purification

An expression construct for mouse sulfite oxidase (pQE-80-mSO) was obtained from Dr. Ralf R. Mendel at the Technical University of Braunschweig, Germany. The cDNA encoding the full mouse sulfite oxidase gene was originally cloned into the pQE-60 expression vector using *Nco*I and *Bgl*II restriction sites, placing a 6X His-tag at the C-terminus of the protein. DNA sequence analysis of the pQE-80-mSO construct verified that the mSO gene with the C-terminal His-tag was transferred to the pQE-80 expression vector using *Eco*RI and *Hind*III restriction sites. This construct was transformed into TP1000 (ΔmobAB) cells, which were grown aerobically in modified 2xYT medium at 30°C for 24 hours after induction with 0.1 mM isopropyl-β-thiogalactoside at low cell density (A600=0.05). Crude lysate from cell lysis / sonication was loaded onto a Ni-NTA column pre-equilibrated with lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole). The column was washed with 2-3 times the bed volume of lysis buffer followed by wash buffer consisting of 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole, until the eluate had an OD<sub>280</sub> less than 0.05. mSO was eluted with 150 mM imidazole lysis buffer. Fractions containing mSO as judged by UV-visible spectroscopy and SDS-PAGE analysis were pooled, concentrated by ultrafiltration and loaded onto a Mono-Q column pre-equilibrated with 50 mM NaH₂PO₄, pH 8.0. Mouse sulfite oxidase was eluted with the same buffer and fractions
were concentrated based on purity (UV-visible spectrum, SDS-PAGE). Concentrated mSO was exchanged into 20 mM Tris-acetate, pH 8.0, 300 mM NaCl by gel filtration on a Sephadex G-25 column and stored at 5°C for immediate use or in liquid nitrogen for long-term storage. Enzyme concentration was determined spectrophotometrically using an extinction coefficient of 100 mM⁻¹ cm⁻¹ at 414 nm, similar to chicken sulfite oxidase (Brody and Hille, 1999). Protein was considered to be pure based on A₂₈₀/A₄₁₄ ratio of ≥ 0.7 and as judged by SDS-PAGE.

**Rat OM Cytochrome b₅ Expression & Purification**

An expression construct for rat outer mitochondrial cytochrome b₅ was obtained from Dr. Manuel Rivera at the University of Oklahoma (Rivera et al., 1992) and prepared as described in the literature with the following modifications. The cloned gene encodes the soluble domain of the protein containing the heme with the membrane anchor domain removed to improve solubility. DNA sequence analysis verified that the gene was cloned into the pET-15b expression vector using the NdeI and BamHI restriction sites. This construct was transformed into BL21(DE3) cells for expression under the strong T7 promoter. Cultures were grown aerobically in modified 2xYT medium with 10 mg/L ampicillin at 37°C to a cell density of 0.8-1.0 OD₆₀₀. IPTG was added to a final concentration of 0.5 mM and the culture was grown for an additional 4 hours at 37°C. Cells were harvested by centrifugation and stored at −80°C until protein purification. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). Lysozyme (0.1 mg/mL) and PMSF (0.1 mM) were added and the cells were incubated at room temperature for 30 minutes. Deoxyribonuclease I was added (1 mg/mL) and the mixture was sonicated. Crude
extract from ultracentrifugation (50,000 x g for 30 minutes at 4°C) was concentrated through centrifugation and diluted with 50 mM Tris-HCl, 10 mM EDTA, pH 7.8. This was loaded onto a DEAE-Sephacel column pre-equilibrated with the same buffer and protein was eluted with a linear NaCl (0-250 mM) gradient. Fractions containing cytochrome b5 judged spectroscopically (A280/A414 ~ 0.3) were pooled, concentrated and loaded onto an S-200 column. Protein was eluted with 50mM Tris-HCl, 1mM EDTA, pH 7.8. Fractions with an A280/A414 ≤ 0.3 were pooled and stored at –80°C for long term storage or at 5°C for immediate use. Enzyme concentration was determined spectrophotometrically using an extinction coefficient of 117 mM⁻¹ cm⁻¹ at 414 nm (Fukushima and Sato, 1973). Protein was considered to be pure based on A280/A414 ratio of ≤ 0.2-0.3 and by lack of contaminating bands from SDS-PAGE.

**Sulfite oxidase Assays**

Assays for mouse sulfite oxidase were carried out at 25°C in 20 mM Tris-acetate, pH 8.0 following reduction of cytochrome c (ε=19,630 M⁻¹ cm⁻¹) at 550 nm (Brody and Hille, 1999).

**Spectroscopic Methods**

UV-visible spectra and kinetic assays were recorded using a Hewlett-Packard 8452A single-beam diode-array spectrophotometer. Reduction of the enzyme was performed using either sodium dithionite or sodium sulfite as circumstances dictated.

Mass spectrometry was carried out on a Bruker Esquire quadrupole ion-trap mass spectrometer (MS). Samples in 20mM Tris-Acetate, pH 8.0 were made to 50% with HPLC grade methanol and directly infused into the MS at a rate of 100 μL/hr with a syringe pump. Samples were analyzed in positive and/or negative ion mode, due to
the apparent formation of proton and sodium adducts in positive ion mode, which resulted in an increase in the molecular mass by $m/z + 1$ or a $+22 \text{m/z}$ increase in sulfate (and Tris) peaks (Smith et al., 1990). Operation in negative ion mode revealed two distinct sulfate peaks: one possessing a proton adduct ($\text{HSO}_4^-$) and another a sodium adduct ($\text{NaSO}_4^-$). Standards of sodium sulfite and sodium sulfate (10 mM each) were prepared in water and 20 mM Tris-acetate, pH 8.0 and used along with the Tris buffer alone as controls for the location of the desired peaks and to verify the likely formation of sodium adducts (Figures 3.3-3.6). Spectra were collected for the specified times (approximately 20 spectra/minute), and averaged for the final spectrum for a given reaction sample. Mass spectra were collected over the range of $m/z$ 50.00 – 2200.00, but were only analyzed from $m/z \sim 50-200$ to focus on the location of the sulfate peaks. H$_2^{18}$O (95+ %) was purchased from Isotec Inc. HPLC grade methanol was purchased from VWR. Horse heart cytochrome c was purchased from Sigma and was used without further purification.

The turnover experiment described here was performed using 16 mg of mouse sulfite oxidase, which was prepared the previous day and lyophilized overnight. Lyophilized enzyme was taken up in 900 µL H$_2^{18}$O, followed by addition of 9 µL of 2 M Tris-acetate, pH 8.0. A 100-fold molar excess of substrate was added through the addition of 7 µL of a 4 M solution of sodium sulfite (final concentration of ~30 mM). This was stirred vigorously for 5 minutes and then concentrated by half through centrifugation. The eluate from this step is Sample #1. The ionic strength of the retentate was increased by adding 8 µL of 2 M Tris-acetate, pH 8.0 to bring the buffering capacity to greater than 50 mM Tris-acetate. A 10-fold molar excess of
cytochrome c (35.7 mg) was added along with a 5-fold molar excess of sulfite (4 µL of the 4 M sodium sulfite solution). The reaction was mixed gently until a color change was observed (red to pink), indicating reduction of cytochrome c. This mixture was concentrated almost to dryness through centrifugation, with the eluate being Sample #2. Both samples were stored at 5°C until diluted with an equal volume of methanol and analyzed on the mass spectrometer.

NMR spectroscopy was carried out on a Bruker DRX 800 MHz NMR spectrometer. Deuterated samples of mouse sulfite oxidase and rat outer mitochondrial membrane cytochrome b₅ were prepared by exchanging concentrated protein solutions into 20 mM Tris-acetate, pD 7.0 by gel filtration on a Sephadex G-25 column. Final concentrations were typically between 400-500 µM. Reduced samples were prepared by addition of a concentrated sodium dithionite solution prepared in anaerobic deuterated buffer directly into the NMR tube with gentle mixing immediately prior to data collection. Reduction of the samples was verified by a color change from red to pink and by spectrophotometric analysis immediately following the acquisition of data. One-dimensional proton NMR spectra were obtained before and after the acquisition of the relaxation time data to verify that the samples were unchanged over the course of data collection.

3.3. Results

Oxidation of sulfite to sulfate using H₂¹⁸O

For the turnover-labeling experiment, mass spectral data for both samples were collected in both positive and negative ion modes to verify the presence and location of sulfate peaks. Sample #1 (Figure 3.7) showed three peaks of varying intensity at m/z
168.8, 170.8 and 172.8, with the $m/z$ 170.8 peak having the highest intensity. These peaks represent sodium adducts of doubly, triply and quadruply labeled sulfate respectively. In negative ion mode, the same sample showed peaks at $m/z$ 100.9, 102.9 and 104.9 and also at $m/z$ 122.8, 124.8 and 126.8, the latter set likely arising from a single sodium ion adduct. The location for sulfate in positive ion mode (Figures 3.4 and 3.5) would be expected to be at approximately $m/z$ 141.9, or if sodiated at $m/z$ 164.9 ($\text{Na}_3\text{SO}_4^+$). In negative ion mode (Figure 3.4 and 3.6), two sulfate peaks would be expected, one at $m/z$ 96.9 (the protonated species – $\text{HSO}_4^-$) and one at $m/z$ 118.9 (the sodiated species – $\text{NaSO}_4^-$). The multiple peaks observed at $m/z$ values higher than expected likely arise from multiple $^{18}\text{O}$ incorporation into sulfate. Due to the addition of excess substrate to the reaction mixture for Sample #1, multiple turnovers of the enzyme may have occurred due to air-reoxidation. Spectra for Sample #2 (Figure 3.8) show the same peaks as Sample #1 but with different peak intensities, the $m/z$ 172.8 and $m/z$ 104.9 $m/z$ 126.8 peaks having the largest intensities for the positive and negative ion modes respectively.

**Protein NMR of mouse sulfite oxidase and rat OM cytochrome b$_5$**

One-dimensional proton NMR spectra were obtained for mouse sulfite oxidase (Figure 3.9) as well as for rat outer mitochondrial membrane cytochrome b$_5$ (Figure 3.10). The spectra for the mouse sulfite oxidase show very little change between the oxidized and reduced states and did not have the sharp peaks such as observed in the cytochrome b$_5$ spectra, raising concern over the experimental conditions used for collection of the data. In addition, the enzyme appeared to form partially soluble aggregates upon reduction with the concentrated sodium dithionite solution. The
spectra for the rat outer mitochondrial membrane cytochrome $b_5$ show well-resolved peaks and significant changes between the oxidized and reduced spectra and did not appear to form any aggregates upon reduction. Spectra for the determination of the T1 and T2 relaxation times were also taken for both enzymes in their oxidized and reduced states (data not shown). It was expected that the heme domain would produce sharper peaks in the mouse sulfite oxidase spectra, allowing us to compare the motion of the smaller domain with that of the larger protein and the rat outer mitochondrial membrane cytochrome $b_5$ as well. The absence of these peaks, along with the micro-aggregation of the mouse sulfite oxidase, suggested that the conditions needed to be modified.

In order to optimize the experimental conditions, spectra were recorded for the mouse sulfite oxidase using deoxygenated deuterated buffers at pH 8.0 and 9.0. Prior to reduction with concentrated sodium dithionite solution (prepared in anaerobic 200 mM Tris-acetate, pH 8.0 or 9.0), aliquots of a 4 M potassium chloride solution were added to achieve concentrations of 250 mM and 500 mM KCl. Reduction was verified by the change in color of the sample from red to pink. The spectra for oxidized and reduced mouse sulfite oxidase at pH 8.0, 250 mM KCl (Figure 3.11) show a significant improvement in the resolution of sharper peaks in the broad protein spectra. In addition, distinct changes are observed upon reduction of the enzyme with sodium dithionite. The spectra of mouse sulfite oxidase at pH 9.0 (data not shown) show fewer peaks than seen at pH 8.0, and less of a difference between the oxidized and reduced states of the enzyme. The observation of sharper peaks in the broader spectrum at pH 8.0 indicates the presence of a smaller domain that may be tumbling at different rates.
than the larger protein, allowing the determination of the relaxation rates and rotational
correlation times under these conditions.

3.4. Discussion

Sulfite oxidation with solvent as the source of oxygen incorporated into product

Turnover experiments with sulfite oxidase in $^{18}\text{O}$-buffer reveal multiple label
incorporation in product, showing that it is solvent that is the source of oxygen
incorporated into product by the enzyme. The multiple labeling itself, however, is an
unexpected result. It is well established that sulfite and sulfate do not appreciably
undergo oxygen exchange in labeled solvent (Radmer, 1972, Betts and Voss, 1970,
Betts and Libich, 1971, Krouse et al., 1991). It can thus be expected that multiple
turnover conditions may have occurred with Sample #1 due to the addition of excess
substrate in the reaction mixture. Further, some oxidation of the enzyme may have
occurred in the time that it took to concentrate the reaction volume by half for Sample
#1, this was not performed anaerobically. Electron transfer from the molybdenum
center to the heme and further on to oxygen may well have occurred, permitting the
enzyme to react with another equivalent of substrate. In the course of reoxidation,
enzyme will have acquired labeled oxygen from solvent. The reaction of $^{18}\text{O}$-labeled
enzyme with substrate would form the Mo$^{\text{IV}}$-product intermediate, with labeled oxygen
being transferred, allowing full labeling of product.

The proposed reaction mechanism for sulfite oxidase involves attack of the
sulfite lone-pair of electrons on the equatorial oxo ligand, forming a bidentate
intermediate (Figure 3.2). This intermediate breaks down in an associative mechanism,
where product is displaced by hydroxide (or water), leaving the reduced LMoO(OH)(S-
Cys) active site. Under single turnover conditions in labeled buffer, it would be expected that sulfate would acquire its oxygen from the molybdenum center, which is unlabeled and not expected to turnover in labeled buffer without substrate. The release of product by hydroxide would then incorporate label at the equatorial position giving LMoO(\textsuperscript{18}OH)(S-Cys). For multiple labeling to occur, one of two possibilities may occur at the intermediate step of the reaction.

First, it is possible that product may react with reduced enzyme, backing up the reaction so that sulfate donates its oxygen back to the molybdenum center. Formation of multiply labeled product would involve formation of labeled product, dissociation from the active site, and rebinding to the reduced molybdenum and back transfer of an unlabeled oxygen. The binding of a singly labeled substrate, to a different, labeled enzyme, could then lead to incorporation of multiple \textsuperscript{18}O into product, although this would require a significant amount of reoxidation of the enzyme by oxygen to provide a sufficient pool of labeled enzyme. Indeed, mouse sulfite oxidase has been observed to slowly reoxidize in the presence of equimolar amount of substrate.

Alternatively, the stability of the Mo\textsuperscript{IV}-product intermediate may permit hydroxide attack at the sulfur atom, replacing unlabeled oxygen with labeled oxygen, prior to product dissociation. In this scheme, the initial turnover would theoretically allow for the incorporation of three labels on the sulfur as the enzyme would provide the fourth oxygen, which is unlabeled. This would account for the large amount of product with three labels seen in Sample #1. Although product might dissociate through hydroxide attack on the sulfur atom, it is known that the enzyme acquires label from solvent during product dissociation. Resonance Raman experiments with A.
*Arabidopsis thaliana* sulfite oxidase (Chapter 2) and human sulfite oxidase (Garton *et al.*, 1997a) have shown exchange of a single oxygen in the molybdenum coordination sphere in the course of turnover in H$_2^{18}$O. Reoxidation of the enzyme, followed by turnover with substrate would lead to generation of fully labeled product, as seen in Samples #1 and #2, as the Mo$^{IV}$-product intermediate would contain labeled oxygen.

It is important to emphasize that even with the multiple labeling of sulfate during the course of turnover, it is apparent that the source of the oxygen incorporated into product is from solvent and not molecular oxygen, supporting the results seen for the model compound chemistry and the other molybdenum enzyme families.

**Protein NMR of mouse sulfite oxidase**

One-dimensional $^1$H NMR spectra of the oxidized and reduced mouse sulfite oxidase are not sufficiently resolved at pD 7 to permit the accurate determination of the relaxation times (Figure 3.9). In analyzing the protein sequence, it was determined that the pI for the protein was ~5.7, indicating that the current experimental conditions may have been less than optimal and it was decided to work at higher pD to avoid possible micro-aggregation in solution. Also, the presence of oxygen (through the use of aerobic buffers) can alter the relaxation times for the protein, thereby misrepresenting the tumbling of the protein in solution. While the spectra and data for the rat outer mitochondrial membrane cytochrome $b_5$ were adequately resolved to allow for the determination of the relaxation rates (Figure 3.10), the lack of quality data for the mouse sulfite oxidase required the establishment of different experimental conditions.

One-dimensional $^1$H NMR spectra for mouse sulfite oxidase were obtained in anaerobic 20 mM Tris-acetate at pD values of 8.0 and 9.0 and also in the presence of up
to 500 μM KCl, which should help to resolve individual peaks which may be attributed to the heme domain. At a pD of 8.0, an increase in the resolution of individual peaks is seen which is slightly enhanced upon the addition of KCl (Figure 3.11). Reduction of the enzyme with a buffered sodium dithionite solution, resulted in the shift of a number of peaks, strengthening the requirement for a higher pD and salt concentration to resolve the proton NMR spectrum. However, at pD 9.0 and regardless of the salt concentration, the individual peaks seen in previous oxidized spectra at lower pD appeared to combine into broader peaks (data not shown). These data suggest that the determination of the relaxation rates for the mouse sulfite oxidase and rat outer mitochondrial membrane cytochrome b$_5$ should be performed in deoxygenated buffer at pD 8.0 and with KCl present.

With the modification of the experimental conditions and resolution of several peaks in the mouse sulfite oxidase spectra, we will be able to determine relaxation rates for both proteins with greater accuracy. Mouse sulfite oxidase, at 110 kDa, is expected to tumble much more slowly than the rat outer mitochondrial membrane cytochrome b$_5$, which is ~12-kDa. However, if the heme domain of mouse sulfite oxidase were sufficiently flexible and dynamic, we would expect to see faster relaxation rates than for the full protein. Determination of the relaxation rates for a variety of peaks in the mouse sulfite oxidase spectra will reveal if the protein tumbles as a whole, or if the heme is in fact mobile, which will be evident by the presence of different relaxation rates. These rates will be further compared to those determined for the rat outer mitochondrial membrane cytochrome b$_5$, confirming the mobility of the heme domain and the likelihood of dynamic motion during catalysis.
Figure 3.1. Chicken sulfite oxidase active site. Representation of the active site of chicken sulfite oxidase as determined by x-ray crystallography. The pyranopterin dithiolate ligands, an apical oxo group, and an equatorial protein-supplied cysteine sulfur coordinate the molybdenum center (shown in pink). Also shown is a water molecule, occupying an equatorial ligand position, and a bound sulfate molecule. The three arginines proposed to form the positively charged binding pocket are also displayed. (From Kisker et al., 1997)
Figure 3.2. Reaction mechanism for sulfite oxidase. The proposed reaction of sulfite oxidase with sulfite showing formation of the Mo$^{IV}$-product intermediate followed by displacement of product by solvent and transfer of electrons to the physiological electron acceptor, cytochrome $c$, through the $b_5$-type heme. Transfer of one electron to the heme domain results in the Mo$^V$ oxidation state, which is conveniently investigated by EPR. (Adapted from Brody and Hille, 1999).
Figure 3.3. Tris-acetate standard mass spectra. Mass spectral analysis of 10 mM Tris-acetate, pH 8.0 / 50% MeOH standard for the mouse sulfite oxidase turnover-labeling experiment. A, Positive ion mode analysis showing representative Tris peaks at m/z 122. B, Negative ion mode analysis showing peaks at m/z 140.9, 147.9, and 189.7, seen in most mass spectra and believed due to the methanol used for sample preparation.
Figure 3.3.
Figure 3.4. Sodium sulfate and sodium sulfite standard mass spectra. Mass spectra of sulfate and sulfite standards in 10 mM Tris-acetate, pH 8.0 / 50% MeOH. A, 10 mM Na₂SO₄ in positive ion mode analysis showing representative peaks at m/z 122.0 (Tris), 143.9 (Tris-Na⁺), and 164.8 (Na₃SO₄⁺). B, 10 mM Na₂SO₃ in positive ion mode analysis showing representative peaks at m/z 148.8 (Na₃SO₃⁺).
Figure 3.4.
Figure 3.5. Sodium sulfate standard (in H₂O) mass spectra. Mass spectra of 10 mM Na₂SO₄ in H₂O / 50% MeOH. A, Positive ion mode analysis showing representative peaks at m/z 164.8 (Na₃SO₄⁺). B, Negative ion mode analysis showing representative peaks at m/z 96.9 (HSO₄⁻) and m/z 118.8 (NaSO₄⁻).
Figure 3.5.
Figure 3.6. Sodium sulfite standard (in H₂O) mass spectra. Mass spectra of 10 mM Na₂SO₃ in H₂O / 50% MeOH. A, Positive ion mode analysis showing representative peaks at m/z 148.8 (Na₃SO₃⁺). B, Negative ion mode analysis showing representative peaks at m/z 79.9 (HSO₃⁻) and 102.9 (NaSO₃⁻). Peaks representative of sulfate (m/z 96.9 and 118.8) are also observed.
Figure 3.6.
Figure 3.7. Sample #1 mass spectra. Mass spectra of Sample #1 in 10 mM Tris-acetate, pH 8.0 / 50% MeOH for the mouse sulfite oxidase turnover-labeling experiment. A, Positive ion mode analysis showing peaks at \( m/z \) 168.8, 170.8, and 172.8 representing doubly, triply, and quadruply labeled sulfate (\( \text{Na}_3\text{SO}_4^+ \)) respectively. B, Negative ion mode analysis showing peaks at \( m/z \) 100.9, 102.9, and 104.9 and \( m/z \) 122.8, 124.8, and 126.8 representing doubly, triply, and quadruply labeled sulfate (\( \text{HSO}_4^- \) and \( \text{NaSO}_4^- \)) respectively.
Figure 3.7.
**Figure 3.8.** Sample #2 mass spectra. Mass spectra of Sample #2 in 10 mM Tris-acetate, pH 8.0 / 50% MeOH for the mouse sulfite oxidase turnover-labeling experiment. *A*, Positive ion mode analysis showing peaks at $m/z$ 168.8, 170.8, and 172.8 representing doubly, triply, and quadruply labeled sulfate ($\text{Na}_3\text{SO}_4^+$) respectively. *B*, Negative ion mode analysis showing peaks at $m/z$ 100.9, 102.9, and 104.9 and $m/z$ 122.8, 124.8, and 126.8 representing doubly, triply, and quadruply labeled sulfate ($\text{HSO}_4^-$ and $\text{NaSO}_4^-$) respectively.
Figure 3.8.
Figure 3.9. NMR spectra of mouse sulfite oxidase at pD 7.0. One-dimensional proton NMR spectra of oxidized (red line) and reduced (black line) mouse sulfite oxidase in 20 mM Tris-acetate, pD 7.0.
Figure 3.10. NMR spectra of rat outer mitochondrial membrane cytochrome $b_5$. One-dimensional proton NMR spectra of oxidized (black line) and reduced (red line) rat outer mitochondrial membrane cytochrome $b_5$. 
Figure 3.11. NMR spectra of mouse sulfite oxidase at pD 8.0. One-dimensional proton NMR spectra of oxidized (black line) and reduced (red line) mouse sulfite oxidase in 20 mM Tris-acetate, pD 8.0, 250 mM KCl.
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**Table 3.1.** Mass spectra standards. Molecular weight of the various chemical species of sodium sulfate, sodium sulfite and tris(hydroxymethyl)aminomethane (Tris) used for the determination of representative peaks in the mass spectrograms from positive- and negative-ion mode analysis for the labeling experiment.
4.1. Introduction

Nitrate reductase catalyzes the rate-limiting pyridine nucleotide-dependent reduction of nitrate to nitrite as the first step of nitrogen assimilation in algae, fungi and higher plants (Solomonson and Barber, 1990). Following this initial two-electron reduction of nitrate, nitrite is further reduced in a six-electron process to ammonia (catalyzed by nitrite reductase), which is then incorporated into organic compounds. The reduction of nitrate, a major source of inorganic nitrogen for these species, has an extremely large free energy change (-34.2 kcal/mol), making the reaction a favorable and essentially irreversible process (Campbell, 1999). Assimilatory nitrate reduction accounts for the transformation of more than 10,000 megatons of inorganic nitrogen per year (Guerrero et al., 1981). The excessive use of fertilizers has led to the presence of high levels of nitrate in the environment and growing concerns regarding its biological impact, making nitrate reductase a focus of intense research. Excess nitrate from drinking water can form toxic N-nitroso compounds and has been implicated in a severe
blood disorder called blue-baby syndrome, which ultimately causes respiratory failure in infants (Saull, 1990). The potential use of nitrate reductase as a means of detecting and/or removing excess nitrates has thus received considerable attention (Kroneck et al., 2002). In addition, deficiency in mammalian NADH:cytochrome b5 reductases, similar to the flavin domain of the assimilatory nitrate reductases, results in two clinically different types of hereditary methemoglobinemia (Shirabe et al., 1992, Shirabe et al., 1994), and can even affect other tissues, causing severe symptoms including mental retardation (Jaffé and Hultquist, 2001). The knowledge gained from investigation of the flavin domain of nitrate reductase can therefore be applied not only to its role in nitrate assimilation in plants, but likely also to its effect on human pathology.

The assimilatory nitrate reductases belong to the eukaryotic oxotransferase family of molybdenum enzymes, along with the sulfite oxidases, which contain LMoO$_2$(S-Cys) active sites (Hille, 1996). By contrast, the dissimilatory nitrate reductases belong to the dimethylsulfoxide reductase family of enzymes, molybdoenzymes that contain two equivalents of the unique pterin cofactor (Hille, 1996). The dissimilatory enzymes, which may contain other redox active centers (such as a 4Fe-4S cluster in the periplasmic nitrate reductase from *D. desulfuricans*, Dias et al., 1999), are components of the respiratory electron transfer chain using nitrate as the terminal electron acceptor, and also in energy conservation and redox balancing (dentrification and ammonification, Moreno-Vivián et al., 1999, Zumft, 1997).

Spinach assimilatory nitrate reductase is an ~110-kDa homodimer with a modular layout of its domains similar to that seen in other molybdoenzymes. It consists
of an N-terminal molybdenum-binding domain, a central $b_{557}$ cytochrome domain and a C-terminal flavin- and NAD(P)H-binding domain (Figure 4.1). Reduction of the enzyme occurs at the flavin center with electrons donated by NADH (or NADPH depending on the source). Transfer of electrons continues through the cytochrome and on to the molybdenum, which is the site of nitrate reduction. Two linker regions connect the three domains, the first (N-terminal) containing a serine residue (Ser 543 in spinach) which is transiently phosphorylated for regulation – this phosphoserine is recognized by a 14-3-3 protein, whose binding reversibly inhibits the enzyme (Su et al., 1996). This inhibition may be due to inhibition of electron transfer from the heme to the molybdenum center (Hille, 1996), possibly altering the reduction potential of the heme (Campbell, 1999).

Owing to the modular nature of nitrate reductase, catalytically active fragments have been purified and characterized following mild proteolysis (Solomonson et al., 1986, Kubo et al., 1988). The FAD/NADH domain is commonly referred to as the cytochrome $b$ reductase fragment (CbR), while the combined flavin and heme domains give the cytochrome $c$ reductase fragment (CcR) (also called the molybdenum-reductase fragment, MoR, Mertens et al., 2000). The full-length protein is formally known as NADH:nitrate reductase, and the individual domains have a variety of electron transfer activities as shown by Figure 4.1. Electron transfer within the enzyme has been extensively studied using these artificial electron donors and acceptors.

The cloning of several nitrate reductase genes has allowed for the generation of the full-length enzyme as well as various individual domains to further examine the specific properties of the distinct redox-active centers (Prosser and Lazarus, 1990,
The recent development of recombinant expression systems for the various fragments of nitrate reductase has yielded significant information regarding the reaction mechanism. The molybdenum domain has been expressed in *E. coli* and shown to exhibit the typical EPR signals and methyl-viologen:nitrate reductase activity of native enzyme (Pollock *et al.*, 2002). A non-functional tungsten analog has also been generated, with the first W$^V$ EPR signals being obtained for any nitrate reductase (Pollock *et al.*, 2002).

Functional expression of the heme domain (Barber *et al.*, 2002), as well as the generation of various chimeric constructs (spinach and corn molybdenum reductase fragments, Mertens *et al.*, 2000; spinach cytochrome *b* reductase fragment, Quinn *et al.*, 1994; spinach cytochrome *c* reductase fragment, Ratnam *et al.*, 1997), has allowed for the investigation of electron transfer from the flavin domain to the heme domain. From an investigation of the recombinant full-length nitrate reductase, it has been proposed that electron transfer from the FAD via the heme to the molybdenum is overall rate-limiting, and specifically that the transfer from the FAD to the heme plays a significant role (Skipper *et al.*, 2001). Transfer of electrons from NADH to the flavin center results in the formation of a long-wavelength charge-transfer complex between reduced flavin and NAD$^+$ (Ratnam *et al.*, 1995). The breakdown of this complex, proposed to be required prior to electron transfer to the heme domain, has been suggested to be the rate-limiting step for that portion of the reaction (Ratnam *et al.*, 1997).

There are two invariant cysteine residues among nitrate reductases from various organisms, one in the molybdenum-binding domain and one in the flavin domain. The cysteine in the molybdenum domain, conserved among the sulfite oxidases as well, has
been shown to be the residue that coordinates the molybdenum (Garde et al., 1995, Su et al., 1997). The cysteine residue in the flavin domain can be protected by binding of pyridine nucleotides and chemical modification of this cysteine by sulfhydryl reagents significantly reduces activity (Barber and Solomonson, 1986).

A recombinant expression system for the flavin domain of spinach nitrate reductase has been established with protein showing similar NADH:ferricyanide activities to native enzyme (Quinn et al., 1996). Initially, all cysteine residues located in the flavin-binding domain were mutated to ascertain their individual roles in NADH:ferricyanide activity (Trimboli et al., 1996). While the results varied, mutation of the conserved cysteine, C240 (C898 in the full-length sequence) of spinach nitrate reductase and C242 of corn leaf nitrate reductase, reduced cytochrome b reductase activity significantly (Trimboli et al., 1996, Ratnam et al., 1995). Further investigation showed that, while playing an important role in catalysis, the cysteine residue is not critical. NADH:ferricyanide activity was reduced by two-thirds upon mutation to serine, and further reduced in a C242A mutant by almost one-tenth the wild-type activity for the corn flavin domain (Dwivedi et al., 1994). This has also been seen for the spinach flavin domain (60% and 30% reduction from wild-type activity for the C240S and C240A mutants respectively, Trimboli et al., 1996) and in reductive half reactions of the corn flavin domain (474 s⁻¹ for wild-type and 68 s⁻¹ for C242S, Ratnam et al., 1995).

The crystal structure for the flavin domain of corn nitrate reductase has been solved for the wild-type fragment (2.5 Å), a C242S mutant (2.5 Å), and for the wild-type fragment co-crystallized with ADP (2.7 Å) (Lu et al., 1995). The structures show
the bound FAD on one side of an active site, adjacent to a putative NADH binding site (Figure 4.2). This interpretation is supported by the positioning of the ADP molecule (in that crystal structure), which is located at the opening to the proposed binding site, as well as modeling studies using the ADP-complex and the structure of the PDR-NADH complex determined by Correll et al. (1992) (Lu et al., 1995). The structure of the C242S mutant shows a change in position of the hydroxyl group of the serine residue compared to the sulfhydryl group of the cysteine residue, with the hydroxyl being oriented away from the active site and possibly interacting with the protein backbone. Given the minimal effects of the C242S mutation on the kinetics of flavin reduction, this result suggests that this conserved cysteine may play more of a role in positioning the nicotinamide ring for efficient electron transfer to the flavin rather than being directly involved in catalysis or electron transfer (Lu et al., 1995).

Also located in the active site are the glycine and proline residues immediately following the conserved cysteine (G243 and P244 in the corn enzyme). The glycine residue is part of the Cys-Gly motif seen for other members of the ferrodoxin NADP+ reductase family (Karplus et al., 1991), but has not been ascribed a specific role as of yet. The proline residue appears to be involved in binding the adenine ring of the ADP molecule at the entrance to the active site, in a hydrophobic environment created partially by an additional Pro (246) and Met (247) (Lu et al., 1995). Pro244 is an alanine in the birch nitrate reductase (Schöndorf and Hachtel, 1995), which is unusual in being able to utilize either NADH or NADPH as substrate, suggesting that the proline in the corn enzyme may play a role in substrate specificity. The development of a recombinant expression system for the flavin domain of spinach nitrate reductase has
allowed us to investigate the catalytic properties of the flavin domain for the wild-type protein, as well as C898, G899, and P900 mutants, with the goal of further understanding structure-function relationships in this protein.

The wild-type and mutant spinach nitrate reductase flavin fragments have been analyzed for the ability to form the long-wavelength charge-transfer complex and to establish its role in catalysis. In addition, kinetic parameters have been determined for all enzymes with the goal of better understanding the mechanistic roles of the various active site residues.

4.2. Materials and Methods

Enzyme Preparation

Recombinant wild-type and mutant (C898S/A; G899S/A; P900S/A) spinach nitrate reductase CbR fragments and the spinach nitrate reductase heme domain fragment were prepared by the laboratory of Dr. Michael Barber, shipped on dry ice as pellets and stored at -80°C until use. Pellets were thawed in ice-cold 0.1 M MOPS, 0.1 mM EDTA, 0.1 M KCl, pH 7.0, concentrated and run over a G-25 gel filtration column equilibrated with the same buffer prior to use. Enzyme concentration was determined spectrophotometrically using an extinction coefficient of 10.6 mM⁻¹ cm⁻¹ at 460 nm for the flavin domain (Quinn et al., 1996) and an extinction coefficient of 130 mM⁻¹ cm⁻¹ at 414 nm for the heme domain (Barber et al., 2002). All chemicals were purchased from Fisher Scientific or Sigma-Aldrich and used without further purification.

Experimental conditions

UV-visible spectra and steady-state kinetic assays were recorded using a Hewlett-Packard 8452A single beam diode-array spectrophotometer. Reductive
titrations and generation of long-wavelength absorbing charge-transfer complexes were performed anaerobically at 25°C. Protein samples were made anaerobic by alternately flushing with argon and evacuating with vacuum every ten minutes over the course of 1.5 hours in a glass tonometer. All reagent solutions used were prepared fresh prior to use, made anaerobic by bubbling under argon for 15 minutes, and added via a gas-tight syringe designed to fit the tonometer. For the reductive titration of the wild-type and C899S mutant, aliquots of a concentrated NADH solution were added to the protein with gentle rocking of the tonometer between aliquots. Generation of the charge-transfer complex was carried out with similarly prepared enzyme, which was then fully reduced through addition of a concentrated sodium dithionite solution, followed by addition of a concentrated NAD$^+$ solution.

Steady-state kinetic assays for the spinach nitrate reductase flavin domain were carried out in 0.1 M MOPS, 0.1 mM EDTA, 0.1 N KCl, pH 7.0 following reduction of potassium ferricyanide (ε=1,020 M$^{-1}$ cm$^{-1}$) at 420 nm (Gardlik and Rajagopalan, 1991) or reduction of cytochrome $b_5$ (ε=27.4 mM$^{-1}$ cm$^{-1}$) at 556 nm (determined empirically based on ε$_{413}$ = 130 mM$^{-1}$ cm$^{-1}$, Barber $et al.$, 2002). Assays were carried out with a 10-fold excess of NADH ($K_m$ = 17 µM) or a 5-fold excess of NADPH ($K_m$ = 520 µM), and a 10-fold excess of ferricyanide ($K_m$ = 35 µM) or a 4-fold excess of cytochrome $b_5$ ($K_m$ = 6 µM) all prepared in the same MOPS buffer (Quinn $et al.$, 1996). Reduction of the cytochrome $b_5$ domain was performed using 30 µM enzyme with 170 µM NADH or 2.5 mM NADPH under the assay conditions. Steady-state kinetic measurements were
performed aerobically at 25°C using a 1.0 cm light path cuvette and a final sample buffer volume of 1.0 ml.

Rapid reaction kinetics were performed using an Applied Photophysics Inc. stopped-flow apparatus. Single-wavelength kinetic transients were measured at 460 nm and 800 nm using a photomultiplier tube and full spectral analysis (250-750 nm) using a diode array detector. Reductive half-reactions were carried out anaerobically at 10°C in 0.1 M MOPS, 0.1 mM EDTA, 0.1 M KCl, pH 7.0 using 7.5 M enzyme and varying NADH concentrations (25-200 µM). Samples were made anaerobic as described above. Kinetic transients were best fit to a double exponential and kinetic constants were determined from double-reciprocal plots of the observed rate versus substrate concentration.

4.3. Results

*Formation of the long-wavelength charge-transfer complex*

Formation of the $E_{\text{red}}$-NAD$^+$ charge-transfer complex was observed for recombinant wild-type and most of the mutants of the flavin domain of spinach nitrate reductase (Figures 4.3-4.6). Anaerobic reduction with sodium dithionite followed by addition of an excess of NAD$^+$ resulted in an increase at long-wavelength, indicative of the charge-transfer complex as seen previously (Ratnam *et al.*, 1995), although to different extents for each mutant. The formation of the charge-transfer complex for the P900S mutant is not shown due to the instability of the protein once it was reduced with dithionite. This was also the case for the G899S mutant to a lesser extent, but formation of the charge-transfer complex was nevertheless verified by aerobic reduction of the flavin center with excess NADH (data not shown). In addition, wild-type enzyme and
the C898S mutant were reduced anaerobically with NADH to show formation of the charge-transfer complex concomitant with flavin reduction, indicating the likely mechanistic relevance of the complex (Figure 4.7).

**Steady-state turnover and substrate specificity**

Steady-state kinetic analysis of the reaction of the wild-type flavin domain with NADH or NADPH as substrate yielded similar rates (k_{obs}, Table 4.1) when compared to V_{max} values seen previously using ferricyanide as electron acceptor (84 \mu mol NADH and 4 \mu mol NADPH oxidized/min/nmol FAD for the flavin domain and 83 \mu mol NADH oxidized/min/nmol FAD for the full spinach nitrate reductase, Quinn et al., 1996). Turnover with cytochrome b_{5} (Figure 4.10.A) as acceptor resulted in much slower kinetics (Table 4.1), with an increase in absorbance at 556 nm which eventually plateaued, most likely due to rapid reoxidation of the heme by oxygen. This is similar to that seen previously with the spinach nitrate reductase flavin and heme domains (V_{max} = 1.2 \mu mol NADH/min/nmol FAD, Quinn et al., 1996). Incubation of cytochrome b_{5} (30 \mu M) with NADH (170 \mu M) and 1.2 nmol of the flavin domain showed reduction of the heme by the flavin domain with concomitant formation of the charge-transfer complex (Figure 4.10.B).

Aerobic reduction of the wild-type flavin domain with NADPH resulted in reduction of the flavin center with formation of the charge-transfer complex, but to a significantly lesser degree than seen for NADH reduction (Figure 4.3.B). Reduction of the P900A mutant with NADPH also showed minimal formation of the charge-transfer complex, similar to that seen for the wild-type enzyme (Figure 4.6.B). Steady state
turnover of the P900A mutant using ferricyanide as electron acceptor showed a
decreased $k_{\text{obs}}$ for NADH (1400 s\(^{-1}\) and 830 s\(^{-1}\) for wild-type and P900A respectively),
but an increase in the $k_{\text{obs}}$ for NADPH (220 s\(^{-1}\) vs. 922 s\(^{-1}\) respectively, Table 4.1).

**Pre-steady state analysis of the reduction of the flavin domain**

Rapid reaction kinetics on the reductive half-reaction of the flavin center yielded
$k_{\text{red}}$, $K_d$, and $k_{\text{red}}/K_d$ values for all enzyme forms (Table 4.2). The wild-type flavin
domain yielded $k_{\text{red}}$ and $K_d$ values significantly higher than those seen previously for the
corn-leaf flavin domain ($k_{\text{red}} = 474$ s\(^{-1}\), $K_d = 3$ $\mu$M at 10\(^\circ\)C, Ratnam *et al.*, 1995) as well
as for the spinach flavin-heme construct ($k_{\text{red}} = 560$ s\(^{-1}\), $K_d = 3$ $\mu$M at 10\(^\circ\)C, Ratnam *et
al.*, 1997). Reduction of the flavin domain was monitored at 460 nm, showing a
decrease in absorbance (Figure 4.8). Formation of the charge-transfer complex was
monitored at 800 nm, which surprisingly showed a decrease in absorbance at a rate
similar to that seen for flavin reduction. This is seen for all flavin domains tested and is
in contrast to the expected increase in absorbance, which indicates formation of the
complex (Ratnam *et al.*, 1995, Ratnam *et al.*, 1997). To confirm formation of the
charge-transfer complex, the same rapid-reaction experiment was carried out on the
stopped-flow instrument using the wild-type fragment and observing the spectral
changes over the range of 350–750 nm. It was apparent that reduction of the flavin was
almost complete within the first few milliseconds of the reaction (as seen at 460 nm),
with concomitant formation of the charge-transfer complex at long-wavelength (at 750
nm, Figure 4.9). The charge-transfer complex was formed within the dead time of the
instrument and remained stable, without apparent breakdown, out to 500 milliseconds.
4.4. Discussion

*Formation of the charge-transfer complex and its implications in electron transfer*

The recombinant wild-type and most of the mutant flavin domain fragments of spinach assimilatory nitrate reductase have been found to form the typical long-wavelength absorbing $E_{\text{red}}$-NAD$^+$ charge-transfer complex, although to different degrees. The complex could be generated either by reduction of the flavin domain with substrate or by anaerobic reduction with dithionite followed by addition of NAD$^+$. It has been proposed that the transfer of electrons from the flavin domain to the heme domain is rate-limited ($12 \text{ s}^{-1}$) by the breakdown of the charge-transfer complex (Ratnam *et al.*, 1997). The ability to form the charge-transfer complex suggests that all forms are catalytically viable to a certain degree at least.

*Steady-state and rapid-reaction kinetics of flavin reduction*

Steady-state turnover of the wild-type flavin fragment showed similar rates to those reported in the literature for reduction with an excess of NADH or NADPH using ferricyanide and cytochrome $b_5$ as electron acceptor (Table 2.1). Electron transfer to ferricyanide using NADH was very rapid ($1400 \text{ s}^{-1}$), while that to cytochrome $b_5$ was much slower ($37 \text{ s}^{-1}$), which was expected based on previous results (Quinn *et al.*, 1996). The lower rates for electron transfer to cytochrome $b_5$ are likely due to the two proteins being separate, an interpretation that is supported by the increase in cytochrome $b_5$ reduction rates observed in a flavocytochrome $b_5$ chimera ($V_{\text{max}}$ of 37 $\mu$mol NADH/min/nmol FAD versus 1.2 for the separate proteins, Quinn *et al.*, 1994, Quinn *et al.*, 1996).
Under pre-steady-state conditions, while flavin reduction occurred as expected (following flavin beaching at 460 nm), the formation of the charge-transfer complex (reflected as an increase in the long-wavelength absorbance) was not seen. Instead, a decrease in long-wavelength absorbance was observed, with rates similar to that of flavin reduction. This is in contrast to the results seen for the corn nitrate reductase flavin domain (Ratnam et al., 1995), where reduction of flavin was accompanied by formation of the charge-transfer complex, observed as an increase in absorbance at 800 nm, with similar rates. However, when the reaction was monitored using the full spectrum, it was apparent that the charge-transfer complex formation was very rapid, almost within the dead-time of the stopped flow instrument. The likeliest explanation is that a long-wavelength absorbing FAD-NADH charge-transfer complex forms prior to reduction of the flavin. Precedent for an $E_{ox}$-NADH charge-transfer complex exists for the NAD(P)H:flavin oxidoreductase from *E. coli* (Nivière et al., 1998) and phthalate dioxygenase reductase (Gassner and Ballou, 1995). Interestingly, the phthalate dioxygenase reductase system is shown to require the release of NAD$^+$ from the charge-transfer complex to have electron transfer to its iron-sulfur center, at a rate of 35 s$^{-1}$ (Gassner et al., 1994), as is thought to be the case with nitrate reductase.

*The roles of Cys 898 and Gly 899 in flavin reduction and/or electron transfer*

Similar to the results seen for the corn leaf flavin domain of nitrate reductase, mutation of the invariant Cys residue to a Ser in the spinach nitrate reductase flavin domain resulted in a modest 2-fold decrease in the rate of reduction of the flavin center, with an unchanged $K_d$ for substrate. Mutation to an alanine resulted in a larger decrease in $k_{red}$ accompanied by a moderate 2-fold increase in $K_d$. It has previously been
proposed that the Cys maintains proper positioning of substrate for efficient electron transfer to the flavin (Lu et al., 1995). The crystal structure of the Ser mutation shows the hydroxyl side chain rotated out of the pocket somewhat and hydrogen-bonded to the protein backbone. This leaves more room in the binding pocket and allows more flexibility for substrate positioning, which could account for the decreased reduction of flavin and increased $K_d$ with the Ala mutation, since less than optimal orientations would be allowed in the larger active site of the mutant.

The specific role of Gly899 is presumably steric. As a consequence, mutation to the bulkier Ala or Ser results in 10- to 100-fold decrease in $k_{\text{red}}/K_d$. The Ser mutation has significant effects on both rate of reduction as well as binding, while the Ala mutation primarily alters the binding of substrate, possibly due to electrostatic and/or steric effects due to the added bulk of each side chain. It is interesting to note that the $K_d$ is affected more with the Ala mutant than with the Ser mutant. It may be possible that the Ser side chain hydroxyl may rotate slightly out of the pocket as the Cys to Ser mutant is seen to do, although this is purely hypothetical at this point.

*NADPH reduction and a role for Pro 900 in substrate specificity*

Reduction of the flavin domain with NADPH resulted in formation of a much weaker charge-transfer complex than was seen with NADH as reflected by the decreased long-wavelength absorbance as compared to NADH reduction of wild-type (Figure 4.3.B) and the P900A mutant (Figure 4.6.B). NADPH is a much less efficient substrate for spinach nitrate reductase ($V_{\text{max}} = 84$ and 4 $\mu$mol substrate/min/nmol FAD for NADH and NADPH respectively, Quinn et al., 1996) and likely does not bind tightly in the active site due to the additional phosphate group on the ribose ring of the
adenine moiety. The amino acid sequence for spinach nitrate reductase contains three successive proline residues immediately following the invariant Cys-Gly motif. The crystal structure of the flavin domain of corn-leaf nitrate reductase shows proline 244 (P900 in spinach nitrate reductase, the first of the three) at the opening to the active site cleft and potentially interacting with the ribose ring of the substrate (Figure 4.2.Bottom). Addition of the phosphate group may cause steric effects that, while allowing reduction of the flavin center to occur, does not allow for tight enough binding of the reduced substrate to form a stable charge-transfer complex.

Interestingly, a nitrate reductase variant in birch, which is bispecific for NADH or NADPH, has an alanine in place of the first proline. Mutation of the alanine to a proline altered the specificity of the birch NAD(P)H-nitrate reductase primarily for NADH as substrate and reduced the ability to efficiently utilize NADPH (Schöndorf and Hachtel, 1995). While significant, it is by no means a definitive answer to the binding of substrates. Other NAD(P)H-nitrate reductases, barley nitrate reductase for instance, have a proline at that position (Friemann et al., 1991). These enzymes also typically have different amino acids in the third position, an Ala in the case of the birch nitrate reductase or a Gly residue for the Neurospora NADPH-NR, which appears to confer their preference for NADPH over NADH. Then again, this is in contrast to the squash nitrate reductase, which contains a proline in the first position and an alanine in the third position, and primarily utilizes NADH as substrate (Hyde et al., 1991). Obviously there is no single determinant for substrate specificity, but is apparent that there is a trend towards a less bulky residue being present to allow for the binding of NADPH with its additional phosphate group.
Steady-state kinetics of the wild-type and P900A mutant show that mutation of the proline to an alanine residue diminishes the ability to utilize NADH as a substrate while greatly enhancing the use of NADPH (Table 4.1), giving behavior similar to that seen for the wild-type birch nitrate reductase. The variance amongst nitrate reductases for residues in any of the three positions suggests that positioning of substrate in the active site and, possibly, the fold of the protein backbone will determine the location and identity of the three residues for effective interaction with substrate.

*The role of the charge-transfer complex*

Steady-state kinetics of the spinach nitrate reductase wild-type and P900A flavin domains with cytochrome \( b_5 \) as electron acceptor resulted in significantly slower rates than that observed with ferricyanide as acceptor (Table 4.1). This is not surprising and has been shown previously with the spinach nitrate reductase flavin and heme domains (\( V_{\text{max}} = 1.2 \, \mu\text{mol NADH/min/nmol FAD} \), Quinn *et al.*, 1996). Indeed, electron transfer between the two domains was significantly enhanced in a flavocytochrome \( b_5 \) chimera (\( V_{\text{max}} = 37 \, \mu\text{mol NADH/min/nmol heme} \), Quinn *et al.*, 1994). It is also possible that the conditions used in this work were sub-optimal for efficient electron transfer between the two domains (a low cytochrome \( b_5 \) concentration compared to \( K_m \)) and the potential competition of oxygen in reoxidation of the heme domain specifically.

Interestingly, the rates observed for reduction of cytochrome \( b_5 \) correlate roughly to the amount of charge-transfer complex formed in each case. It is possible that the role of the charge-transfer complex is to stabilize the interaction between the two domains of nitrate reductase, increasing the efficiency of electron transfer between the two. This has been seen by Meyer *et al.* with the human cytochrome \( b_5 \) reductase-
cytochrome \( b_5 \) system (Meyer et al., 1995). These workers have shown that the presence of NAD\(^+\) stabilizes the protein-protein interaction and increases the rate of electron transfer. They also suggest that the presence of NAD\(^+\) (and the formation of the charge-transfer complex) may prevent autooxidation of the flavin semiquinone species after transfer of one electron to the heme (Meyer et al., 1995), which has also been seen with pig hepatic NADH:cytochrome \( b_5 \) reductase (Iyanagi et al., 1984). However, Ratnam et al. (1997) saw that electron transfer from the flavin to the heme was limited by the breakdown of the charge-transfer complex and that NAD\(^+\) did not appear to stabilize the flavin semiquinone, similar to that seen for adrenodoxin reductase (Kobayashi et al., 1995). Release of NAD\(^+\) from the charge-transfer complex in phthalate dioxygenase reductase is also required for electron transfer to occur (Gassner et al., 1994, Gassner and Ballou, 1995). The results herein suggest that nitrate reductase may work more like the former system, stabilizing the protein-protein complex and accelerating electron transfer between the two domains.

The work presented here demonstrates that mutations in the conserved Cys-Gly motif, as well as the following proline, of spinach nitrate reductase has a significant effect on the kinetics of formation of the long-wavelength charge-transfer complex seen in this enzyme, although clearly additional factors are involved in determining both reaction rate and substrate specificity. Static anaerobic reductive titrations of the spinach flavin domain with NADH (or sodium dithionite in the presence NAD\(^+\)) show that recombinant wild-type and all mutants form the charge-transfer complex although to slightly different extents. The alteration of the kinetic parameters \( k_{\text{red}}, K_d^{\text{NADH}}, \) and \( k_{\text{red}}/K_d \) for flavin reduction in each mutant indicate roles for each in the transfer of
electrons from substrate to the flavin center to varying degrees, manifested primarily as substrate binding effects (increases in $K_d$ of up to 2 orders of magnitude). Rapid reaction kinetics on all recombinant proteins has yielded $k_{\text{red}}$ and $K_d$ values for the reduction of the flavin cofactor by NADH, with a reduction in $k_{\text{red}}/K_d$ for all. Interestingly, the most pronounced effects were seen with the glycine mutations, with the G899S mutation showing the largest decrease in $k_{\text{red}}/K_d$ (almost a full 2 orders of magnitude lower than wild-type) and the G899A mutation resulting in a significant increase in the $K_d$ for NADH. These results suggest a role for the conserved glycine in this family of enzymes, specifically the requirement of no amino acid side-chain in the NAD(P)H binding site to avoid steric hindrance at that position. Full spectral analysis showed that formation of the charge-transfer complex happened rapidly as flavin was reduced. The specific activity for the spinach nitrate reductase flavin domain was previously observed to be approximately twice that of the corn nitrate reductase flavin domain (Quinn et al., 1996), supporting the rapid rates of flavin reduction observed in the kinetic studies.
Figure 4.1. Assimilatory nitrate reductase redox-active centers. Organization of the redox centers in spinach assimilatory nitrate reductase showing the substrate and product reactions and overall electron transfer from FAD to heme to molybdenum. Also shown are the various artificial electron acceptors and donors used for kinetic investigation and the location of the reversibly phosphorylated serine in hinge 1 which plays a role in regulation.
Figure 4.2. Nitrate Reductase Flavin Domain Crystal Structures. Shown are the crystal structures for the flavin-binding domain of wild-type (top), C242S mutant (middle), and wild-type with ADP bound (bottom) spinach assimilatory nitrate reductase. Ball and stick representations are of the bound FAD and the location of the three mutants investigated in this work (C242, G243, and P244 which correspond to C898, G899, and P900 respectively in the full-length protein), as well as the bound ADP molecule.
Figure 4.3. Formation of the wild-type sNR-FAD charge-transfer complex with NADH and NADPH. A, Generation of the long-wavelength charge-transfer complex for the wild-type flavin domain with NADH showing oxidized (solid line), reduced with sodium dithionite (dotted line), and following addition of excess NAD$^+$ (dashed line). B, Reduction with NADPH showing oxidized (solid line) and reduced (dotted line) with minimal long-wavelength charge-transfer complex formation upon reduction of the flavin center.
Figure 4.3.
**Figure 4.4.** Formation of the C898 mutant sNR-FAD charge-transfer complexes. Generation of the long-wavelength charge-transfer complex of, *A*, the C898S flavin domain, and *B*, the C898A flavin domain showing oxidized (solid line), reduced with sodium dithionite (dotted line), and following addition of excess NAD$^+$ (dashed line).
Figure 4.4.
Figure 4.5. Formation of the G899 mutant sNR-FAD charge-transfer complexes. Generation of the long-wavelength charge-transfer complex of, $A$, the G899S flavin domain, and $B$, the G899A flavin domain showing oxidized (solid line), reduced with sodium dithionite (dotted line), and following addition of excess NAD$^+$ (dashed line).
Figure 4.5.
Figure 4.6. Formation of the P900A mutant sNR-FAD charge-transfer complex with NADH and NADPH. A, Generation of the long-wavelength charge-transfer complex for the P900A flavin domain with NADH showing oxidized (solid line), reduced with sodium dithionite (dotted line), and following addition of excess NAD$^+$ (dashed line). B, Reduction with NADPH showing oxidized (solid line) and reduced (dotted line) with minimal long-wavelength charge-transfer complex formation upon reduction of the flavin center.
Figure 4.6.
Figure 4.7. Formation of the charge-transfer complex of wild-type and C898S mutant sNR-FAD with NADH. Anaerobic reductive titration of, A, wild-type, and B, C898S mutant, with NADH showing formation of the long-wavelength charge-transfer complex concomitant with reduction of the flavin center.
Figure 4.7.
Figure 4.8. Single wavelength rapid reaction kinetics of C898A mutant flavin reduction by NADH. Stopped flow rapid-reaction kinetics of reduction of the flavin center at 460 nm at 10°C. NADH reduction of the flavin center is ~75% complete within the dead time of the instrument (~2 milliseconds).
Figure 4.9. Full spectrum rapid reaction kinetics of flavin reduction by NADH. Stopped flow rapid-reaction kinetics of NADH reduction of the wild-type flavin domain showing almost complete reduction and formation of the charge-transfer complex within 2-3 milliseconds at 10°C using the photo-diode array.
**Figure 4.10.** Synthetic spinach assimilatory nitrate reductase heme domain. *A*, UV-visible spectra of oxidized (solid line) and dithionite reduced (dotted line) heme domain from spinach nitrate reductase. *B*, NADH reduction of the heme domain showing oxidized (solid line), addition of NADH (dotted line) and addition of wild-type sNR-FAD (dashed line) with an increase in the $\alpha$ and $\beta$ bands. The inset shows the increase in long-wavelength absorbance indicative of charge-transfer complex formation.
Figure 4.10.
Table 4.1. Steady-state kinetics of the wild-type and P900A flavin domains. Reduction of the wild-type and P900A flavin domains of spinach assimilatory nitrate reductase with NADH or NADPH monitoring ferricyanide reduction at 420 nm and cytochrome $b_5$ (spinach nitrate reductase heme domain) reduction at 556 nm.

<table>
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<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_{obs}$ ($s^{-1}$)</th>
<th>$V_{max}$ (μmol NADH/min/nmol FAD)</th>
<th>$k_{obs}$ ($s^{-1}$)</th>
<th>Cyt $b_5$</th>
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<tr>
<td></td>
<td></td>
<td>$K_3$Fe(CN)$_6$</td>
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<td></td>
<td>Cyt $b_5$</td>
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<tr>
<td>Wild-type</td>
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<td>84</td>
<td>37.7 ± 0.46</td>
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<tr>
<td></td>
<td>NADPH</td>
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<td>0.97 ± 0.10</td>
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<tr>
<td>P900A</td>
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<td>830 ± 126</td>
<td>50</td>
<td>15.2 ± 0.19</td>
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<tr>
<td></td>
<td>NADPH</td>
<td>922 ± 44</td>
<td>55</td>
<td>0.98 ± N.D.</td>
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<tr>
<td>sNR-FAD</td>
<td>NADH</td>
<td>-</td>
<td>84</td>
<td>20 ($V_{max}$ = 1.2)</td>
<td></td>
</tr>
<tr>
<td>(Quinn, 1996)</td>
<td>NADPH</td>
<td>-</td>
<td>4</td>
<td>-</td>
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Table 4.2. Rapid-reaction kinetic parameters for the wild-type and mutant flavin domains. $k_{\text{red}}$ and $K_d$ values for reduction of the flavin domain of wild-type, C898S/A, G899S/A, and P900S/A mutants, monitored at 460 nm at 10°C. cNR-CbR is the cytochrome $b_5$ reductase domain (flavin) from corn nitrate reductase and sNR-CcR is the cytochrome $c$ reductase domain (flavin and heme, also called the molybdenum reductase domain) from spinach nitrate reductase.

<table>
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<tr>
<th></th>
<th>$k_{\text{red}}$ (s$^{-1}$)</th>
<th>$K_d^{\text{NADH}}$ (µM)</th>
<th>$k_{\text{red}}/K_d$ (M$^{-1}$ s$^{-1}$)</th>
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<tr>
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<tr>
<td>sNR-CcR</td>
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CHAPTER 5

CLONING AND MUTAGENESIS OF THE cDNAs FOR HUMAN AND
CHICKEN XANTHINE DEHYDROGENASE

5.1. Introduction

The hydroxylase family of molybdenum-containing enzymes, typified by xanthine oxidoreductase (XOR), have active sites that consist of LMoOS(OH) centers, with L representing the pyranopterin cofactor, and with a bound hydroxy/water molecule in the case of xanthine oxidase (XO). Members of this family of enzymes catalyze hydroxylation reactions, generally (but not always) involving proton abstraction at an activated carbon center. The use of solvent as the ultimate source of oxygen and the ability to generate reducing equivalents during the course of the reaction distinguish these enzymes from true monooxygenases, which utilize molecular oxygen as the source of oxygen incorporated into product and consume reducing equivalents (Hille, 1996). Members of this family include bovine xanthine oxidoreductase (bXOR), chicken xanthine dehydrogenase (cXDH), *Rhodobacter capsulatus* xanthine dehydrogenase (RcXDH), *Oligotropha carboxydivorans* carbon monoxide dehydrogenase (CODH) and *Desulfovibrio gigas* aldehyde oxidoreductase (AOR).
Xanthine oxidoreductase from cow’s milk is one of the most studied molybdoenzymes. While lack of XOR activity is not as critical as loss of sulfite oxidase activity in humans, it is still of interest due to its potential role in ischemia-reperfusion injury and generation of reactive oxygen species (ROS), and its importance as a therapeutic target. During ischemia, the breakdown of ATP (and other nucleotides) leads to the accumulation of hypoxanthine and xanthine, and at the same time xanthine dehydrogenase is converted to the oxidase form. Following reperfusion and reintroduction of oxygen, superoxide and hydrogen peroxide are generated by the oxidase through its reaction with hypoxanthine and xanthine. Transfer of electrons to molecular oxygen results in high levels of damaging oxygen species, most notably $O_2^-$ and $\cdot$OH (from $H_2O_2$ through $Fe^{2+}$-catalyzed Fenton reactions). The involvement of XOR in the generation of reactive oxygen species has been highly debated with significant research on both fronts (Beetsch et al., 1998, Cighetti et al., 1994 as selected examples). It remains to be definitively established whether xanthine oxidase is truly responsible or if reperfusion injury is a combination of cellular effects. Nitric oxide synthase, for example, is a possible source due to the generation of peroxynitrite, a powerful oxidizing agent formed from the reaction of nitric oxide with superoxide. It has also been suggested that peroxynitrite may down-regulate XOR through inactivation of its molybdenum center, thereby reducing the amount of superoxide generated (Lee et al., 2000), although this is in contrast to previous results (Fukahori et al., 1994).

Hyperuricemia is a condition where uric acid, the primary product of xanthine oxidase, accumulates due to the inability to convert it to allantoin by the enzyme uricase (in humans). This has been assigned a role in hypertension, intrarenal vascular disease,
and renal injury (Kang et al., 2002). The administration of the drug allopurinol, a substrate of xanthine oxidase, is the most common method for treating hyperuricemia and gouty arthritis. Allopurinol is converted to oxypurinol (alloxanthine), which is a tight-binding and very specific inhibitor of XOR, reducing xanthine oxidase activity and lowering uric acid levels in the blood. However, the increase of detrimental side effects with allopurinol has led to the investigation into other potential inhibitors for XO.

Loss of function of xanthine oxidase manifests itself clinically as type I or type II xanthinuria (Raivio et al., 2001), with most patients showing no symptoms for either. Type I xanthinuria results in a loss in xanthine oxidase activity due to a lesion in the structural gene, which is not usually of great concern as substrates can occasionally be metabolized by aldehyde oxidases. Type II xanthinuria results in loss of both xanthine oxidase and aldehyde oxidase and is attributed to mutation of human molybdenum cofactor sulfurase (Ichida et al., 2001), which transfers a sulfur to the molybdenum center. Both conditions typically require modified diets and high fluid intake to avoid the accumulation of xanthine (Raivio et al., 2001).

Xanthine oxidoreductase is an approximately 300-kDa homodimeric molybdenum enzyme found in a virtually all organisms. Its redox-active centers consist of a pair of 2Fe-2S centers, a flavin adenine dinucleotide center and a molybdenum center (Figure 5.1). The gene encoding the enzyme is modular in nature, with the domains for each center encoded in individual regions and folded separately in the native protein (Ichida et al., 1993, Sato et al., 1995, Enroth et al., 2000). XO from vertebrate sources exists as an α₂ homodimer in solution, although other members of its family adopt a variety of tetrameric conformations. In bacteria, RcXDH forms an (αβ)₂
dimer while CODH is an (αβγ)₂ dimer. The visible spectrum of these enzymes is dominated by the flavin and iron-sulfur centers, with a broad absorption maximum at 450 nm and a shoulder around 550 nm (Figure 5.2.A, Hille, 1996, Ryan et al., 1995). Although it is convenient to investigate the enzyme with regards to these two centers, the small spectral signal for the molybdenum center makes it difficult to observe chemistry attributed to that center (Ryan et al., 1995).

While the two iron-sulfur centers (Fe/S I and II) of bXOR are indistinguishable in the visible spectrum (Hille et al., 1985), there are distinct differences between the two. The redox potentials for the two centers are dissimilar, with Fe/S I poised at –310 mV and Fe/S II at –235 mV (Hunt et al., 1993). In addition, the Fe/S I center is of the spinach ferredoxin-type with a typical fold for those proteins while Fe/S II has a unique fold not previously seen for other 2Fe-2S centers. Differences in the temperature dependent EPR characteristics have been observed (Palmer and Massey, 1969, Hille et al., 1985) and definitively assigned to the individual centers through mutagenesis and EPR of the rat XDH (Iwasaki et al., 2000) and also through EPR of CODH with and without the associated flavin domain (Gremer et al., 2000). These results have shown that the C-terminal Fe/S domain (Fe/S I) is the one closer to the molybdenum center. One of the cysteine residues from this iron-sulfur center is hydrogen-bonded by the amino group of the pyranopterin, forming a path for electron transfer out of the molybdenum through the pyranopterin. Electron transfer continues through the other iron-sulfur center (Fe/S II) and on to the flavin center.

The folds seen for the FAD domain for bXDH, the FAD-binding portion of the □-subunit of ReXDH and the M-subunit of CODH are very similar and akin to that
seen in vanillyl-alcohol oxidase and other FAD-binding oxidoreductases (Fraaije and Mattevi, 2000). The flavin domains are of interest due to the altered reactivity between NAD$^+$ and oxygen at the flavin site upon conversion of the dehydrogenase form of the enzyme to the oxidase. The dehydrogenase form passes electrons from the flavin center primarily to NAD$^+$, although reactivity with oxygen is seen (Harris and Massey, 1997). The oxidase form of the enzyme reacts only with oxygen and can no longer bind NAD$^+$ at the flavin site. The dehydrogenase to oxidase conversion occurs reversibly through disulfide bond formation or irreversibly through limited proteolysis. The latter process results in irreversible conversion to the oxidase, essentially cleaving the monomer into three separate domains that interact as a heterotrimer. This conversion is due to a rearrangement of a binding-site loop in the flavin domain that shifts upon conversion to the oxidase form and blocks binding of NAD$^+$ at the flavin center (Enroth et al., 2000).

This also results in a change in the electrostatic environment of the flavin center, giving it a more positive potential. Not surprisingly, in hindsight, modification of a tyrosine residue (Tyr 419 in cXDH) in the binding loop with 5’-p-fluorosulfonylbenzoyladenosine results in decreased dehydrogenase activity due to steric effects and reduced NAD$^+$ binding (Nishino and Nishino, 1989).

The molybdenum-binding domains contain a single molybdenum atom coordinated to a single equivalent of the pterin cofactor via its dithiolene sidechain. Catalysis at the molybdenum center is proposed to be initiated through base catalysis via proton abstraction from the Mo-OH group by an active site glutamate residue (Figure 5.2.B). Subsequent attack of the oxy group on substrate forms a Mo-O-C$_8$xanthine (substrate) intermediate with concomitant transfer of a hydride group to the
molybdenum sulfido group (Manikandan et al., 2001). Displacement of product by solvent water is followed by transfer of electrons out of the molybdenum center to the iron-sulfur centers and finally to the flavin center, where they pass to the oxidizing substrate. As indicated above, transfer of an electron from the molybdenum center to the first iron-sulfur center is apparently directed through the pyranopterin cofactor, which is hydrogen bonded to the sulfur of a coordinating cysteine of the proximal Fe/S center. The distance between the redox centers is 14.7 Å from the molybdenum center to Fe/S I, ~13 Å from Fe/S I to Fe/S II, and ~8 Å from Fe/S II to FAD (Enroth et al., 2000). Transfer of electrons from the first iron-sulfur center to the other centers has no obvious through-bond pathways and likely occurs via a tunneling method (Page et al., 1999). The occurrence of molybdoenzymes with bis-pterin systems, as in dimethylsulfoxide (DMSO) reductase poses the question as to the role of the second pterin cofactor in this latter group of enzymes. If one pyranopterin is involved in electron transfer into or out of the molybdenum center, perhaps the other pterin mediates the electronic properties of the molybdenum. It is the lack of other redox-active centers that makes the A. thaliana sulfite oxidase a good source for investigation of a molybdenum center with a single pyranopterin cofactor.

The protocols used to obtain the full cDNAs encoding both the human and chicken xanthine dehydrogenase are presented. In addition, several active site mutants for human xanthine dehydrogenase have been generated to ascertain their roles in catalysis.
5.2. Materials and Methods

General Cloning Reagents:

The gene sequences for the cDNA encoding human xanthine dehydrogenase (Ichida et al., 1993) and chicken xanthine dehydrogenase (Sato et al., 1995) were used as a basis for the development of the cloning strategy. Human liver tissue was acquired from The Ohio State University Tissue Procurement Services, a part of the Cooperative Human Tissue Network. Chicken liver tissue was acquired from Park Farms, Inc. in Canton, OH. Livers were frozen in liquid nitrogen immediately upon procurement. Total RNA was purified from the tissue using TRI Reagent from Sigma Chemical Company according to the included protocol. Total RNA was either used as a template for reverse-transcription-polymerase chain reaction (RT-PCR) experiments or for synthesis of first-strand cDNA. First strand cDNA was generated according to the protocol included with the reverse-transcriptase and was used for standard polymerase chain reaction experiments (PCR). Oligonucleotides were purchased from Integrated DNA Technologies and were resuspended in nuclease-free water upon receipt as a 100 O.D./mL stock solution. A working solution of each oligonucleotide was prepared by the appropriate dilution to 10 O.D./mL and used directly in RT-PCR, PCR and cDNA synthesis reactions. Enhanced avian myoblastosis virus reverse-transcriptase (eAMV-RT) was purchased from Sigma. DeepVent DNA polymerase was purchased from MBI Fermentas, Inc. Pfu and PfuTurbo DNA polymerases were purchased from GibcoBRL. Taq DNA polymerase and the Access RT-PCR Kit were purchased from Promega. A-tailing of PCR products was carried out by addition of Taq DNA polymerase to the reaction mixture and incubation for an additional 30 minutes at the desired extension
temperature (typically 72°C). The desired products were typically gel-purified from 0.7% agarose gels and the DNA cleaned using an Amicon Gel-nebulizer/spin-filter in a microcentrifuge. pGEM-3Z and pGEM-T Easy cloning plasmids were purchased from Promega. All restriction endonucleases were purchased from either MBI Fermentas or New England BioLabs. T4 DNA Ligase and Calf Intestine Alkaline Phosphatase were purchased from New England Biolabs. The TOPO-TA Cloning Kit was purchased from Invitrogen (with TOP10 E.coli electrocompetent cells). JM109 E. coli cells were purchased from Fischer Scientific. BL21, BL21(DE3) and BL21(DE3)pLysS E. coli cells were purchased from Novagen. Plasmid DNA constructs were transformed into electrocompetent E.coli cells using the Invitrogen Electroporator II at 1250V, with a 5 ms pulse decay according to the manufacturer’s protocol. Colonies from the transformation plates were selected based on PCR-screening results and used to inoculate 5ml of modified 2xYT culture media (10g tryptone, 10g yeast extract and 5g NaCl) and grown with shaking overnight at 37°C. Plasmid DNA was purified using the Promega Mini-Prep SV Plus Kit and sequenced using automated BigDye Terminator Fluorescence Sequencing. Desired constructs were purified using the Promega Maxi-Prep Wizard Kit for use in further cloning. All microbiology reagents were purchased from Fischer Scientific.

Mutagenesis was carried out using a two-step process in most cases (Figure 5.3.A). External primers were designed based on the location of unique restriction sites around the mutation to be made and internal primers were designed at the site of the mutation. The first round of PCR generated two overlapping products, each containing the desired mutation. The second round of PCR used the gel-purified products from the
first reaction as template and a 5- to 10-fold excess of the external primers. The desired product from the second round was gel-purified, cloned into the TOPO-TA vector and sequenced for correctness. For the correction of the hXDH mutation, the two PCR products generated in the first round were used as external primers with the hXDHp20 cDNA as the template for the second round of PCR. The first round products were designed to revert the mutation with one reaction and to change the XhoI/SacI restriction sites with the other reaction. PCR with these products as primers resulted in full-length product with the desired corrections/mutations. Table 5.1 lists the corrections and mutations presented in this work.

RT-PCR/PCR conditions:

**hXDH cloning**

The polymerase chain reaction (PCR) conditions for amplification of the cDNAs encoding the individual domains for human xanthine dehydrogenase (GenBank accession No. D11456) were carried out under the conditions given below. The gene-region encoding the first of the two iron-sulfur domains was amplified using a recursive PCR technique and six overlapping primers (Figure 5.3B). The primers are as follows:

#12: 5’-TGC AGT GTC GAA TTC AGC ATA TGA CAG CAG ACA AAT TGG TTT TCT TTG TGA ATG GCA GAA AGG TGG TGG AGA AAA; #13: 5’-CCA ACT TTC TTC TCA GGT AGG CCA AAA GGG TTG TCT CTG GAT CTG CAT TTT TCT CCA CCA CCT TTC TGC C; #14: 5’-GGC CTA CCT GAG AAG AAA GTT GGG GCT GAG TGG AAC CAA GCT CGG CTG TGG AGA GGG GGG CTG C; #15: 5’-GGA CGA TCT TGT TCT GCA GAC GAT CAT ACT TGG AGA GCA TCA CTG TGC AAG CCC CGC AGC CCC CCT CTC CAC A; #16: 5’-CGT
CTG CAG AAC AAG ATC GTC CAC TTT TCT GCC AAT GCC TGC CTG GCC
CCC ATC TGC TCC TTG CAC CAT G; #17: 5'-TAC CAA GCT TCG CTC GAG
TTA TTA GGA TCC TAT TCC TTC CAC AGT TGT CAC TGG CAA CAT GGT
GCA AGG AGC AGA TGG. PCR was carried out with a 30-second denaturation
step, a 2-minute, 50°C annealing step and a 2-minute extension step. The bold print
indicates the overlapping regions of the primers. Primers for the gene-region encoding
the second iron-sulfur domain were: #41: 5'-TGG AAG GAA TAG GAT CCA CCA
AGA CG and #42: 5'-TCC TCT GGT TTA AAT AAA GAT GGC GAG. PCR
conditions were a 1-minute denaturation step, a 2-minute, dual- temperature annealing
step at 40°C for 10 cycles and then 50°C for an additional 20 cycles and a 3-minute
extension step. Primers for the gene-region encoding the flavin adenine dinucleotide
domain were: #43: 5'-TCG CCA TCT TTA TTT AAA CCA GAG GAG and #44: 5'
TCG TAG CGA GG A ATA TC G TCA CAG. PCR conditions were a 1-minute
denaturation step, a 2-minute, 55°C annealing step and a 2.5-minute extension step.
Primers for the gene-region encoding the first of the molybdenum-binding domains
were: #45: 5'-TAC TGT GAC GAT ATC CCT CGC TAC GAG and #46: 5'
-TGA AAC CCT C AA GCT T CT GGT TGA AG. PCR conditions were the same as for the FAD
domain. Primers for the gene encoding the second molybdenum-binding domain were:
#64: 5'-AGT GCT GGA TGA GTG AAG TTG and #65: 5'-CGT GAA TTC CTC GAG
CTT TAG ACC CTC ACA GAC CAG GGT TTG CAG TTT TCT GGG ACA CCA
GTG ACA CAC AGG. PCR conditions were a 1-minute denaturation step, a 2-minute,
48°C annealing step and a 2.5-minute extension step. All PCR reactions were similar
with regard to their denaturation steps (94°C) and extension steps (72°C) and were
carried out for a total of 30 cycles. The FeSI, FAD and Mo1 genes were amplified by RT-PCR using human liver total RNA as template with a 45-minute, 48°C reverse-transcription step and a 2-minute, 94°C denaturation step to inactivate the reverse-transcriptase prior to the PCR reaction. The Mo2 gene was amplified by PCR using first-strand cDNA generated from human liver total RNA. Italics in each primer represent the restriction sites incorporated into each amplified DNA for cloning purposes.

Mutagenesis for the correction of the I1008V single-base mutation was carried out with the following sets of primers. The first set of primers to revert the I1008V mutation were: #115: 5’-ATT GCC GAG TGC TGG ATG AGT and #116: 5’-GCT TAT TCC GAA CTT GGT GGG A. The second set of primers to remove the internal XhoI and SacI restriction sites were: #114: 5’-CTC GGG CAC AGC ACA CAG GTA A and #113: 5’-ATC TCA GTG GTG GTG GTG GT. Bold type indicates the single base change to generate the desired mutation and the underlined regions represent the affected triplet codon.

Primers used for the mutagenesis of active site residues are as follows. For the E1261Q mutation, the external primers used were #115 and #113 listed above. The internal primers were: #144: 5’-TTG GAC AGC CGC CCC TCT TC and #145: 5’-GAA GAG GGG CGG CTG TCC AAC AG. For the E802Q and Q767E mutations, the external primers used were #45 and #46 listed above. The internal primers for the E802Q mutation were: #146: 5’-CAA GCA GAC CCG GAG CAC TGT G and #147: 5’-TCC GGG TCT GCT TGC CTC CAA AG. The internal primers for the Q767E mutation were: #148: 5’-GTG TCT ACA GAG AAC ACC ATG AAG ACC and #149:
5’-TGG TGT TCT CTG TAG ACA CAA AGA GC. Bold type indicates the single base change to generate the desired mutation and the underlined regions represent the affected triplet codon. All reactions were carried out using DeepVent DNA polymerase and using the hXDHp20 construct as template with a 30-second, 94°C denaturation step, 1-minute, 55°C annealing step and 1:10, 72°C extension step for 30 cycles. Second round PCR was carried out with the same conditions using a 1:30 extension step. All products were A-tailed as described earlier and cloned into the TOPO-TA vector for sequence analysis.

cXDH cloning

For the chicken XDH gene (GenBank accession No. D13221), the polymerase chain reaction (PCR) conditions for amplification of the cDNAs were carried out as described below. All reactions were run for 30 cycles using a 30-second, 94°C denaturation step, a 1-minute, 50°C annealing step and 1:40 minute, 72°C extension step using either Pfu Turbo or DeepVent DNA polymerase. The Mo1 cDNA was amplified under similar conditions, but using a 45°C annealing step. Primers for the gene-region encoding both iron-sulfur domains were: #22: 5’-TGG AAG GTG TGC CAT ATG GCT CCA CCT GAG and #96: 5’-CAG CA A CCG GTG CCA TTG G. Primers for the gene-region encoding the flavin adenine dinucleotide domain were: #97: 5’-CAC CGG TTG CTG TCA TAG TAA G and #85: 5’-GTG GGA TAT CAT CAC AGT AAA CGG. Primers for the gene-region encoding the first of the molybdenum-binding domains were: #86: 5’-GAT ATC CCA CAC TAT GAG AAC and #119: 5’-TAG AGT AAA TCC CTC GAG TT. Primers for the gene-region encoding the second molybdenum-binding domain were: #88: 5’-ACT CGA GGG ATT TAC TCT ACG GC and #89: 5’-
ACT GTT TCT CGA GGA GAG TTT GC. Italics in each primer represent the restriction sites incorporated into each amplified DNA for cloning purposes. All DNAs were amplified from first-strand cDNA generated from chicken liver total RNA.

Mutagenesis to correct the A499S (SacI-SacI piece) and I924S (HincII-XhoI piece) mutations was carried out with following sets of primers. Primers for the gene-region encoding the SacI-SacI portion of the FAD/Mo1 domains were: #132: 5’-AGA GCT CGC TGG CAG AGA CTG GAA TGA G and #121: 5’-TCC TGA ATT GTG ACA ATC GGT TT TGC. PCR was carried out using first strand cDNA (generated from chicken liver tissue total RNA as described in Materials and Methods) as template. Reaction conditions were a 30-second, 94°C denaturation step, a 1-minute, 58°C annealing step, and a 1:10, 72°C extension step for 30 cycles. Primers for correction of the I924S mutation were as follows using the two-step PCR protocol as described in Materials and Methods. The external primers were: #122: 5’-GAG ATG GAA CTC TTT GTG TCA ACC C and #125: 5’-TCC CTC GAG TTT TTG GTT AAA ATG AGT C. The internal primers were: #124: 5’-ATG TCA GCA TCA TGG GCT TTA TAT GCA AG and #123: 5’-TAT AAA GCC CAT GAT GCT GAC ATT GGG GAT GTT G. First round PCR was carried out using the original cMo1 cDNA as template with 30-second, 94°C denaturation step, a 1-minute, 54°C annealing step, and a 1-minute, 72°C extension step for 30 cycles. Products from these reactions were used for the second round PCR under the same conditions with no template. Italics represent the restriction sites used (the HincII site is downstream of primer #122), bold type indicates the single base change to generate the desired mutation and the underlined regions represent the affected triplet codon. All products were A-tailed as described
earlier and cloned into the TOPO-TA vector for sequence analysis prior to further cloning.

5.3. Results

\textit{hXDH Cloning:}

Due to the modular structure of the xanthine dehydrogenase gene, the cDNAs for the corresponding protein domains were cloned individually prior to generating the full-length cDNA (Figure 5.4). This allowed for the incorporation of unique restriction sites (some exceptions will be noted below) for the general cloning plan and also for the generation of expression constructs for individual redox center protein/domains if so desired. The cDNA encoding the first of the two 2Fe-2S cluster domains (FeSII) was cloned using a recursive-PCR technique (Prodromou and Pearl, 1992). Six oligonucleotide primers were designed with overlapping regions of 15-25 bases and incorporation of an N-terminal \textit{NdeI} restriction site and a C-terminal \textit{BamHI} restriction site. PCR was carried out using a proofreading DNA polymerase (DeepVent) with an ~10-fold excess of the outside primers. Purified DNA was sub-cloned into the pGEM-T Easy vector and transformed into JM109 cells. The cDNAs for the second 2Fe-2S cluster domain (FeSI), the flavin adenine dinucleotide (FAD) and the first of the molybdenum-binding domains (Mo1) were cloned using the Access RT-PCR Kit from Promega. Human liver total RNA was prepared as described in Materials and Methods and used as template for the reactions. Unique restriction sites were incorporated at the junction of the FeSI and FAD domains (\textit{DraI}) as well as the junction of the FAD and Mo1 domains (\textit{Eco321/EcoRV}). The FeSI DNA was sub-cloned into the pGEM-T Easy vector and transformed into JM109 cells and the FAD and Mo1 DNAs were sub-
cloned into the pCRII-TOPO-TA vector and transformed into TOP10 cells. The cDNA for the second molybdenum domain (Mo2) was cloned from first-strand cDNA generated from human liver total RNA using Enhanced Avian-Myoblastosis Virus (eAMV) reverse transcriptase. PCR was carried out using *Tfl* DNA polymerase from the Access RT-PCR kit and incorporated C-terminal *Eco*RI and *Xho*I restriction sites. Purified DNA was sub-cloned into the pCRII-TOPO-TA vector and transformed into JM109 cells. Once all of the cDNAs were amplified and the DNA constructs purified the cDNAs encoding the individual domains were digested and ligated together according to the cloning plan shown in Figure 5.4. The cloning was carried out with the presence of a single base mutation (resulting in I1008V) in the second molybdenum-binding domain cDNA and was corrected after the full cDNA was pieced together.

Initially, the FeSII cDNA was cloned in the pET-20b (hFeSIIp20) and pET-32a expression vectors for synthesis of the first iron-sulfur domain for EPR studies. Both vectors are T7-based expression plasmids, utilizing T7 RNA polymerase, which has been incorporated in a variety of bacterial expression strains as a DE3 lysogen. Induction of these genes with IPTG allows for the synthesis of the T7 RNA polymerase, which in turn translates the cDNA, which is cloned behind the T7 promoter of these plasmids. The pET-20b vector is a standard T7 expression plasmid and the pET-32a vector contains the gene for thioredoxin as well as several affinity tags for purification simplification. This allows for the generation of a fusion protein, the first 12 kDa being the thioredoxin protein, which aids in the overall stability of the expressed construct, and an additional 6 kDa consisting of two different affinity tags. Initial expression trials produced a partially soluble protein from the thioredoxin-fusion but not with the iron-
sulfur domain alone (the implications of which we be discussed later, Figure 5.5A). Expression of both iron-sulfur domains from the corresponding chicken cDNA was carried out in a similar manner, although it resulted primarily in insoluble protein (Figure 5.5B). Formation of the iron-sulfur cluster was not apparent in either protein as probed by UV-visible and EPR spectroscopy. Protocols for the reconstitution of the iron-sulfur clusters for either protein were unsuccessful.

To continue the cloning, the FeSII-FeSI (hFeSp20) and FeSI-FAD (hFFp20) cDNAs were cloned together in the pET-20b vector. hFeSp20 was generated from hFeSIIp20 using \textit{Bam}HI and \textit{Dra}I restriction sites, removing the \textit{Dra}I restriction site by ligating it with the blunt end generated from digest of the vector with \textit{Eco}321. The initial idea was to clone the FeSII cDNA into the FeSI-FAD-p20 construct using the \textit{Nde}I and \textit{Bam}HI restriction sites. However, due to multiple \textit{Bam}HI sites in the FAD domain not considered at the outset of the original project goals, an alternate approach was developed. The FeSII-FeSI-FAD-p20 construct (hFFFp20) was generated by digesting both constructs with \textit{Nde}I and \textit{Nco}I restriction enzymes followed by ligation of the FeSII-FeSI insert and the FeSI-FAD-p20 vector pieces.

The cDNAs encoding the two molybdenum-binding domains were similarly cloned into the pET-20b vector. Due to internal \textit{Hind}III and \textit{Xho}I restriction sites, the Mo2 cDNA was digested from the TOPO-TA vector (pCRII) using the \textit{Not}I restriction sites from the vector. This carried over a portion of the TOPO vector DNA to enable cloning into the pET-20b vector, generating hMo2p20. This construct, along with the Mo1 DNA from the TOPO vector, was digested using \textit{Pst}I and \textit{Not}I restriction enzymes. Because of an additional \textit{Pst}I restriction site on the opposite side of the pET-
20b vector, a partial digest was performed to isolate a linear vector for ligation with the Mo1 insert. The *Pst*I digest removed the extra DNA from the TOPO vector at the N-terminal of the Mo2 cDNA, leaving only the additional vector DNA at the C-terminal end. This extra DNA was used only for subcloning purposes, since the hXDH cDNA did not contain a *Not*I restriction site, and does not interfere with the generation of future constructs or with expression of the full-length protein. Cloning of the Mo1 cDNA into the hMo2p20 vector generated the hMo1Mo2p20 construct. Digestion with *Nde*I and *Eco*321 restriction enzymes and ligation of the hFFFp20 insert and hMo1Mo2p20 vector generated the full hXDHp20 construct. This construct was complete with the additional DNA carried over from the TOPO vector at the C-terminal end of the Mo2 gene and the internal error (resulting in an I1008V mutation in the protein sequence) in the Mo2 gene referred to above. An alignment of the protein sequence translated from this construct showed no deviation from the published hXDH protein sequence (Figure 5.6).

Mutagenesis was carried out to correct the single-base mutation location and also to remove the internal *Xho*I restriction site (as well as a *Sac*I restriction site immediately following the *Xho*I site). Amplification of the gene was carried out according to the protocol outlined in the Materials and Methods section, and the resulting product was cloned into the TOPO-TA vector. Following sequence analysis that confirmed the desired changes, the corrected cDNA was cloned into the hXDHp20 construct using *Hinc*II (*Hind*II) and *Not*I restriction sites. The resulting construct completed the cloning of hXDH and also allowed for the shuttling of the entire gene between plasmids using the *Nde*I and *Xho*I restriction sites. Specifically, this allows
transfer of the entire cDNA to a modified pTrcHis2-A vector, which contains the trc promoter, a strong, IPTG-inducible promoter used for expression of genes in any bacterial strain (i.e. without the need for the T7 RNA polymerase strains). This construct is suitable for use in TP1000 cells, a ΔmobAB strain of E. coli used for expression of proteins containing the non-dinucleotide form of the molybdopterin cofactor seen in eukaryotes (Temple et al., 2000).

In addition to the cloning of the hXDH gene, several mutants of potential interest were generated to investigate the catalytic roles of several active site residues. The E1261Q, E802Q and Q767E mutants were generated, using the specific primers and the two-step PCR protocol in Materials and Methods, and ligated into the hXDHp20 construct, using HinclI/NotI and MunI/SphI restriction sites for the E1262Q and the E802Q and Q767E mutants respectively. E1261 is proposed to be involved in catalysis, while E802 and Q767 are proposed to be involved in substrate binding, with Q767 also having a potential role in mediating the protonation state of the molybdenum sulfido group (see Figure 5.1, the implications of these mutations will be discussed later).

cXDH Cloning:

As with human xanthine oxidoreductase, the chicken XDH cloning plan was based on the modular nature of the gene. Primers were designed to individually amplify the gene fragments encoding the two iron-sulfur domains (designated FeSII/I), the flavin adenine dinucleotide domain (designated FAD) and the molybdenum-binding domains (designated Mo1 and Mo2). The individual cDNAs were cloned into the TOPO-TA vector, transformed into TOP10 cells and sequenced. The cloning of the
cDNAs for the individual domains of cXDH resulted in a few mutations that altered the original cloning plan. The first mutation (A499S) was located in the middle of the FAD cDNA (immediately following a SacI restriction site) and appeared to be a legitimate mutation due to the inability to isolate a correct clone through various PCR amplifications. This was corrected (to correspond to the published sequence) by the amplification of a new region of DNA between the SacI restriction sites located in the FAD and Mo1 cDNAs as described in Materials and Methods. A second mutation (I924S) was located in the first molybdenum-binding domain gene slightly downstream of the SacI restriction site located in that region. Primers were designed to correct this mutation through the two-step amplification of a smaller product, using internal primers at the site of the mutation and external primers at the N-terminal of the cMo1 cDNA and using the C-terminal Mo1 primer (see Materials and Methods). These pieces were then incorporated into a revised cloning plan for generation of the entire cXDH gene (see Figure 5.7), removed the Eco321 restriction site that was going to be incorporated in the original plan to ligate the FAD and Mo1 cDNAs together.

The entire FeSII/I cDNA was originally cloned in the pET-20b vector for expression trials, but the revised cloning plan used a new AgeI restriction site incorporated just upstream of the C-terminal of the FeS cDNA. The cDNA for the Fe/S II/I binding domain was PCR amplified, cloned into the TOPO-TA vector, sequenced and further sub-cloned into the pET-20b vector. This construct, along with the FAD cDNA in the TOPO-TA vector, was digested with AgeI and SacI and the resulting pieces (the FeS vector and the FAD insert) were ligated together to generate the cFF-
1p20 (cFAD*-1) construct. This represented the first half of the FAD cDNA from the AgeI restriction site to the SacI restriction site.

The original Mo1 cDNA was cloned into pET-20b using SacI restriction sites, bringing some of the TOPO-TA vector along in the process, to create cMo1*p20 (not shown in Figure 5.7). The error observed in the Mo1 region was corrected as described above and the resulting product was cloned into cMo1*p20 using HincII and XhoI restriction sites. This generated a corrected cMo1p20 (cMo1*-2) construct, removing the excess DNA from the TOPO vector and representing the second half of the Mo1 cDNA from the SacI restriction site to the C-terminal XhoI restriction site.

The cFF-1p20 and cMo1p20 constructs were digested with NdeI and SacI, the insert from cFF-1p20 and the vector from cMo1p20 were ligated together to form the cFF*Mo1p20 construct lacking the SacI-SacI piece from the FAD domain to the Mo1 domain. The SacI cDNA fragment was PCR amplified to correct the error in the FAD domain and was digested with SacI for ligation with the SacI digested and alkaline-phosphatase (AP) treated cFF*Mo1p20 vector. The resulting cFFMo1p20 construct was digested with XhoI, AP treated and ligated with the cMo2-XhoI digested cDNA to generate the complete cXDH cDNA pET-20b construct (cXDHp20). This construct was also generated in the modified pTrcHis2A-NdeI vector by digesting the cFF*Mo1p20 construct and vector with NdeI and XhoI, followed by ligation, transformation and sequencing. Correct constructs (identified through sequence analysis) were digested with XhoI, AP treated and ligated with the same cMo2-XhoI DNA used previously. Screening for directionality in both cases yielded full clones of the cXDH cDNA in both vectors, which allows for expression of the protein in either
system. As with the hXDH cDNA, the alignment of the protein sequence derived from the cDNA from this construct showed no deviation from the published protein sequence for cXDH (Figure 5.8).

5.4. Discussion

The cDNAs encoding the human and chicken xanthine oxidoreductase proteins have been cloned and established in expression vectors for generation of protein. Several active site mutations have also been made for the hXDH protein (Figure 5.9, Table 5.1). Investigation into the nature of the distinct Fe/S EPR signals seen for bovine xanthine oxidase led us to clone and generate cDNA constructs for the expression of either the pair of iron-sulfur domains of cXDH or the first iron-sulfur domain of hXDH. Unfortunately, suitable expression of proteins containing intact iron-sulfur clusters for either construct (as well as the inability to reconstitute those clusters) was not forthcoming, which resulted in the cloning of each full-length cDNA for eventual mutagenesis. PCR was used to amplify distinct regions of the cDNA from total RNA purifications from liver tissues of both organisms. Digestion and ligation of the various pieces resulted in the generation of full cDNA clones.

Low-level expression of rat XDH has been established in baculovirus (Iwasaki et al., 2000), and other molybdoenzymes have been expressed in T7-based systems (Garrett and Rajagopalan, 1994, Hitlon et al., 1996), but only low-level expression and/or reduced levels of active enzyme were obtained. However, high-level expression of human sulfite oxidase in TP1000 cells has shown that eukaryotic enzymes, and the eukaryotic form of the cofactor, specifically, can be obtained in a bacterial expression system (Temple et al., 2000). Most bacteria will generate the pyranopterin cofactor as
one or more dinucleotide forms, whereas all eukaryotic enzymes utilize the mononucleotide form. Interestingly, the XDH isolated from *Rhodobacter capsulatus* does not contain a dinucleotide addition. *E.coli* TP1000 cells, discussed in earlier chapters, have a deletion of the mobAB genes, which catalyze synthesis of the principal dinucleotide form of the cofactor seen in *E. coli*, MGD (the guanine dinucleotide conjugated cofactor). Transfer of the cDNAs for human and chicken XDH into an appropriate expression vector for use with TP1000 cells is an essential step in the generation of an effective recombinant system for these enzymes. It is with this eventual goal in mind that we have begun with the cloning of these genes.

While the cloning itself has been the bulk of the work presented in this chapter, the ultimate goal is the development of efficient expression systems for both proteins. This will be a complicated process involving the establishment of suitable growth conditions, optimal purification protocols, etc. The cDNAs were cloned complete with the termination codon inherent in each gene. This will result in the expression of protein without attached tags (most notably a His-Tag) such as are typically used for ease of purification. While this may appear at the outset to be an oversight, the presence of well-established purification schemes for both proteins from their native hosts provides a solid framework for the development of optimal conditions for the purification of the bacterially expressed proteins.

Expression of the first iron-sulfur domain from hXDH was possible only with the attachment of a thioredoxin-fusion tag to the N-terminal of the sequence (pET-32a). Expression of this construct was seen at the expected increased molecular weight, but was lacking any spectral signature attributable to the formation of an iron-sulfur cluster.
Expression of the iron-sulfur domain alone in the pET-20b system did not appear to be successful perhaps because the protein is too small (~10kDa) to accurately observe by typical SDS-PAGE, and was also unobserved with Tricine gels. Analysis of the sequence at the beginning of the cDNA for hXDH reveals several codons that are not optimal for expression in bacterial systems, most specifically the second codon, which is ACA, a very rare codon in bacteria. It is possible that these rare codons result in the early termination of expression due to stalling and release of the ribosome with the pET-20b construct, but are less deleterious in the pET-32a construct due to the addition of the N-terminal tag. Current expression of the full hXDH cDNA from the pTrcHis2A/TP1000 system has not yielded protein at the correct apparent molecular weight as judged by SDS-PAGE, suggesting that the occurrence of rare codons may be the problem. It may be that these codons will need to be changed to more common codons for bacteria to express viable protein. Expression of the chicken Fe/S II/I domains from pET-20b or pET-32a constructs resulted primarily in insoluble protein. Solubilization from inclusion bodies and reconstitution lacked observable iron-sulfur cluster formation.

Regarding the inability to assemble the iron-sulfur clusters, it is possible that the truncated proteins from human and chicken have difficulties in retaining a stable cluster. The locations of the clusters in each domain are close to the protein surface (approximately 12 Å from the surface), protected by the interaction of the flavin- and molybdenum-binding domains in the holoenzyme. The role of the flavin domain in stabilizing the iron-sulfur clusters may not be important (the aldehyde oxidase from *Desulfovibrio gigas* has a similar fold for its Fe/S and Mo domains, but lacks the flavin
domain seen in the XDHs), but the molybdenum-binding portion may be crucial. The related carbon monoxide dehydrogenase from *Oligotropha carboxydivorans* is a heterotrimer, each cluster located in individual subunits, but it is unknown whether the Fe/S subunit (L-subunit) from this enzyme can be isolated in a stable form. In any case, the establishment of reliable expression of human and chicken holo-XDH in the TP1000 cells will allow for further investigation into this potential problem.

Development of a suitable expression protocol for both proteins will allow the generation of mutant enzymes for investigation of residues proposed to play pivotal roles in the chemistry or stability of the enzymes and their cofactors (See Table 5.1). The reaction of xanthine oxidase with substrate is postulated to occur through base-catalysis, specifically involving a glutamate residue located in the active site (E1261 in the bovine protein). Proton abstraction from the Mo-OH group by this glutamate results in attack on substrate with concomitant hydride transfer from substrate to the sulfido group of the molybdenum. The Mo$^{IV}$-OR intermediate thus obtained is displaced by a solvent water/hydroxy molecule, and electrons are passed sequentially to the iron-sulfur centers and on to the flavin, regenerating the oxidized Mo$^{VI}$ oxidation state. If the proposed mechanism is correct, mutagenesis of Glu1261 should result in a dramatic decrease in the catalytic activity of the enzyme - the E1261Q mutant has been made and is awaiting expression and kinetic investigation.

Two additional mutations in the active site of hXDH have been constructed, Q767E and E802Q, which are also awaiting expression in the pTrcHis2A/TP1000 system. Both residues are highly conserved amongst xanthine dehydrogenases. Q767 is located near the molybdenum sulfido group and is proposed to play a role in
moderating the protonation of that group in the reduced Mo$^{IV}$ state (Truglio et al., 2002). E802 is located above the substrate-binding pocket, suggesting a role in hydrogen bonding or electrostatic interactions with substrate (although it is not conserved in the aldehyde oxidoreductases, Figure 5.1). In the \textit{R. capsulatus} XDH crystal structure the homologous residue, E232, appears to interact with one of the carboxylate groups of bound alloxanthine (a potential and specific enzyme inhibitor), but in the bovine crystal structure it is positioned towards the hydrophobic portion of the salicylate ring (Truglio et al., 2002). This residue may also act in conjunction with an arginine residue (R880) on the opposite side of the substrate binding pocket, together forcing substrate into a particular tautomeric state and thereby assisting catalysis. The proximity of E802 to Q767 (and somewhat near the molybdenum sulfido group) may also suggest a concerted role between these two residues during catalysis. An E802Q/Q767E double mutant has also been generated and is to be cloned into the hXDH construct prior to expression. The reversal of these two residues may result in a quasi-normal functioning enzyme, supporting a catalytic synergy between them.

As indicated above, xanthine oxidoreductase can exist as one of two forms in vertebrates, a dehydrogenase form and an oxidase form, although in some species (e.g. chicken or \textit{R. capsulatus}), it is limited to the dehydrogenase form. The dehydrogenase utilizes NAD$^+$ as oxidizing substrate while the oxidase uses molecular oxygen, generating the reactive oxygen species O$_2^-$ and H$_2$O$_2$. Conversion of the dehydrogenase to the oxidase form has recently been examined using crystallography and has revealed much about the nature of the loop shift (Enroth et al., 2000). These modifications cause a change in conformation of an access loop near the FAD center, resulting in the loss of
the NAD binding site due to steric hindrance, leaving room only for oxygen to bind. Specifically, the rearrangement causes a replacement of Asp 429 with Arg 426, near C6 of the flavin ring and apparently alters the electrostatic potential of the center (Enroth et al., 2000) responsible for loss of the NAD$^+$ binding site. The differences between the two forms can be investigated more thoroughly with the cloning of both the human and chicken XDHs. In addition, mutagenesis of critical residues in hXDH might generate protein that is unable to be converted, reducing the need for experimental conditions required to maintain the dehydrogenase form.

Generation of additional mutants, specifically substrate and cofactor binding residues implicated from crystallography, could lend significant insight into their roles in the chemistry of catalysis. Of critical importance is the function of the pyranopterin cofactor and its role in mediating electron potential of the molybdenum center and the transferal of electrons to the other redox-active centers. The cloning of human and chicken XDH and the establishment of a suitable expression system will enable both protein forms to be investigated, with specific regard for structure-function relationships and the roles of critical amino acid residues.
Figure 5.1. Xanthine oxidase redox centers. Redox centers from xanthine oxidase crystal structure showing linear path of electrons from molybdenum (in pink, right side) to the iron-sulfur centers (iron in green, sulfur in yellow) and on to the flavin adenine dinucleotide. Salicylate, Glu 1261, Glu 802 and Gln 767 are also shown in the molybdenum active site / binding pocket.
**Figure 5.2.** UV-visible spectra and proposed reaction mechanism of xanthine oxidase.  
*A*, Spectra of xanthine oxidoreductase showing, top, contribution of individual redox centers to overall absorbance features (Hille, 1996), and, bottom, spectral changes associated with each center (taken from Ryan *et al.*, 1995).  
*B*, Proposed reaction mechanism for xanthine oxidase showing base catalysis as the initiating step in catalysis.
Figure 5.2.
Figure 5.3. XDH cloning PCR methods. A, The two-step protocol for mutagenesis of base changes incorporated during cloning and for alteration of active site residues. B, Modified recursive PCR technique used to generate the hXDH FeSII cDNA (based on Prodromou and Pearl, 1992).
Figure 5.3.
Figure 5.4. hXDH cloning plan. Cloning plan for human xanthine dehydrogenase (D11456) showing plasmid constructs generated in route to completion of full cDNA clone.
Figure 5.4. (Cont.)
Figure 5.5. Expression of hXDH and cXDH. SDS-PAGE analysis of the expression of, A, the human FeSII domain and, B, the chicken FeSII/I domains in *E. coli* BL21(DE3) cells. Lanes are represented as follows: M = molecular weight marker, T# = Time points (in hours) when an aliquot was taken, S = soluble fraction, I = insoluble fraction.
Figure 5.5.
Figure 5.6. hXDH sequence alignment. Protein sequence alignment of human xanthine dehydrogenase (GenBank D11456) and hXDHp20 showing complete identity. Sequence aligned using ClustalW 1.8 program from BCM Search Launcher website.
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**Figure 5.6.**
Figure 5.7. cXDH cloning plan. Cloning plan for chicken xanthine dehydrogenase (D13221) showing plasmid constructs generated in route to completion of full cDNA clone.
Figure 5.7.
Figure 5.8. cXDH sequence alignment. Protein sequence alignment of chicken xanthine dehydrogenase (GenBank D13221) and cXDHp20 showing complete identity. Sequence aligned using ClustalW 1.8 program from BCM Search Launcher website.
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Figure 5.9. Alignment of select XDH protein sequences. Alignment of amino acid sequences for *Homo sapiens* (Human), *Bos taurus* (Bovine) and *Gallus gallus* (Chicken) xanthine dehydrogenases. A pound sign (#) indicates mutation location in the human cDNA and an asterisk (*) indicates mutation locations in chicken cDNA. Triangles (△) represent active-site mutations made for human XDH.
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Figure 5.9.
Figure 5.9. (Cont.)
Figure 5.9. (Cont.)
Table 5.1. hXDH and cXDH corrections and mutations. The hXDH cDNA (D11456) and hXDHp20 numbering begins with the initial CAT codon (for NdeI restriction endonuclease incorporation) prior to the initiator methionine. The cXDH cDNA (D13221) numbering is based on the cDNA sequence obtained from GenBank, while the cXDHp20 numbering is based similar to the hXDH numbering. Underlined regions represent the codon change to affect the desired amino acid mutation.
CHAPTER 6

SUMMARY

Mononuclear molybdenum enzymes are prevalent in nature and play critical roles in global sulfur, nitrogen, and carbon cycles (Stiefel, 2002). These enzymes carry out a variety of oxygen atom transfer reactions utilizing any number of redox-active centers for electron transfer processes. In this work, a variety of molybdenum-containing enzymes have been studied with the goal of better understanding the chemistry of catalysis and electron transfer amongst the various redox-active centers these enzymes contain. The research presented here provides for greater characterization of a novel plant sulfite oxidase, which possess a single equivalent of the molybdopterin cofactor and no additional redox-active centers. Catalytic turnover experiments with mouse sulfite oxidase lend insight into the chemistry of oxygen atom transfer for the LMoO₂ family of enzymes. Kinetic investigation of mutations in the flavin-binding domain of spinach assimilatory nitrate reductase has revealed functions for those residues in substrate-binding and clues to the role of the charge-transfer complex seen for these domains. Finally, the cloning of the cDNAs for human and chicken xanthine dehydrogenase, and the generation of a few active site mutants, will
enable future investigation of catalysis in these enzymes yielding a clearer understanding of the hydroxylation chemistry for these enzymes.

*Arabidopsis thaliana sulfite oxidase*

The kinetic and spectroscopic characterization of this novel molybdenum enzyme has definitively established it as a true sulfite oxidase with many characteristics common to sulfite oxidases from vertebrates. The lack of additional spectroscopically confounding redox-active centers allows direct investigation of the molybdenum center with a variety of spectroscopic methods. Kinetic investigation yielded a similar pH dependence for catalysis, with a significantly faster reductive half-reaction than seen for the chicken enzyme. The EPR data show similar features as other sulfite oxidases with a few exceptions, suggesting subtle differences in the active site geometry of this enzyme. Resonance Raman data of oxidized enzyme shows peaks representative of an $\text{LMoO}_5(\text{S-Cys})$ active site coordination, with the presence of a single pyranopterin cofactor of the $\square$-delocalized variety. Catalytic turnover in the presence of $^{18}\text{O}$-labeled water resulted in exchange of a single oxygen in the molybdenum coordination sphere, similar to previous results seen for human sulfite oxidase (Garton *et al.*, 1997a) and supporting the oxygen atom transfer chemistry proposed for this family of enzymes (Brody and Hille, 1999).

Generation of the C97S mutant, the protein ligand to the molybdenum, is the first step in utilizing the power of site-directed mutagenesis to investigate the function of various residues in catalysis, modulation of the oxidation-reduction potential of the molybdenum center, and the role of the pyranopterin in electron transfer from the molybdenum. It also remains to be determined what the physiological electron acceptor
for this enzyme is. Lack of transfer to various heme proteins suggests either an alternate redox-partner or a requirement for the linker region seen in other sulfite oxidases for efficient electron transfer between the two domains.

Mouse sulfite oxidase

$^{18}$O-labeling experiments presented demonstrate unequivocally that the source of oxygen incorporated into product is derived from solvent, and not from dioxygen for this family of enzymes (LMoO$_2$S-Cys). This is in agreement with results for the xanthine oxidase (LMoOS(OH), Hille and Sprecher, 1987) and dimethysulfoxide reductase (L$_2$MoO(X), Schultz et al., 1995) families of molybdenum enzymes and also with various inorganic model compound studies (Xiao et al., 1992, Schultz et al., 1993, Pietsch and Hall, 1996, Thomson and Hall, 2001). The presence of multiple labeling of substrate is an interesting result that deserves attention and may reveal significant information regarding intermediates formed during catalysis and the chemistry involved thereof.

Preliminary NMR results have yielded experimental conditions that will allow for the determination of relaxation rates for both mouse sulfite oxidase and rat outer mitochondrial membrane cytochrome $b_5$ to understand the dynamics of sulfite oxidase based on oxidation state. The presence of sharp peaks among the broad NMR spectra of mouse sulfite oxidase suggests the presence of a domain that may be tumbling at a faster rate than the larger protein. A comparison of the rates seen for mouse sulfite oxidase with those for rat outer mitochondrial membrane cytochrome $b_5$ will hopefully yield information regarding the dynamic nature of the heme domain of sulfite oxidase. In addition, the development of the expression system for the molybdenum domain of
mouse sulfite oxidase will allow a comparison of the results observed for the full-length and molybdenum proteins, providing for information on the effect of the heme domain. Kinetic and spectroscopic investigation of the molybdenum domain of mouse sulfite oxidase will also act as a direct comparison to the results observed for the *A. thaliana* sulfite oxidase.

*Flavin-binding domain of spinach assimilatory nitrate reductase*

The kinetic and spectroscopic investigation of the wild-type and mutant flavin-binding domains of spinach assimilatory nitrate reductase have provided a further understanding of substrate binding and electron transfer from substrate, NADH, to the flavin center and further on to the heme domain. The roles of the three residues in the substrate-binding pocket, C898, G899, and P900, have been postulated based on the kinetic results. C898, as previously determined by other labs, is involved in the positioning of substrate in the active site for efficient electron transfer. G899 appears to indirectly control steric conditions in the binding pocket, mutations to larger residues had significant kinetic impacts, primarily on *Kₐ*. P900 is involved in substrate specificity, distinguishing between NADH and NADPH, similar to that seen for a variety of assimilatory nitrate reductases.

The charge-transfer complex formed between reduced flavin and NAD⁺ was shown to exist for all variants investigated, although to different degrees. The proposed role in the rate-limiting transfer of electrons from the flavin to the heme has not been further solidified. On the contrary, steady-state kinetics using the spinach assimilatory nitrate reductase heme domain showed an interesting correlation to the amount of charge-transfer complex formed and the rate of electron transfer to the heme domain.
While this finding is similar to results seen for human NADH:cytochrome c reductase, these results are preliminary and need to be continued to establish their significance. The lack of a tether between the domains is known to reduce electron transfer efficiency between the two domains, which may complicate interpretation of the present data. Rapid-reaction kinetics will provide the means of determining accurate rates of electron transfer from the flavin to the heme and hopefully shed light on the true nature of the charge-transfer complex.

**Cloning of human and chicken xanthine dehydrogenase**

The cDNAs encoding the full-length human and chicken xanthine dehydrogenase have been cloned. In addition, the E1261Q, E803Q, and Q768E active site mutants have been generated and hopefully will provide information regarding the proposed base-catalysis mechanism (E1261) and the roles in catalysis and substrate binding (E803 and Q768). Long-term, these expression systems will allow further investigation of the molybdenum center, as well as the two 2Fe/2S centers and the FAD center using site-directed mutagenesis and through the development of individual expression constructs for each domain.

In conclusion, the research presented here provides a solid framework for future experiments towards a more complete understanding of the nature of the various catalytic mechanisms for mononuclear molybdenum enzymes. Investigation of the single-pyranopterin system seen in the *A. thaliana* sulfite oxidase will provide a strong complement to the data obtained for the bis-pyranopterin enzymes, such as DMSO reductase, revealing distinct roles for each cofactor in electron transfer and modulation of the reduction potentials of the molybdenum centers. It will also be beneficial to
understand the nature of electron transfer between the various domains seen in these enzymes, the sulfite oxidase and nitrate reductase systems described above providing a few interesting examples, as well as the recombinant human and chicken XDH systems and the ability to use site-directed mutagenesis to target specific redox-active centers.


Henderson, P. (1986) *Inorganic Geochemistry*


