Title
Spectroscopic and Kinetic Studies of Arabidopsis thaliana Sulfite Oxidase

Permalink
https://escholarship.org/uc/item/06v99497

Author
Byrne, Robert Stephen

Publication Date
2010

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA, RIVERSIDE

Spectroscopic and Kinetic Studies of *Arabidopsis thaliana* Sulfite Oxidase

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Biochemistry and Molecular Biology

by

Robert Stephen Byrne

December 2010

Dissertation Committee:
Dr. Russ Hille, Chairperson
Dr. Richard Debus
Dr. Li Fan
The Dissertation of Robert Stephen Byrne is approved:

Chairperson

University of California, Riverside
Acknowledgements

First and foremost, I would like to thank Dr. Hille for all of his support and guidance throughout this entire process. I would also like to thank several of my colleagues, without whom this would have taken much longer:

Craig Hemann for helping me to get this project started. Dr. Ralf R. Mendel and Dr. Florian Bittner for providing us with our cell line and overexpression systems. The entire EPR group at the University of Arizona: Dr. John Enemark, Dr. Arnold Raitsimring, Dr. Andrei Astashkin, Dr. Kayunta Johnson-Winters, and Dr. Eric Klein for their continued collaborations and assistance. All of my fellow graduate students, past and current, that have been in the trenches with me. My copious amount of undergraduate researchers through the years, specifically Jeff Whitman who provided a second set of reliable hands to help with this project.

The text of this dissertation, specifically chapter 3, in part or in full, is an adaptation or copy of the material as it appears in: “Direct demonstration of the presence of coordinated sulfate in the reaction pathway of Arabidopsis thaliana sulfite oxidase using $^{33}$S labeling and ESEEM spectroscopy.”


The co-author John H Enemark listed in that publication directed and supervised the research which forms the basis for this chapter. The remaining authors assisted in this work based on their relative fields of expertise in EPR spectroscopy and computational chemistry
The text of this dissertation, specifically chapter 4, in part or in full, is an adaptation or copy of the material as it appears in: “Oxidative half-reaction of *Arabidopsis thaliana* sulfite oxidase: generation of superoxide by a peroxisomal enzyme.” Byrne RS, Hänsch R, Mendel RR, Hille R. J. Biol. Chem. 2009 Dec 18; 284(51):35479-84.

The co-author Russ Hille listed in that publication directed and supervised the research which forms the basis for this chapter. Robert Hänsch and Ralf Mendel graciously constructed the plasmid and cell line that allowed us to perform this research.
ABSTRACT OF THE DISSERTATION

Spectroscopic and Kinetic Studies of Arabidopsis thaliana Sulfite Oxidase

by

Robert Stephen Byrne

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology University of California, Riverside, December 2010

Dr. Russ Hille, Chairperson

The sulfite oxidases are a family of enzymes characterized by the nature of their molybdenum centers. There are three classes of sulfite oxidizing enzymes: vertebrate sulfite oxidases, plant sulfite oxidase and bacterial sulfite dehydrogenases. All these proteins catalyze the oxidation of sulfite to sulfate - as part of sulfite detoxification in plant and animals and during chemolithotrophic growth in bacteria. While sharing a common overall reaction, each family has distinct characteristics both in the overall structure and in the reaction mechanism. Here we have set out to examine the reaction mechanism of sulfite oxidase from Arabidopsis thaliana, a protein that is unique compared to the other sulfite oxidizing enzymes. Previous work, utilizing continuous wave electron paramagnetic resonance (CW-EPR), has shown that a unique signal is seen in plant sulfite oxidase. The nature of this signal has been studied here using two different pulsed EPR techniques, two-pulse electron spin echo envelope modulation (ESEEM) and two-dimensional hyperfine sublevel correlation (2D-HYSCORE). In these studies we show that this signal is due to the formation of a sulfate-bound Mo(V) intermediate, a finding of major mechanistic implication since it implies that plant sulfite oxidases are catalytically active while previous studies have shown that vertebrate and
bacterial sulfite oxidase become trapped in this form yielding a catalytic dead end. We also show here kinetic evidence of superoxide production by this enzyme during its oxidative half-reaction with oxygen, a reaction not shared with the other sulfite oxidizing enzymes. Through rapid-reaction kinetic studies, we show that this reaction involves two sequential one-electron transfers, each generating an equivalent of superoxide. Finally, we have examined the reaction of plant sulfite oxidase with dimethylsulfite and shown that a substrate lone pair attack at the equatorial oxo group of the molybdenum center must be responsible for initiation of catalysis. The interpretation of these results and their implications are discussed in the context of a modified reaction mechanism to explain the behavior of this protein during the oxidative half-reaction.
Table of Contents

Chapter 1: General Introduction
1.1 Molybdenum: General Background .................................................. 1
1.2 Molybdenum in biology and the pyranopterin cofactor ...................... 2
1.3 Pyranopterin cofactor synthesis and insertion ..................................... 5
1.4 The mononuclear molybdenum enzymes ........................................... 13
1.5 The mononuclear molybdenum enzymes and human pathology .......... 22

Chapter 2: Introduction to the sulfite oxidases
2.1 General introduction to the sulfite oxidases ....................................... 29
2.2 The active site of the sulfite oxidases ................................................. 33
2.3 The catalytic mechanism of sulfite oxidation by sulfite oxidases .......... 47
2.4 The purpose of this work ..................................................................... 49

Chapter 3: Evidence of a Sulfate-bound Mo(V) Species in the Reaction of Plant Sulfite Oxidase
3.1 Introduction ....................................................................................... 52
3.2 Materials and Methods ....................................................................... 57
3.3 Continuous Wave EPR ....................................................................... 59
3.4 Two-Pulse ESEEM spectroscopy ......................................................... 62
3.5 Simulations from ESEEM data ............................................................ 67
3.6 Two-dimensional HYSCORE experiments and simulations .................. 76
3.7 Discussion .......................................................................................... 81

Chapter 4: The production of superoxide during aerobic turnover of plant sulfite oxidase
4.1 Introduction ........................................................................................ 88
4.2 Materials and Methods ....................................................................... 91
4.3 Rapid-reaction kinetics of reduced enzyme with oxygen ..............................97

4.4 Steady state studies following oxygen consumption .................................100

4.5 Observation of superoxide production by steady state reduction of cytochrome c ...........................................................................104

4.6 Discussion ..........................................................................................110

Chapter 5: The reaction of plant sulfite oxidase with the substrate analog dimethylsulfite
5.1 Introduction ..........................................................................................119

5.2 Materials and methods ........................................................................122

5.3 Steady state kinetics with sulfite and dimethylsulfite ...............................124

5.4 Temperature dependence of the steady state kinetics of plant sulfite oxidase ......................................................................................126

5.5 pH effects on catalysis in plant sulfite oxidase with sulfite and dimethylsulfite .................................................................130

5.6 Discussion ..........................................................................................132

Chapter 6: General summary and future direction
6.1 ESEEM studies with $^{33}$S-labeled sulfite .............................................141

6.2 Studies of the oxidative half-reaction of plant sulfite oxidase ...............144

6.3 The reaction of plant sulfite oxidase with dimethylsulfite ........................146

6.4 Future direction ..................................................................................149

Works Cited: ..........................................................................................151
List of Tables

Table 1.1: Human disorders of the molybdenum containing enzymes.........................24

Table 2.1: Structural alignment data comparing several sulfite oxidase family members........................................................................................................................................30

Table 2.2: Sequence identity of the molybdenum cofactor domains of representative sulfite oxidase family proteins.................................................................38

Table 3.1: Explanation of the numerical values given in Tables 3.2 through 3.5........71

Table 3.2: The simulation parameters for Figure 3.8......................................................72

Table 3.3: The simulation parameters for Figure 3.9......................................................73

Table 3.4: The simulation parameters for Figure 3.10...................................................75

Table 3.5: The simulation parameters for Figure 3.11...................................................76

Table 3.6: Possible sets of $hfi$ constants $A = (A_x, A_y, A_z)$ as estimated from analysis of HYSCORE spectra for use in the simulations shown in figure 3.14........80

Table 4.1: pH Dependence of Superoxide Production by A. thaliana sulfite oxidase........................................................................................................................................108

Table 4.2: Temperature Dependence of Superoxide Production in A. thaliana sulfite oxidase.....................................................................................................................108

Table 5.1: Temperature dependence of the steady-state reaction of plant sulfite oxidase with sulfite and dimethylsulfite...............................................................130

Table 5.2: pH dependence of the steady state reaction of plant sulfite oxidase with sulfite and dimethylsulfite.................................................................131
List of Figures

Figure 1.1. The molybdenum cofactors .................................................................4

Figure 1.2. Hydrogen bonding Interactions of the molybdenum cofactor ..............5

Figure 1.3. The molybdenum cofactors of the mononuclear molybdenum enzymes......10

Figure 1.4: Synthesis of the molybdenum cofactor ...........................................11

Figure 1.5. Crystal structure of xanthine oxidase and DMSOR ..........................19

Figure 1.6. Crystal structures of the sulfite oxidases ............................................20

Figure 1.7. Diagram of domain arrangement in the Sulfite Oxidase Family .......21

Figure 1.8. Metabolism of sulfur containing amino acids ......................................27

Figure 2.1. Sequence alignment of the molybdenum domains of the sulfite oxidase family .........................................................................................................................40

Figure 2.2. Active site overlay of chicken and plant sulfite oxidase ....................41

Figure 2.3. R138Q chicken sulfite oxidase point mutation ....................................42

Figure 2.4. Conformation change of R374 (R450) in an overlay of crystal structures from chicken and plant sulfite oxidases .........................................................43

Figure 2.5. Active site residues of Y322F chicken sulfite oxidase point mutation ...44

Figure 2.6. Active site residues of G375A chicken sulfite oxidase point mutation ...45

Figure 2.7. Active site residues of A186D chicken sulfite oxidase point mutation ...46

Figure 2.8. General mechanism for the oxidation of sulfite to sulfate as catalyzed by sulfite oxidases .................................................................................................48

Figure 2.9. Proposed mechanisms for coordination of sulfite to the molybdenum cofactor during catalysis in sulfite oxidases .......................................................51

Figure 3.1. Predicted coordination of the Mo(V) centers from the sulfite oxidases ...53

Figure 3.2. Conformation change of R374 (R450) in an overlay of crystal structures from chicken and plant sulfite oxidases .......................................................56
Figure 3.3. CW EPR spectra of lpH$^{32}$S-At-SO, top and $^{33}$S-At-SO, bottom.............60

Figure 3.4. General pulse sequence for two-pulse ESEEM and diagram of the process of making a primary echo.................................................63

Figure 3.5. Two-pulse ESE field-sweep spectrum..............................................64

Figure 3.6. Normalized Primary ESEEM trace of $^{32}$S- and $^{33}$S-sulfite reduced plant sulfite oxidase at low field turning point ($g_1$)........................................65

Figure 3.7. Zeeman splitting diagram for an $M_s = 1/2 M_I = 3/2$ system (splitting not to scale)..............................................................67

Figure 3.8. Amplitude FTs of two-pulse ESEEM traces recorded for lpH$^{33}$S-At-SO (solid traces) and $^{32}$S-At-SO (dashed traces) at the EPR turning points, as indicated by the labels “$g_3$”, “$g_2$” and “$g_1$”.............68

Figure 3.9. Cosine FTs of two-pulse ESEEM traces recorded for lpH$^{33}$S-At-SO at the EPR turning points, as indicated by the labels “$g_3$”, “$g_2$” and “$g_1$”.................................................................70

Figure 3.10. Simulated amplitude FT traces for anisotropic $h\tilde{f}i$ and an axial $nqi$........72

Figure 3.11. Simulated amplitude FT traces for varied anisotropic $h\tilde{f}i$ and orientation of the $nqi$ tensor (which was kept axial in this case) with respect to the $h\tilde{f}i$ tensor.................................................................73

Figure 3.12. Simulated amplitude FT traces for varied anisotropic $h\tilde{f}i$ and orientation of the $nqi$ tensor (which was kept rhombic in this case) with respect to the $h\tilde{f}i$ tensor.................................................................75

Figure 3.13. Simulated amplitude FT traces for an unbound, closely situated sulfite or sulfate.............................................................................76

Figure 3.14. 2D-HYSCORE experiments and simulations for the $^{33}$S-sulfite reduced low pH form of plant sulfite oxidase........................................79

Figure 4.1. The reaction mechanisms of vertebrate and plant sulfite oxidases...........91

Figure 4.2. Oxidized and reduced absorbance spectra of A. thaliana sulfite oxidase...98

Figure 4.3. The dependence of observed rate constant versus [$O_2$] for the reoxidation of A. thaliana sulfite oxidase.........................................................99
Figure 4.4. The reduction of cytochrome c in the course of the oxidative half-reaction of A. thaliana sulfite oxidase……………………………….100

Figure 4.5. Superoxide dismutase concentration dependence on the reduction of oxygen consumption by the pSOx reaction monitored by oxygraph….103

Figure 4.6. Effect of cytochrome c and superoxide dismutase on the steady-state rate of oxygen consumption by the plant sulfite oxidase reaction as monitored oxymetrically…………………………………………………..106

Figure 4.7. Effects of cytochrome c and SOD on the rate of oxygen consumption by the xanthine oxidase reaction as monitored by oxygraph………………107

Figure 4.8. Effect of superoxide dismutase on cytochrome c reduction by A. thaliana sulfite oxidase reaction as monitored spectrophotometrically………………………………………………………………..109

Figure 4.9. Effect of superoxide dismutase on cytochrome c reduction by A. thaliana sulfite oxidase as monitored spectrophotometrically………………110

Figure 4.10. Effect of superoxide dismutase on cytochrome c reduction by xanthine oxidase reaction as monitored by UV-Visible spectrophotometer………………………………………………………………111

Figure 4.11. Temperature dependence of the overall oxidative half reaction of A. thaliana sulfite oxidase…………………………………………………..114

Figure 4.12. Overall mechanism for the reaction of A. thaliana sulfite oxidase with sulfite………………………………………………………………………………117

Figure 5.1. The structures of sulfite and dimethylsulfite…………………………121

Figure 5.2. Dependence of A. thaliana sulfite oxidase activity on dimethylsulfite concentration……………………………………………………………………125

Figure 5.3. Dependence of plant sulfite oxidase activity on sulfite concentration…..125

Figure 5.4. Hydrolysis of dimethylsulfite in aqueous solution……………………….127

Figure 5.5. Temperature dependence of the steady-state reaction of A. thaliana sulfite oxidase with sulfite…………………………………………………..128

Figure 5.6. Temperature dependence of the steady state reaction of A. thaliana sulfite oxidase with dimethylsulfite……………………………………..129
Figure 5.7. The pH dependence of $k_{cat}$ for the reactions of plant sulfite oxidase with sulfite and dimethylsulfite………………………………………134

Figure 5.8. The pH dependence of $k_{cat}/K_m$ for the reactions of plant sulfite oxidase with sulfite and dimethylsulfite………………………………………135

Figure 5.9. Electrostatic interactions and distance measurements for bound sulfate in the crystal structure of chicken sulfite oxidase (1SOX)………..139
**Chapter 1: General Introduction**

1.1 Molybdenum: general background

Molybdenum is an element counted among the transition metals of the \( d \)-block of the periodic table. It is found below chromium (Cr) and above tungsten (W) and has an atomic number of 42. It was discovered by Carl Wilhelm Scheele, a Swedish chemist, in 1778, as molybdenite (\( \text{MoS}_2 \)) (Elmsley, 2001) and it was first purified in its elemental form by Peter Jacob Hjelm in 1781 (Urdang, 1942). In its pure, elemental form, molybdenum has the sixth highest melting point of all the elements, has mid-level hardness (5.5 on Moh’s hardness scale (Brittanica, 2010)) and has a low coefficient of thermal expansion (CRC Handbook of Chemistry and Physics 90th ed., 2009). This, along with its ability to readily form carbides, makes it a very useful element in many steel and chromium alloys. There are many isotopes of molybdenum ranging in atomic mass from 83 to 117 with seven, naturally occurring, stable forms 92 (14.84% natural abundance), 94 (9.25%), 95 (15.92%), 96 (16.68%), 97 (9.55%), 98 (24.13%), and 100 (9.63%). Along with its variety of isotopic abundances, molybdenum can exist in oxidation states ranging from -2 to +6 (except -1).

Transition metals often have the ability to achieve high coordination numbers, due in part to their larger \( d \)-orbitals (Crabtree, 1994). Such is the case with molybdenum, which can coordinate up to eight ligands in stable configurations. In nature, molybdenum is always found in one of these coordinated compounds, most notably, \( \text{MoS}_2 \) in the crust of the Earth and the molybdate di-anion (\( \text{MoO}_4^{2-} \)) in the oceans (Henderson, 1984). While it
only composes about 0.0003% of the earth’s crust, it is the most abundant transition metal in the oceans due to the high water-solubility of the molybdate anion. Its importance in synthetic chemistry has culminated recently with the 2005 Nobel Prize for Chemistry being awarded to Richard Schrock, Robert Grubbs and Yves Chauvin for their work on the olefin metathesis process using molybdenum based catalysts.

1.2 Molybdenum in biology and the pyranopterin cofactor

There are very few organisms known that do not require molybdenum; yeasts of the Saccharomyces species are a notable eukaryotic exception. Those that do not use molybdenum, typically use tungsten instead, which shares many chemical properties with molybdenum (Hille R., Molybdenum and Tungsten in Biology, 2002). Tungsten utilizing organisms are typically anaerobic archaea or bacteria that live near geothermal vents in the depths of the oceans. The stability of molybdenum complexes along with the multiple oxidation states available to the metal makes molybdenum useful in biologically important chemical reactions. Specifically, molybdenum is an important redox-active (oxidation/reduction) metal under physiological conditions, easily undergoing redox reactions between the +IV, +V and +VI oxidation states, allowing for molybdenum to act as an oxidant and a reductant for both obligatory one- and two-electron systems (Hille R., 2002). The molybdenum-containing proteins participate in oxidation-reduction reactions with a wide variety of electron donors and acceptors depending on the protein, the cellular localization and the organism. Some molybdenum-dependent proteins can catalyze carbon hydroxylation reactions while others are involved in more simple oxygen
atom transfers reactions (Hille R., 2005). Also, molybdenum is most often found covalently linked to one or two pyranopterin cofactors which provide an appropriate coordination and electrochemical environment for catalysis (Mendel & Bittner, Cell Biology of Molybdenum, 2006). The only known exception is the MoFe$_7$ center of nitrogenase, the enzyme responsible for the formation of bio-available ammonia from nitrogen (N$_2$) (Figure 1.1A) (Schwarz, Mendel, & Ribbe, Molybdenum cofactors, enzymes and pathways, 2009).

The general structure of the pyranopterin cofactor remains the same throughout all molybdenum enzymes (Figure 1.1B) (Mendel, Smith, Marquet, & Warren, 2007), which consists of a pyran ring linked to a pteridine ring system to give a tricyclic structure. Molybdenum is coordinated to the cofactor through a dithiolene bond (Burgmayer, Pearsall, Blaney, Moore, & Sauk-Schubert, 2004, pp. 59-66). The cofactors, frequently called molybdopterin (MPT) in the literature are frequently extended as a dinucleotide of an adenosine, guanosine, cytosine, or inosine in bacterial systems, whereas eukaryotic molybdenum proteins only have a phosphate as shown (Hille, Retey, Bartlewski-Hof, Reichenbecher, & Schink, 1999) (Figures 1.1B and 1.1C).

There can also be variations as to the groups coordinated to the molybdenum in that there can be multiple combinations of oxo-, sulfo-, or hydroxyl groups along with the possibility of protein coordination through side chain sulfur, selenium or seleno-cysteine bonds. Finally, there can be one or two cofactors coordinated to the molybdenum, as noted above (Figure 1.1C). In any specific protein, the cofactor is typically found with many hydrogen bonds and polar coordination bonds formed between the many charged
species on the cofactors and the side chains of the peptide backbone (Figure 1.2) (Burgmayer, Pearsall, Blaney, Moore, & Sauk-Schubert, 2004). This ultimately leads to a positionally-fixed pyranopterin cofactor with its coordinated molybdenum held firmly in place relative to the active site.

Figure 1.1. The molybdenum cofactors. A. The MoFe$_7$ cofactor from nitrogenase. B. The pyranopterin cofactor from xanthine oxidase and sulfite oxidase families. The molybdenum is coordinated as an LMoOS(OH) in xanthine oxidase and LMoO$_2$(S-cys). C. The pyranopterin cofactor from the dimethyl sulfoxide reductase family. -R denotes either Adenosine, Guanosine, Cytosine or Inosine.
Functionally, it has been suggested that the cofactor modulates the reduction potential of the molybdenum in such a way that it is optimal for catalysis (Westcott, Gruhn, & Enemark, 1998). Additionally, it is believed that the cofactor may serve to facilitate electron transfer, passing electrons to or from the molybdenum and other redox-active centers. One notable, and well documented, example of this is seen in xanthine oxidase where it is believed that the cofactor assists in the transfer of electrons from the molybdenum to a nearby iron-sulfur center (Hille R., Molybdenum and Tungsten in Biology, 2002).

![Figure 1.2. Hydrogen bonding Interactions of the molybdenum cofactor. Shown is the molybdenum cofactor taken from the crystal structure of chicken sulfite oxidase (1SOX) (Kisker, et al., 1997). Hydrogen bonds calculated from the crystal structure are shown in black dashed lines.](image)

1.3 Pyranopterin cofactor synthesis and insertion

There are more than 80 known molybdenum-containing enzymes (Hille R., Molybdenum and Tungsten in Biology, 2002). These can be divided into the iron-
molybdenum center of nitrogenase and three different groups of pyranopterin-containing enzymes. Unlike the multinuclear molybdenum center of nitrogenase, all well-characterized molybdenum-pyranopterin enzymes are mononuclear, containing a single atom of molybdenum in each active site (the single exception is carbon monoxide dehydrogenase (CODH) which contains a molybdenum-copper complex (Dobbek, Gremer, Kiefersauer, Huber, & Meyer, 2002)). The mononuclear molybdenum enzymes are classified according to their cofactor coordination. Each family of molybdopterin proteins has a unique molybdenum coordination sphere. In all cases, the addition of at least one sulfur (or selenium, in the case of some DMSOR family members), either in the form of a sulfo-group, or a ligation through a cysteine or serine residue, is necessary for proper activity of the cofactor.

Each of the three molybdopterin families are easily distinguished from each other by the nature of the molybdenum coordination and the types of reactions catalyzed by the enzymes (Hille R., The mononuclear molybdenum enzymes, 1996). The xanthine oxidase family is named after its long-studied member, xanthine oxidase from bovine milk. The structure of the active site of this family of enzymes has an LMoVLS(OH) coordination in its oxidized state with a single pyranopterin ring system coordinated (designated L) (Figure 1.3A). The coordination sphere of the oxidized form has distorted square-pyramidal geometry with the molybdenum atom slightly out of the equatorial plane toward the apical position (Hille R., The mononuclear molybdenum enzymes, 1996). The sulfite oxidase family includes vertebrate sulfite oxidases, plant sulfite oxidases, bacterial sulfite dehydrogenases, plant nitrate reductase, and a few other
proteins that are structurally related to the other sulfite oxidases but remain functionally unique. Members of the sulfite oxidase family provide a conserved cysteine, in the active site of the apoprotein, thus allowing the cofactor to be ligated to the protein. The oxidized form has a single cofactor with an LMoVI02(S-cys) coordination (Figure 1.3B) with a distorted square-pyramidal geometry. The third family, the dimethyl sulfoxide reductase (DMSOR) family, has members with a variety of structures and functions, but all of these proteins have a single molybdenum coordinated to two equivalents of pyranopterin ring cofactors. They are generally oxotransferases with a molybdenum center having an L2MoVI0(X) coordination where X can be a serine, a cysteine, a selenocysteine, or a hydroxyl/water group (Figure 1.3C). In this case, the coordination sphere has a trigonal-prismatic geometry (Kaupp, 2004). As noted above, some organisms utilize tungsten instead of molybdenum. Tungsten-containing enzymes, such as formate dehydrogenase (FDH), most closely resemble members of the DMSOR family of molybdenum enzymes (Hille R., The mononuclear molybdenum enzymes, 1996) (Hille R., Molybdenum and Tungsten in Biology, 2002) (Andreesen & Makdessi, 2008). In fact, they utilize the exact same pyranopterin cofactor. Other tungsten-dependent proteins, such as aldehyde:ferredoxin oxidoreductase, form a separate family of tungsten-containing enzymes (Mukund & Adams, 1991).

The overall biosynthetic pathway for the pyranopterin cofactor is well conserved phylogenetically (Figure 1.4) and can be divided into four steps based on the formation of three intermediates: cyclic pyranopterin monophosphate (cPMP), metal binding pterin (MPT) and an Adenosine monophosphate (AMP) coordinated MPT (Schwarz, Mendel, &
Ribbe, Molybdenum cofactors, enzymes and pathways, 2009). This pathway has been characterized in detail in plants, bacteria and humans (Schwarz & Mendel, Molybdenum cofactor synthesis in molybdenum enzymes, 2006) (Schwarz, Hagedoorn, & Fischer, Molecular microbiology of heavy Metals, 2007) (Reiss & Johnson, 2003). In prokaryotes, a final step frequently involves the addition of a second nucleotide to the structure, as mentioned above.

The process starts with a molecule of guanosine triphosphate (GTP) being converted to cPMP (also known as precursor Z) (Hanzelmann & Schindelin, 2006). This reaction is catalyzed by two proteins. The first protein is MoaA (bacterial nomenclature will be used for the duration of the cofactor synthesis description as it is best characterized), which is responsible for the production of a radical S-Adenosyl methionine (SAM). MoaA utilizes two four-iron-four-sulfur clusters [4Fe-4S], located at either terminus, to move the reaction forward. The N-terminal cluster is involved in the production of the SAM radical species while the C-terminal cluster is utilized in substrate binding. The second protein, MoaC is a hexameric accessory protein that is involved in the release of pyrophosphate from GTP.

The next step in the pathway involves the conversion of cPMP to MPT (Gutzke, Fischer, Mendel, & Schwarz, 2001). This is accomplished through the addition of two sulfur atoms to the cPMP, forming the dithiolate portion of the ring system and is catalyzed by MPT synthase. This heterotetrameric protein has two small (MoaD) and two large (MoaE) subunits. The MoaD subunits carry a thiocarboxylate moiety, as the
sulfur source, with each subunit responsible for one sulfuration event. The homologous eukaryotic mechanism, as well as the proteins involved, has been examined (Schwarz G., Molybdenum cofactor biosynthesis and deficiency, 2005). The enzymes responsible for adding the sulfur to each subunits are MoeB which adenylylates MoaD, activating it for sulfide transfer, and cysteine desulfurase and rhodanase which act together to transfer the sulfur to the appropriate glycine residue (Schwarz, Mendel, & Ribbe, Molybdenum cofactors, enzymes and pathways, 2009).

At this point the pterin ring system is coordinated to a copper atom, which must ultimately be replaced with a molybdenum atom. In bacteria, this reaction occurs via a multistep process involving two separate proteins, MoeA and MogA. In plants and animals, this same reaction is performed by a single, two-domain protein, Cnx1 (in A. thaliana) and gephyrin (in humans), respectively. The first step in the reaction involves the addition of AMP to the MPT, forming a MPT-AMP intermediate (Bevers, Hagedoorn, Santamaria-Araujo, Magalon, Hagen, & Schwarz, 2008). This is performed by MogA (or the G- domain of plant and animal protein homolog). The next step is catalyzed by MoeA (or the E- domain of plant and animal protein homolog), in the presence of molybdate and divalent cations, and results in the hydrolysis of the MPT-AMP bond and displacement of the copper bound to the dithiolene bond by molybdenum to yield MPT. Recent work suggests that this molybdenum carries with it two oxo groups and one protonated hydroxyl group (Schwarz, Mendel, & Ribbe, Molybdenum cofactors, enzymes and pathways, 2009). Formation of a dinucleotide, from MPT, is catalyzed by proteins such as MobA which converts MPT to molybdenum-bis-MPT guanine...
dinucleotide (MGD) in the presence of GTP (Stevenson, Sargent, Buchanan, Palmer, & Lawson, 2000).

Figure 1.3. The molybdenum cofactors of the mononuclear molybdenum enzymes. A. The molybdenum cofactor of the Xanthine Oxidase Family. B. The molybdenum cofactor of the Sulfite Oxidase Family. C. A representative molybdenum cofactor of the DMSOR family where R can be adenine, guanine, cytosine, or inosine and X can be selenocysteine, serine, cysteine or a hydroxyl/water.
Figure 1.4: Synthesis of the molybdenum cofactor. Shown is a general representation of the overall pathway for the production of the molybdenum cofactor. This pathway is well conserved across all species for which it has been characterized. Bacterial nomenclature is used for the enzymes indicated.
Although the mature cofactor has now been synthesized, further modifications may be necessary depending on the nature of the protein into which the cofactor is being inserted. For those proteins belonging to the xanthine oxidase family, sulfuration of the molybdenum is required for activity, a reaction catalyzed by a specific sulfurase (Bittner, Oreb, & Mendel, 2001): Aba3 in plants, human molybdenum cofactor sulfurase (HMCS) in humans and XdhC in E. coli (Neumann, Schultz, Junemann, Stoklein, & Leimkuhler, 2006). The eukaryotic mechanism of sulfuration involves abstraction of a sulfur atom from a cysteine residue with the result being a persulfide cysteine intermediate. This occurs at an N-terminal NifS-like domain in a pyridoxal phosphate (PLP) dependent manner (Heidenreich, Wollers, Mendel, & Bittner, 2005). NifS is a PLP-dependent cysteine desulfurase that forms a protein bound cysteine persulfide with the sulfur eventually donated to NifU during the process of Iron-Sulfur cluster formation. (Smith, et al., 2005) (Johnson, Dean, Smith, & Johnson, 2005). Upon formation of a persulfide on a conserved cysteine in the N-Terminal domain of Aba3, the sulfur is transferred to a second cysteine in the C-Terminal domain and finally to the molybdenum cofactor (Wollers, et al., 2008). This human sulfurase is believed to act in the same manner (Schwarz, Mendel, & Ribbe, Molybdenum cofactors, enzymes and pathways, 2009).

The molybdenum cofactor is very unstable by itself and therefore requires transporter proteins to shuttle it from the point of synthesis to the point of insertion. To date, these chaperone proteins have been characterized for bacteria and archaea. In these systems, uptake of molybdenum into the cell and its subsequent transport is carried out by high-affinity ATP binding cassette (ABC) transporters (Schwarz, Mendel, & Ribbe,
Molybdenum cofactors, enzymes and pathways, 2009). Multiple proteins have been found for both algal and plant mechanisms of uptake and insertion, although it is not known how many other proteins may be involved (Sargent, 2007). Of the animal systems, no proteins have been identified as molybdenum cofactor transporters or insertion related proteins.

Insertion of the mature cofactor into the apoprotein is a poorly understood process that, in most cases, likely occurs before protein folding is completed due to the fact that the cofactors are usually embedded deep within the holoenzyme. For prokaryotes, and more so for eukaryotes, much is still unknown about the role the chaperone proteins play in cofactor insertion. It is believed, however, that this mechanism could be quite complex as the apoprotein must be protected from degradation while it is held in an open conformation so that cofactor can be inserted (Sargent, 2007). One notable exception to this complex system could be the sulfite oxidase family of proteins whose cofactor generally lies very near the surface of the molecule in an easily accessible portion of the protein. Little else is known about cofactor insertion into the sulfite oxidases, but it can be assumed that the conversion of the tri-oxo cofactor into a di-oxo cysteinyl ligand is a relatively simple reaction involving the loss of one of the oxygens as water (Llamas, Otte, Multhaup, Mendel, & Schwarz, 2006).

1.4 The mononuclear molybdenum enzymes

Perhaps the best studied of the molybdenum proteins are those of the xanthine oxidase family, especially xanthine oxidoreductase, whose discovery and characterization
as an aldehyde oxidase dates back nearly 100 years (Dixon & Thurlow, 1924). Members of this family most usually catalyze the hydroxylation of carbon centers, although, exceptions do exist (Hille R., Molybdenum and Tungsten in Biology, 2002). One such example is carbon monoxide dehydrogenase (CODH), a unique enzyme responsible for catalyzing the oxidation of CO to CO$_2$. Although clearly related to other family members on the basis of its overall protein architecture, the active site of this enzyme is unique in having a copper atom coordinated to the molybdenum. (Dobbek, Gremer, Meyer, & Huber, 1999) (Dobbek, Gremer, Kiefersauer, Huber, & Meyer, 2002). Most members of this family catalyze the hydroxylation of aromatic or aliphatic compounds utilizing water as a source of oxygen and generate, rather than consume, reducing equivalents in the process (Hille R., Molybdenum-containing Hydroxylases, 2005). Members of this family tend to have multiple redox-active centers. Each subunit of the homodimeric xanthine oxidase has a total of four centers: the molybdenum center, two [2Fe-2S] clusters and flavin adenine dinucleotide (FAD) (Figure 1.5A).

Humans possess two proteins from this family, xanthine oxidoreductase and several species of aldehyde oxidase (Hille R., Molybdenum-containing Hydroxylases, 2005). The xanthine oxidoreductase gene is generally expressed as a xanthine dehydrogenase under physiological conditions but, in mammals, the expressed protein can be converted to xanthine oxidase by reduction of cysteine residues creating disulfide bonds or through proteolytic cleavage (Okamoto, Matsumoto, Hille, Eger, Pai, & Nishino, 2004). Conversion to the oxidase form takes place under pathophysiological conditions such as ischemia reperfusion injury.
This major structural difference between these two forms is the position of a peptide loop (Loop A) relative to the FAD cofactor. In changing from the dehydrogenase to the oxidase form, this loop is partially displaced, not only blocking the NAD binding site, but also allowing oxygen to access the FAD (Enroth, Eger, Okamoto, Nishino, Nishino, & Pai, 2000). This loop movement is also accompanied by an electrostatic change in the localized environment of the FAD that results in a large reduction potential change for the redox-active portion of the cofactor (Hunt, Massey, Dunham, & Sands, 1993) (Nishino, Okamoto, Eger, Pai, & Nishino, 2008). The overall result of this movement is a change in reactivity toward the two physiological electron accepting substrates, NAD$^+$ and oxygen. The dehydrogenase form has very high reactivity toward NAD but shows limited activity with oxygen. The conversion to the oxidase form, because of the changes at the FAD site, results in a reversal of these activities with oxygen becoming the primary acceptor (Saito & Nishino, 1989).

The DMSOR family of molybdenum proteins is a very complex family in that its members have a great deal of variation in active site coordination, overall structure, composition of redox-active centers and reactions catalyzed. This diversity is likely due to the fact that this family is evolutionarily ancient as evidenced by the high sequence similarities throughout the archaeal and bacterial domains (Hille R., The mononuclear molybdenum enzymes, 1996). An example of this diversity is seen in the DMSO reductases from \textit{R. sphaeroides} and \textit{R. capsulatis}. In both cases, the protein is a soluble monomeric protein with the only redox-active center being the molybdenum center.
(Figure 1.5B). This same protein, however, from *E. coli*, is a heterotrimer with one subunit containing an iron-sulfur cluster (Rothery, Workun, & Weiner, 2008).

Despite the diversity among members of this family, these proteins typically catalyze oxygen atom transfer reactions with the protein alternating between an oxidized, mono-oxo Mo(VI) form and a reduced des-oxo Mo (IV) form. The distinguishing feature of this family is the coordination of the molybdenum to two equivalents of pyranopterin designated as P and Q on the basis of their disposition in the polypeptide. Crystallographic studies of DMSOR (from *Rhodobacter capsulatis* (Schindelin H., Kisker, Hilton, Rajagopalan, & Rees, 1996) and *Rhodobacter sphaeroides* (Schneider, Lowe, Huber, Schindelin, Kisker, & Knablein, 1996) (Bailey, McAlpine, McEwan, & Shaw, 1997)) have shown that the Q-pterin alternates between a typical dithiolene coordination and a detached form, where the cofactor is completely dissociated from the molybdenum, replaced by a second oxo group in the metal coordination sphere. This dissociation, which seems to be unique to the *Rhodobacter spp.* DMSORs, appears to be dependent on the oxidation state of the molybdenum and is completely reversible through a complete reduction of the enzyme followed by reoxidation (Cobb, Condrads, & Hille, 2005).

The sulfite oxidase family consists of the eukaryotic oxotransferases, as well as some prokaryotic sulfite oxidizing proteins (Hille R., The mononuclear molybdenum enzymes, 1996). This family contains at least five types of proteins, three of which are generally capable of catalyzing the oxidation of sulfite to sulfate. The five types of
proteins are: sulfite oxidase from vertebrate sources (Figure 1.6A), plant sulfite oxidase (Figure 1.6B), bacterial sulfite dehydrogenases (Figure 1.6C), YedY from E. coli, and the assimilatory nitrate reductases that are ubiquitous among higher plants, algae, yeast and fungi. The reaction that occurs at the molybdenum cofactor is a straightforward oxygen atom transfer, and is very simple compared to the majority of reactions catalyzed by many xanthine oxidase and DMSOR family members (Hille R., The mononuclear molybdenum enzymes, 1996) (Hille R., 1994). As discussed previously, these proteins have a single molybdenum cofactor that is liganded to the protein through a highly-conserved cysteine residue.

The proteins of the sulfite oxidase family can have multiple redox-active centers, though some members have only the molybdenum cofactor (Figure 1.7). The homodimeric vertebrate sulfite oxidases not only have a molybdenum center in each monomer but also contain a $b_5$-type heme, which is used to shuttle electrons from the active site molybdenum to a terminal electron acceptor, typically cytochrome $c$ (Figure 1.7A) (Kessler & Rajagopalan, 1972). The heterodimeric bacterial sulfite dehydrogenases have, instead, a $c_{552}$ type heme subunit which serves the same purpose (Figure 1.7B) (Kappler & Bailey, 2005). The homodimeric nitrate reductases of this family contain three redox-active centers in each monomer: a molybdenum cofactor, a small $b$-type heme and an FAD domain that contains an NAD(P)$^+$ binding site (Figure 1.7C) (Kubo, Ogura, & Nakagawa, 1988). The simplest members of this family are the homodimeric plant sulfite oxidases which contain only a molybdenum center. (Figure 1.7D) (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003). The final type of
sulfite oxidase is YedY(Z), a heterodimeric protein of relatively unknown function. This protein, the first molybdenum-pyranopterin containing protein isolated from E. coli, is a soluble, periplasmic protein that strongly associated with a membrane-bound heme protein YedZ (Brokx, Rothery, Zhang, Ng, & Weiner, 2005). The sulfite oxidases, as with other proteins containing multiple redox-active centers, have their prosthetic groups located in separate, independently folding domains of the protein.

Only one protein from the sulfite oxidase family has been confirmed to exist in humans, sulfite oxidase itself. Xanthine oxidoreductase and aldehyde oxidase, both from the xanthine oxidase family, and a newly identified human molybdenum enzyme, mitochondrial amidoxime reducing component, designated hmARC (Gruenewald, et al., 2008), are the other known molybdenum enzymes in humans. Although it is unknown to which family mARC belongs, due to its size (~35kDA), it is more likely that it belongs to the sulfite oxidase family than to the xanthine oxidase family. This mARC catalyzes the reduction of N-hydroxylated compounds in a reaction that is dependent upon not just mARC but also NADH cytochrome b$_5$ reductase and cytochrome b$_5$. Two forms have been found in humans, hmARC1 and hmARC2, though only hmARC1 has been characterized to any extent. As of this report, it is unknown what, if any, the catalytic differences are between these two forms of the protein. Preliminary work suggests that mARC may represent a new member of the sulfite oxidase family (unpublished data from Ralf R Mendel, Technical University of Braunschweig and Russ Hille, University of California, Riverside).
Figure 1.5. Crystal structure of xanthine oxidase and DMSOR. **A.** The crystal structure of xanthine oxidase (PDB 3ETR) (Pauff, Cao, & Hille, 2009). **B.** The crystal structure of DMSOR (PDB 1E18) (Stewart, Bailey, Bennett, Charnock, Garner, & McAlpine, 2000).
Figure 1.6. Crystal structures of the sulfite oxidases. A. Crystal structure of chicken sulfite oxidase (PDB 1SOX) (Kisker, et al., 1997). B. Crystal structure of plant sulfite oxidase from A. thaliana (PDB 1OGP) (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003). C. Crystal structure of sulfite dehydrogenase from Starkeya novella (2CA3) (Bailey, Rapson, Johnson-Winters, Astashkin, Enemark, & Kappler, 2009)
Figure 1.7. Diagram of domain arrangement in the Sulfite Oxidase Family. The arrangements shown are N-terminal to C-terminal A. The domain arrangement of vertebrate sulfite oxidases with the heme domain and molybdenum cofactor domains indicated. B. Bacterial sulfite dehydrogenases are heterodimers made of a single-domain molybdenum cofactor containing protein and a c-type cytochrome subunit. C. The assimilatory nitrate reductases have four domains: one containing a molybdenum cofactor, a dimerization domain, a $b_5$-type heme domain, and an FAD containing domain. D. Plant sulfite oxidases have only a molybdenum cofactor containing domain and a dimerization domain.
1.5 The mononuclear molybdenum enzymes and human pathology

The human molybdenum enzymes have been of great interest for some time to the health care community. The wide diversity of substrates of these proteins has led many to believe that these proteins play an important role in detoxification in the human body. Xanthine oxidoreductase has been of great interest since it causes the most common of the molybdoenzyme-related disease, hyperuricemia (the disorders of xanthine oxidoreductase are summarized in Table 1.1) (Kasper, Braunwald, Fauci, Hauser, Longo, & Jaminson, 2008). Xanthine oxidoreductase is responsible for the final two steps in purine metabolism with the result being the production of uric acid (Hille R., Molybdenum-containing Hydroxylases, 2005) (Hille R., 2006). If too much uric acid is produced or the uric acid is not excreted fast enough (due to renal disorders), uric acid can build up and crystallize in the joints and extremities, leading to severe rheumatic problems such as gout and gouty arthritis (Falasca, 2006). The drug allopurinol, one of the most widely prescribed drugs in the US today, is used to treat hyperuricemia. Its development and use as an xanthine oxidoreductase inhibitor led to the Nobel Prize in Physiology or Medicine being awarded to Sir James W. Black, Gertrude Elion and George Hitchings in 1988. Xanthine oxidoreductase has also been linked to ischemia reperfusion injury by studies that suggesting that an increased conversion of xanthine dehydrogenase to xanthine oxidase leads to increased levels of reactive oxygen species that can subsequently damage tissue and/or slow the healing process (Nishino T., The conversion of xanthine dehydrogenase to xanthine oxidase and the role of the enzyme in
reperfusion injury, 1994) (Hille & Nishino, Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase, 1995).

There are several forms of disease associated with mutations in the genes of molybdenum proteins, including three that are associated with mutations in the proteins responsible for cofactor assembly and insertion. The earliest conditions of this type that were described were the xanthinurias (Raivio, Saksela, & Lapatto, 2001). The first type, called type I Xanthinuria, is caused by a mutation in the xanthine oxidoreductase gene itself that results in a partial or total loss of xanthine dehydrogenase and xanthine oxidase activity. The condition is mild and usually asymptomatic. The second type, called type II Xanthinuria, is caused by mutations in the gene for the protein sulfurase that is responsible for modifying the cofactor for insertion into xanthine oxidase and aldehyde oxidase (Figure 3), and results in loss of activity in both enzymes. Again, somewhat surprisingly, loss or decrease in the function of these two proteins is asymptomatic in most cases.

The third type of disorder, called isolated sulfite oxidase deficiency (ISOD), is due to mutations in the gene coding for sulfite oxidase and results in a decrease in, or loss of, sulfite oxidase activity (Garrett R. M., Johnson, Graf, Feigenbaum, & Rajagopalan, 1998). The phenotype for this condition is severe with extensive neurological effects, especially in the brain and central nervous system, typically resulting in early childhood death (Raivio, Saksela, & Lapatto, 2001). Magnetic Resonance Imaging data has shown severe damage of cortical neurons and dramatic atrophy of white matter in the brain. The
disease is very rare with about a thousand cases or less estimated over the last half century and displays an autosomal inheritance pattern (Gumus, et al., 2010).

Table 1.1: Human Disorders of the Molybdenum Containing Enzymes

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Cause</th>
<th>Result</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperuricemia (gout)</td>
<td>Over-active xanthine oxidase/renal disorders</td>
<td>Over-production of uric acid</td>
<td>Uric acid crystal buildup in joints and extremities</td>
</tr>
<tr>
<td>Ischemia reperfusion injury</td>
<td>Conversion of XDH to XO</td>
<td>Production of Reactive oxygen species</td>
<td>Increased cellular damage/Decreased healing capability</td>
</tr>
<tr>
<td>Xanthinuria Type I</td>
<td>Mutation in XOR gene</td>
<td>Increased substrate concentration (hypoxanthine, xanthine)</td>
<td>Usually asymptomatic</td>
</tr>
<tr>
<td>Xanthinuria Type II</td>
<td>Mutation in Sulferase protein (see Figure 3)</td>
<td>Increased substrate concentration (hypoxanthine and xanthine)</td>
<td>Usually asymptomatic</td>
</tr>
<tr>
<td>Isolated Sulfite Oxidase Deficiency (ISOD)</td>
<td>Mutations in SUOX (sulfite oxidase) gene</td>
<td>Increased concentration of sulfite and S-sulfocysteine/Decreased concentration of sulfate and sulfatide</td>
<td>Seizures, retarded CNS development, white matter atrophy, mental retardation, developmental delays, death</td>
</tr>
<tr>
<td>Combined Molybdenum Cofactor Deficiency (MoCD)</td>
<td>Type A: mutations in MOCS 1A and 1B (moaA and moaC)</td>
<td>Type A: decreased cPMP levels/decreased cofactor levels</td>
<td>Symptoms are a combination of those seen with ISOD and Xanthinuria.</td>
</tr>
<tr>
<td></td>
<td>Type B: mutations in MOCS 2A and 2B (moaD and moaE)</td>
<td>Type B: increased cPMP levels/decreased cofactor levels</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type C mutations in GephyrinG or Gephyrin E (moeA and mogA)</td>
<td>Type C: decreased cofactor levels</td>
<td></td>
</tr>
</tbody>
</table>
The severe phenotype associated with this disease is due to a buildup of toxic sulfite and a loss of sulfate that are necessary for the production of the sulfatide lipids used in the myelin sheath of neurons (Tan, et al., 2005). The buildup of sulfite happens rapidly since sulfite oxidase catalyzes the last step sulfur compound catabolism, including the amino acids cysteine and methionine, ultimately converting sulfite to sulfate (Figure 1.8) (Schindelin, Kisker, & Rajagopalan, 2001) (Enemark & Cosper, Molybdenum enzymes and sulfur metabolism., 2002). The increased sulfite levels have been linked to impaired ATP synthesis (Zhang, vincent, Halliwell, & Wong, 2004). Additionally, sulfite has been shown to reduce cysteine to S-sulfocysteine which has been suggested as an agonist of glutamate receptors (Tan, et al., 2005). This could explain some of the other symptoms commonly seen with ISOD: seizures, convulsions and uncontrolled muscle contractions, disordered autonomic function, altered muscle tone, dysmorphic facial features and progressive cerebral palsy (Veldman, et al., 2010).

Unfortunately, *in utero* detection of this disease is unreliable since the mother typically provides enough sulfite oxidase activity to oxidize sulfite from the fetal circulation. This maternal effect can last well into the first months of the child’s life which often masks symptoms, thereby delaying diagnosis. Even if there were a set treatment, of which there is none, much damage is usually done by the time diagnoses are made. Any treatments devised have only worked on a case-to-case basis with no obvious connections between the different treatments and their effects. Ultimately, these children die very young, with very few living past three years of age. The lifespan of these
children largely depends on the severity of the phenotype, the age of detection and the effectiveness of any treatment.

The fourth and final condition related to molybdenum enzymes is combined molybdenum cofactor deficiency (MoCD). This condition results from mutations that hinder the cofactor synthesis steps common to all the enzyme families. Cofactor deficiency is further divided into three types based on which step in the cofactor synthesis has been affected (Figure 1.4): type A results from mutations in human MOCS1A and MOCS1B (moaA and moaC in bacteria), affecting the conversion of GTP to cPMP in the synthesis pathway, type B results from mutations in MOCS2A and MOCS2B (moaD and moaE in bacteria) leading to accumulation of cPMP due to hindered conversion to MPT, and type C, of which very few cases have been reported, is due to mutations in the human protein gephyrin (mogA and moeA in bacteria) which is responsible for converting MPT into the molybdenum cofactor (Schwarz, Mendel, & Ribbe, Molybdenum cofactors, enzymes and pathways, 2009). All three of these deficiency types have the same overall effect on the molybdenum enzymes since all three proteins require the molybdenum cofactor. All three of these types present with the same symptoms as ISOD, presumably due to the loss of sulfite oxidase function (Raivio, Saksela, & Lapatto, 2001).
Figure 1.8. Metabolism of sulfur containing amino acids. Shown here is a diagram of a simplified methionine and cysteine metabolism. Only the steps pertinent to the discussion of this paper are shown. Structures for each of the compounds are also shown.

As a result of the significant amount of research into cofactor synthesis and molybdenum enzyme associated disease, an early diagnosis was made on a child with Type A MoCD and a treatment plan developed, ultimately saving the child’s life (Veldman, et al., 2010). The child was started on an intravenous substitution therapy of cPMP at 36 days of age and immediately showed improvement in condition. Within weeks, the child’s molybdenum enzyme function was nearly normal and the harmful effects of the disease were halted if not reversed. While it is very unlikely that this child
will fully recover, this case gives hope that, upon development of a method of early
detection, molybdenum cofactor associated diseases may be treatable.
Chapter 2: Introduction to the sulfite oxidases

2.1 General introduction to the sulfite oxidases

There are three overall reaction types catalyzed by members of the sulfite oxidase family. The first involves the oxidation of sulfite to sulfate via the general reaction, shown in reaction (i), as catalyzed by the vertebrate sulfite oxidases, plant sulfite oxidases and bacterial sulfite dehydrogenases. The second is reduction of nitrate to nitrite, catalyzed by the assimilatory nitrate reductases, via the mechanism shown in reaction (ii). The third type of reaction catalyzed is exemplified by the Escherichia coli YedYZ system which shows limited activity towards sulfite as a substrate, instead reducing various S- and N- oxides (Loschi, et al., 2004) (Brokx, Rothery, Zhang, Ng, & Weiner, 2005). This last, recently discovered enzyme is interesting because it is the only known molybdenum protein in E. coli that utilizes the MPT (mononucleotide) form of the pyranopterin cofactor.

\[
\text{SO}_3^{2-} + \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + 2\text{H}^+ + 2e^- \quad (i)
\]

\[
\text{NO}_3^- + \text{NADH} + 2\text{H}^+ \rightarrow \text{NO}_2^- + \text{NAD}^+ + \text{H}_2\text{O} \quad (ii)
\]

As discussed briefly above, the structures of these enzymes can have some variability in oligomerization (monomeric, heterodimeric and homodimeric), the types of redox-active centers present (cyt\text{b}-type, cyt\text{c}-type, FAD) and also the order of the respective cofactor domains in the proteins (presumably due to gene shuffling in recent evolution). Nevertheless, the molybdenum-binding domains of these proteins are highly conserved with regard to protein fold (Workun, Moquin, Rothery, & Weiner, 2008). For
example, the molybdenum cofactor binding domains of YedY (sulfite oxidase from *E. coli*) and nitrate reductase (*Pichia angusta*) have high structural identity, with 69% of the Cα-carbons aligning, despite having only 11.5% sequence identity.

Table 2.1: Structural alignment data comparing several sulfite oxidase family members

<table>
<thead>
<tr>
<th>PDB accession number</th>
<th>Description</th>
<th>Resolution (Å)</th>
<th>RMSD&lt;sup&gt;a&lt;/sup&gt; (Å)</th>
<th>Number of overlapping C-α&lt;sup&gt;b&lt;/sup&gt; (no. of C- α in structure)</th>
<th>C- α&lt;sup&gt;b&lt;/sup&gt; Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1XDQ</td>
<td><em>E. coli</em> YedY</td>
<td>2.6</td>
<td>0</td>
<td>262 (262)</td>
<td>100</td>
</tr>
<tr>
<td>1OGP</td>
<td>A. <em>Thaliana</em> sulfite oxidase</td>
<td>2.6</td>
<td>2.02</td>
<td>185 (388)</td>
<td>71</td>
</tr>
<tr>
<td>2BLF</td>
<td><em>S. novella</em> SDH</td>
<td>1.8</td>
<td>1.85</td>
<td>173 (373)</td>
<td>66</td>
</tr>
<tr>
<td>2BII</td>
<td><em>P. angusta</em> nitrate reductase</td>
<td>1.7</td>
<td>2.00</td>
<td>182 (415)</td>
<td>69</td>
</tr>
<tr>
<td>1SOX</td>
<td><em>G gallus</em> sulfite oxidase</td>
<td>1.9</td>
<td>1.95</td>
<td>186 (466)</td>
<td>71</td>
</tr>
</tbody>
</table>

<sup>a</sup>The comparisons are made with respect to the YedY structure.

<sup>b</sup>The C-a identity is calculated by comparing only those carbons of the molybdenum containing domain and not of the entire protein.

This figure is adapted from the information in the work by Workun et al. (2008)

In the case of the vertebrate sulfite oxidases, there are three domains per monomer, as discussed above, one with the molybdenum center, a second dimerization domain and a third, cytochrome b containing domain with each monomer having a mass of 50-55 kDa (100-110 kDa for the dimer) (Figure 1.6A and 1.7A). The combined molybdenum-containing and dimerization domains are approximately 45kDa, and the heme-domain approximately 10kDa (MacLeod, Farkas, Fridovich, & Handler, 1961) (Cohen & Fridovich, 1971) (Cohen & Fridovich, 1971) (Rajagopalan & Kessler, 1972).
Primary sequence and crystallographic data show that the heme-domain is attached to the molybdenum domain through a short peptide linker of ten to fourteen amino acid residues (Kisker, et al., 1997).

There is some evidence to suggest that the heme domain may be highly mobile and that this mobility is required for catalysis. Crystal structures of chicken sulfite oxidase show the two heme domains of the dimeric protein in two different positions relative to their respective molybdenum-containing domain. The same structure shows that the iron of each heme domain is ~32Å from the molybdenum, a distance that is far too great for electron transfer at the reported rates of ~500s\(^{-1}\) for human sulfite oxidase and ~1400s\(^{-1}\) for chicken sulfite oxidase (Feng C., et al., Role of conserved tyrosine 343 in intramolecular electron transfer in human sulfite oxidase, 2003) (Kisker, et al., 1997) (Karakas, et al., 2005) (Page, Moser, Chen, & Dutton, 1999). It has also been shown that upon increasing the viscosity of the solution, electron transfer from the molybdenum to the heme is slowed down in a manner proportionate to the viscosity increase (Feng, Kedia, Hazzard, Hurley, Tollin, & Enemark, 2002). Together, the data suggests that after reduction of the heme, the heme domain swings away, to a proximal position, from the molybdenum cofactor so that it can pass its electron on to cytochrome c (Kessler & Rajagopalan, 1972). The heme domain orientation seen in the chicken sulfite oxidase structure was different from that seen in the bacterial SDH which showed the heme subunit strongly associated with the molybdenum subunit in such a way that the redox-active centers are within 16Å (Bailey & Kappler, 2005) (Figure 1.6C).
Bacterial sulfite dehydrogenases are believed to work in a generally similar manner as seen with the vertebrate forms of the protein where electrons are transferred out of the active site occurs to a separate heme domain (a separate \(c_{552}\)-type subunit in bacterial SDH) and then on to the terminal electron acceptor (Kappler, et al., 2000). The two subunits typically co-isolate as a single 45-50kDa protein, although it is unknown if the two subunits are permanently associated \textit{in vivo} or whether disassociation is necessary for completion of catalysis to occur. SDH is a soluble, periplasmic protein with strong structural homology, in the molybdenum subunit, to the molybdenum domains of the other sulfite oxidases. Like vertebrate sulfite oxidases, SDH has a solvent accessible tunnel leading directly to the active-site, however, this channel is partially obstructed by the associated heme subunit. Binding and orientation of substrate is presumed to be much the same as in the other sulfite oxidases due to sequence homology of the active site. The eventual destination of the electrons, just like with the vertebrate sulfite oxidases, is cytochrome \(c\) (Kappler & Bailey, 2005). Physiologically, however, while the vertebrate sulfite oxidases serve to remove sulfite as part of a detoxification process, the bacterial SDH catalyzes the oxidation of sulfite during chemolithotrophic growth when thiosulfate is utilized as an energy source (Aguey-Zinsou, Bernhardt, Kappler, & McEwan, 2003).

The most simple of the sulfite oxidases, is the enzyme from plants, such as \textit{Arabidopsis thaliana}, in which he molybdenum center is the sole redox-active cofactor (Figure 1.6B and 1.7D). These proteins are \(\sim\)43.3 kDa (for the monomer) and have very high sequence homology to the molybdenum cofactor domains of the vertebrate sulfite
oxidases (Eilers, et al., 2001). There is some disparity in the published literature as to whether this protein is monomeric or dimeric. FPLC size exclusion chromatography indicated that the majority of the protein purified as a monomer (Eilers, et al., 2001), although the protein may be dimeric in vivo since it has a well conserved dimerization domain like those seen in the other dimeric sulfite oxidases. Plant sulfite oxidases have been shown to be localized in the peroxisomes and, since it does not have a heme domain was originally thought to pass its electrons onto a b-type cytochrome within the peroxisomes (Nowak, et al., 2004) (Eilers, et al., 2001). The protein showed little reactivity, however, toward the horse heart cytochrome c- or b-type cytochrome from rat outer mitochondrial membrane as terminal electron acceptors (Hemann, et al., 2005) (Eilers, et al., 2001). On the basis of its crystal structure, substrate binding and orientation in the A. thaliana sulfite oxidase is nearly identical to that expected for the chicken form of the enzyme. A comparison of the crystal structures indicates that the active sites, of the two forms, are highly conserved (discussed in detail below) (Karakas, et al., 2005) (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003).

2.2 The active site of the sulfite oxidases

Overall, there exist only a dozen or so residues that are absolutely conserved across the sulfite oxidase family (38 residues if the E. coli protein, YedY, is not included in the analysis (Figure 2.1). As would be expected, the eukaryotic sulfite oxidases tend to have much higher sequence identity towards each other than toward their prokaryotic relatives (Table 2.2). Nowhere is this more obvious than in a comparison of the residues
that are involved in substrate binding and those involved in orienting the cofactor in the active site (Figure 2.2). Four residues are universally conserved across the family in the vicinity of the molybdenum center (within 5 Å), one of which is the cysteine (C98, A. thaliana enzyme) that directly coordinates the molybdenum to the protein. The importance of this residue will be discussed in more detail below. There are also three arginines (R51, R103, and R374, A. thaliana enzyme) near the active site that play a role in substrate binding and orientation. Additional residues exist that are well conserved among the eukaryotic proteins and have been shown to play a role in either substrate binding, catalysis, or both. These include a tryptophan, a tyrosine and a histidine (W117, Y241, and H53, respectively, A. thaliana enzyme).

The first suggestion of a coordinating cysteine residue came from extended x-ray absorption fine structure (EXAFS) studies which indicated that the molybdenum of rat liver sulfite oxidase was coordinated to two oxo groups as well as two or three sulfur groups (Cramer, Dori, & Gray, The molybdenum site of sulfite oxidase. Structural information from the x-ray absorption spectroscopy, 1979) (Cramer, Wahl, & Rajagopalan, Molybdenum sites of sulfite oxidase and xanthine dehydrogenase. A comparison by EXAFS., 1981). Sequence alignment studies confirmed the existence of a strictly conserved cysteine residue across the entire sulfite oxidase family (Barber & Neame, 1990). It was later shown, using site-directed mutagenesis, in which each cysteine was individually mutated to a serine, that a specific cysteine (C207 in rat sulfite oxidase for these experiments) (C207 in the human protein, C185 in the chicken protein and C98 in A. thaliana) was necessary for catalytic activity of the protein with the serine
mutant showing a 2000-fold decrease in specific activity (Garrett & Rajagopalan, Site-directed mutagenesis of recombinant sulfite oxidase: Identification of cysteine 207 as a ligand of molybdenum, 1996). Additionally, EXAFS studies of this mutant showed distinctly different structural parameters from those of the wild-type indicating that a trioxo-molybdenum center (LMoO$_3$) had been formed (George G. N., Garrett, Prince, & Rajagopalan, 1996). These results indicated that this cysteine residue was necessary for coordination of the molybdenum cofactor. Additional magnetic circular dichroism (MCD) spectroscopic studies of the cysteine to serine mutant showed that the cysteine ligand may also serve a role in poising the reduction potential of the molybdenum making electron transfer more efficient (Helton, Pacheco, McMaster, Enemark, & Kirk, 2000). Finally, confirmation of a cysteine-coordinated molybdenum came in the form of a solved crystal structure for chicken sulfite oxidase in which the presence of a cysteine-molybdenum coordination was undoubtedly present (Kisker, et al., 1997).

Perhaps the most studied active site residue is an arginine (R160 in the human protein, R138 in the chicken protein, R51 in the A. thaliana protein) (Figure 2.3). Interest in this residue arose from the identification of a sulfite oxidase deficient patient who expressed sulfite oxidase with a R160Q (arginine to glutamine) point mutation (Garrett R. M., Johnson, Graf, Feigenbaum, & Rajagopalan, 1998) (Johnson, et al., 2002) (Iam, et al., 2002). Kinetic studies with a recombinant mutant showed an increase in $K_m$ from 17$\mu$M to 17mM and a decrease in $k_{cat}$ from 16s$^{-1}$ to 2.4s$^{-1}$ with the overall effect of decreasing $k_{cat}/K_m$ by nearly 1000 fold, thus accounting for the lack of sulfite oxidase activity in the patient. Studies of the rate intramolecular electron transfer between the
molybdenum and the heme of the mutant enzyme, utilizing laser flash photolysis, showed that the electron transfer rate in the mutant is lowered by three orders of magnitude (411s$^{-1}$ in the wild type to 0.64s$^{-1}$ in the mutant form) relative to the wild-type protein (Feng C., et al., 2003). Density functional theory and x-ray crystallographic studies have shown the existence of a hexacoordinate molybdenum where this active site glutamine (160), instead of participating in substrate coordination and binding, is coordinated to the molybdenum at the second axial position opposite the apical oxo group (Doonan C. J., et al., 2007). These studies showed that only after reduction does the molybdenum resume its normal pentacoordinate form.

This additional ligand could explain the kinetic characteristics of this mutant since the increased electron density around the molybdenum could result in a decreased reduction potential. Another explanation is that since, according to Marcus theory, intramolecular electron transfer rates are directly related to reorganization energy, the need to rearrange the active site from a six- to a five-coordinate molybdenum would result in slowed electron transfer (Gray & Ellis, 1994). Additionally, based on studies of the arginine to lysine mutant where there was considerably less activity lost, it has been suggested that the loss of the positively charged arginine could alter the ability of the protein to properly bind and orient substrate in the active site (Garrett R. M., Johnson, Graf, Feigenbaum, & Rajagopalan, 1998). Alternatively, it has been suggested that it is not necessarily the loss R160, per se, but rather the conformational changes of other residues near the active site (notably R450 in the human protein, R428 in the chicken protein, and R374 in A. thaliana protein) that is the basis for loss of activity (Karakas, et
A conformational change in this second arginine was first noted upon comparison of the A. thaliana enzyme and chicken enzyme (product bound) crystal (Figure 2.4) (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003). A comparison of the crystal structures of resting state chicken sulfite oxidase (oxidized protein) with sulfate bound chicken sulfite oxidase (reduced protein) confirmed this conformational change as being potentially catalytically relevant (Karakas, et al., 2005).

Another mutant that has been extensively studied, although it has not been associated with an ISOD patient to date, is a tyrosine to phenylalanine mutant (Y343F in the human protein, Y322 in the chicken protein and Y241 in the A. thaliana protein) (Figure 2.5). This residue sits near the molybdenum in the active site and is within hydrogen-bonding distance of the equatorial oxygen of the cofactor. It has been suggested that this residue is involved in proton shuttling to and from the molybdenum during coupled electron proton transfer (CEPT) (Pacheco, Hazzard, Tollin, & Enemark, 1999). In this mechanism, the hydrophilic side chain of the tyrosine assists in attracting water (or hydroxy anions) to the molybdenum center where proton transfer from the equatorial oxygen, with an associated electron transfer, leads to reoxidation of the molybdenum.

This mutant showed only a small attenuation in $k_{cat}/K_m$, a 22-fold decrease, compared to the wild-type (from $1.5 \times 10^7 \text{M}^{-1}\text{s}^{-1}$ to $6.6 \times 10^{-5} \text{M}^{-1}\text{s}^{-1}$) (Feng C., et al., Role of conserved tyrosine 343 in intramolecular electron transfer in human sulfite oxidase, 2003). The intramolecular electron transfer rate, however, was only ten percent that of the wild-type protein, dropping from $411 \text{s}^{-1}$ to $46 \text{s}^{-1}$, suggesting that this tyrosine
contributes very little in substrate binding and orientation, acting more as an electron shuttle out of the active site.

Table 2.2: Sequence Identity of the Molybdenum Cofactor Domains of Representative Sulfite Oxidase Family Proteins

<table>
<thead>
<tr>
<th></th>
<th>PSOX</th>
<th>HSOX</th>
<th>MSOX</th>
<th>CSOX</th>
<th>PNR</th>
<th>SDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSOX</td>
<td>100</td>
<td>46</td>
<td>46</td>
<td>47</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>HSOX</td>
<td>46</td>
<td>100</td>
<td>89</td>
<td>68</td>
<td>37</td>
<td>32</td>
</tr>
<tr>
<td>MSOX</td>
<td>46</td>
<td>89</td>
<td>100</td>
<td>68</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>CSOX</td>
<td>47</td>
<td>68</td>
<td>68</td>
<td>100</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>PNR</td>
<td>30</td>
<td>37</td>
<td>37</td>
<td>38</td>
<td>100</td>
<td>23</td>
</tr>
<tr>
<td>SDH</td>
<td>28</td>
<td>32</td>
<td>31</td>
<td>30</td>
<td>23</td>
<td>100</td>
</tr>
</tbody>
</table>

Shown are the percents of amino acid sequence identity of representative sulfite oxidase family proteins. Only the molybdenum cofactor binding domains were used for this calculation. The sequences used are those shown in Figure 2.1.

Two additional mutants, a glycine to aspartate mutant and an alanine to aspartate mutant, each identified in at least one ISOD patient (Johnson, et al., 2002) (Karakas & Kisker, 2005). The first mutant (G473D in the human protein, G451 in the chicken protein, and G375 in A. thaliana protein) showed a large effect on catalysis and substrate binding with $k_{\text{cat}}$ falling from 26.9 s$^{-1}$ in the wild type recombinant human enzyme to 0.54 s$^{-1}$ in the mutant form and $k_{\text{cat}}/K_m$ decreasing from $3.26 \times 10^6$ M$^{-1}$s$^{-1}$ to 265 M$^{-1}$s$^{-1}$ (Figure 2.6) (Feng C., et al., 2005). Sedimentation studies of this mutant indicated that this mutation may have an effect on dimerization of the protein (Wilson, Wilkinson, & Rajagopalan, 2006). The suggested reasoning for this was that introduction of a bulky, charged residue into this position resulted in changes of the local folding in or near the dimerization domain. Further mutational analysis, in the form of flash photolysis,
showed that perturbation of this glycine residue has effects on intramolecular electron transfer rates (411s\(^{-1}\) in the wild type to 0.17s\(^{-1}\) for the mutant). (Wilson, Wilkinson, & Rajagopalan, 2006).

The second mutation was A208D (A186 in the chicken protein and A99 in the A. thaliana protein) (Figure 2.7). This alanine lies near the active site, is thought to form part of a hydrophobic pocket which allows for proper orientation of the substrate relative to the molybdenum. Mutation of this alanine resulted in changes in \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) similar to those seen in the glycine mutant (26.9s\(^{-1}\) to 0.75s\(^{-1}\) and 3.26 x 10\(^6\) M\(^{-1}\)s\(^{-1}\) to 539 M\(^{-1}\)s\(^{-1}\), respectively). Additionally, intramolecular electron transfer was slowed 4000-fold to 0.10s\(^{-1}\) in the mutant (Feng C., et al., 2005).
Figure 2.1. Sequence alignment of the molybdenum domains of the sulfite oxidase family. Shown is a sequence alignment of the molybdenum domains of plant (*A. thaliana*), chicken, human and mouse sulfite oxidases as well as plant nitrate reductase (*P. angusta*) and bacterial sulfite dehydrogenase (*S. novella*). Strictly conserved residues are highlighted in red.
Figure 2.2. Active site overlay of chicken and plant sulfite oxidase. Shown is an overlay of significant active site residues and the molybdenum cofactor from plant sulfite oxidase (Green) and chicken sulfite oxidase (Grey). Residue numbers shown are for the plant sulfite oxidase with numbers for chicken sulfite oxidase in parentheses. The plant sulfite oxidase data is from the pdb 1OGP (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003). The chicken sulfite oxidase data is taken from the pdb 1SOX (Kisker, et al., 1997).
Figure 2.3. R138Q chicken sulfite oxidase point mutation. Shown here is the crystal structure of the active site of chicken sulfite oxidase. Because of the high sequence and structural identity between the vertebrate forms of the sulfite oxidases, the crystal structures of resting state chicken sulfite oxidases were used to represent the human mutant. The upper panel shows the wild-type active site of recombinant chicken sulfite oxidase (PDB 2A99) (Karakas, et al., 2005) while the lower panel shows the crystal structure of the active site from the R138Q mutant from recombinant chicken sulfite oxidase (PDB 2A9B) (Karakas, et al., 2005) with the glutamine indicated.
Figure 2.4. Conformation change of R374 (R450) in an overlay of crystal structures from chicken and plant sulfite oxidases. Shown is an overlay of significant active site residues and the molybdenum cofactor from plant sulfite oxidase (Green) and chicken sulfite oxidase (Grey). The arginine is labeled with its appropriate plant sulfite oxidase residue number and the chicken sulfite oxidase residue number in parentheses. The plant sulfite oxidase data is from the PDB 1OGP (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003). The chicken sulfite oxidase data is taken from the PDB 1SOX (Kisker, et al., 1997).
Figure 2.5. Active site residues of Y322F chicken sulfite oxidase point mutation. Shown here is the crystal structure of the active site of chicken sulfite oxidase. Because of the high sequence and structural identity between the vertebrate forms of the sulfite oxidases, the crystal structures of resting state chicken sulfite oxidases were used to represent the human mutant. The upper panel shows the wild-type active site of recombinant chicken sulfite oxidase (PDB 2A99) (Karaka, et al., 2005) while the lower panel shows the same crystal structure with the tyrosine to phenylalanine point mutant present.
Figure 2.6. Active site residues of G375A chicken sulfite oxidase point mutation. Shown here is the crystal structure of the active site of chicken sulfite oxidase. Because of the high sequence and structural identity between the vertebrate forms of the sulfite oxidases, the crystal structures of resting state chicken sulfite oxidases were used to represent the human mutant. The upper panel shows the wild-type active site of recombinant chicken sulfite oxidase (PDB 2A99) (Karakas, et al., 2005) while the lower panel shows the same crystal structure with the glycine to alanine point mutant present.
Figure 2.7. Active site residues of A186D chicken sulfite oxidase point mutation. Shown here is the crystal structure of the active site of chicken sulfite oxidase. Because of the high sequence and structural identity between the vertebrate forms of the sulfite oxidases, the crystal structures of resting state chicken sulfite oxidases were used to represent the human mutant. The upper panel shows the wild-type active site of recombinant chicken sulfite oxidase (PDB 2A99) (Karakas, et al., 2005) while the lower panel shows the same crystal structure with the alanine to aspartic acid point mutant present.
2.3 The catalytic mechanism of sulfite oxidation by sulfite oxidases

The generally accepted mechanism for the reaction catalyzed by the sulfite oxidases, shown in Figure 2.8, was originally proposed by Hille in 1994 (Hille R., 1994). A large solvent access channel allows for direct contact of the active site with the bulk solution. After entering the active site, proper orientation of the substrate is achieved via electrostatic interactions between the oxo groups of sulfite and the three active site arginines discussed previously. Once substrate is properly oriented in the active site, the sulfite lone pair is in position for attack at the equatorial oxygen of the molybdenum center (Figure 2.9A). Other possibilities for the mechanism, by which sulfite is coordinated to molybdenum, have been proposed. One such mechanism consists of a sulfite initiated, oxyanionic attack directly at the molybdenum followed by a conformation change resulting in sulfite bound at the equatorial oxygen (Figure 2.9B) (Pal, Chaudhury, & Sarkar, 2007). The formation of a hexacoordinate intermediate has also been proposed (Figure 2.9C) (Astashkin A. V., et al., 2008) (Doonan C. J., et al., 2008) while some evidence has also pointed to the possibility that the lone pair attack could take place at the axial oxygen (Figure 2.9D) (Astashkin, Feng, Raitsimring, & Enemark, 2005).

Despite the evidence that exists for these mechanisms, overwhelming evidence exists, in the form of theoretical studies and kinetic studies with the substrate analog dimethyl sulfite, supporting the lone pair attack mechanism. Theoretical studies utilizing a dioxo-molybdenum model compound showed that, in a reaction where the substrate
analog trimethylphosphine was utilized, it was necessary for the lone pair of the phosphine to attack the equatorial oxygen in order for catalysis to occur (Pietsch & Hall, 1996). Kinetic studies with chicken sulfite oxidase in the reaction with dimethylsulfite showed that, while $k_{\text{cat}}$ for the reaction went relatively unchanged ($79s^{-1}$ with sulfite and $89s^{-1}$ with dimethylsulfite), $K_m$ was increased from 28µM to 6.3mM, thus indicating that the oxyanionic groups of the sulfite were required only for substrate orientation and not for initiation of catalysis (Brody & Hille, 1995).

Figure 2.8. General mechanism for the oxidation of sulfite to sulfate as catalyzed by sulfite oxidases.

Once substrate has been properly oriented and the lone pair attack has occurred, a two electron reduction of the molybdenum center occurs subsequently changing the oxidation state of the molybdenum from +VI to a +IV. In the process of the molybdenum reduction, the sulfite is oxidized via an oxygen atom transfer. The final
product release involves a nucleophilic attack by either a hydroxyl group or a water molecule at the equatorial oxygen. This releases the product leaving the molybdenum in a reduced oxidation state. At this point, the similarity between the reaction mechanisms the plant and vertebrate sulfite oxidases ceases since their modes of reoxidation differ.

While the reductive half reaction of plant sulfite oxidase is well understood, important questions exist about the oxidative half-reaction. Since the plant sulfite oxidases lack a heme domain and it has been shown that they have very limited reactivity toward any cytochromes yet tested (Hemann, et al., 2005), initial studies focused on determining the physical electron acceptor for of A. thaliana sulfite oxidase. Given that the enzyme is localized to the peroxisomes (Nowak, et al., 2004), the possibility that this might be oxygen (O₂) was explored. Through a series of in vitro and in vivo studies, it was determined, not only that oxygen is being utilized as an effective electron acceptor, but that hydrogen peroxide is extensively generated in the course of the oxidative half reaction (Hansch, et al., 2006) (Hemann, et al., 2005).

2.4 The purpose of this work

Here we aim to examine the nature of the oxidative half-reaction of A. thaliana sulfite oxidase through a series of spectroscopic and kinetic studies. Utilizing continuous-wave and pulsed electron paramagnetic resonance in the presence of ³³S labeled sulfite, we have examined the electronic and coordination environment of a proposed sulfate bound intermediate that can be formed by a two electron reduction with sulfite followed by a one electron oxidation with ferricyanide. This species shows a
unique EPR signal with no nearby exchangeable protons observed, similar to the signals previously seen under anion inhibiting conditions with chicken sulfite oxidase. Analysis of previous data, combined with knowledge gained from the crystal structures of both plant and chicken sulfite oxidase has led to this proposed structure.

Utilizing rapid-reaction and steady-state kinetics, we have also explored the reaction kinetics of the oxidative half-reaction of A. thaliana sulfite oxidase in its reaction with oxygen. The thermodynamics of this reaction with oxygen have also been tested and appropriate constants determined. The oxidative half-reaction studies also include steady-state experiments which will allow us to determine the catalytic production of reactive oxygen species during turnover, specifically we have examined the rate of superoxide production at the active site of the protein.

Based on the results obtained, and combined with the conclusions from the pulsed EPR work, we have proposed a bifurcated oxidative half-reaction in which product can be released from the active site before or after the first electron transfer. Taking into account new findings, we also discuss the mechanistic implications of the unique ability of plant sulfite oxidase to utilize oxygen as a terminal acceptor.

Finally, we have performed steady-state experiments, similar to those performed with chicken sulfite oxidase, in which dimethyl sulfite is used as substrate. Here we sought to not only confirm that A. thaliana sulfite oxidase acts in the same manner as its vertebrate homologs, during its reaction with substrate, but also to address small concerns with the initial experiments. The pH and temperature dependence of this reaction has
also been examined in order to obtain more information about substrate orientation in the active site and the overall kinetics of the protein.

Figure 2.9. Proposed mechanisms for coordination of sulfite to the molybdenum cofactor during catalysis in sulfite oxidases. **A.** The generally accepted mechanism in which the lone pair electrons on sulfite attack at the equatorial oxygen. **B.** An oxyanionic attack directly at the molybdenum results in a hexacoordinated intermediate which immediately undergoes a conformational change in which the sulfite is transferred from a direct molybdenum coordination to a coordination with the equatorial oxygen. **C.** An oxyanionic attack at the unoccupied axial position results in a hexacoordinated octahedral intermediate which then undergoes a conformational change. **D.** The sulfite lone pair attacks at the axial oxygen.
Chapter 3: Evidence for a Sulfate-bound Mo(V) Species in the Reaction of Plant Sulfite Oxidase

3.1 Introduction

One feature of molybdenum enzymes that allows for easily performed studies of the electronic structure of the active site is the existence of a thermodynamically stable, EPR-active Mo(V) species. While sometimes difficult, proper interpretation of spectra obtained from these species can yield great insight into the coordination geometry of the molybdenum. EPR and related studies have been among the most useful and informative tools for studying the nature of the molybdenum enzymes. Such is the case for the sulfite oxidases, which have been studied extensively, utilizing both continuous wave and pulsed EPR techniques.

A variety of EPR signals can be obtained with sulfite oxidases depending upon pH, the method of reduction and the presence of anions (Kessler & Rajagopalan, 1972) (Bray, Gutteridge, T, & Wilkinson, 1983) (Gutteridge, Lamy, & Bray, 1980) (Astashkin, et al., 2005). For vertebrate sulfite oxidases, three major signal giving species have been characterized (Figure 3.1): a high pH form that is generated at pH 9.0 or higher, a low pH form generated at a pH below 7.0, and a phosphate (or arsenite) inhibited form generated at low pH (Bray, Gutteridge, T, & Wilkinson, 1983). The nature of the Mo(V) species generated, in these cases, was independent of the reductant utilized: sulfite, photochemical reduction or titanium citrate (Lamy, Gutteridge, & Bray, 1980) (Bray, Gutteridge, T, & Wilkinson, 1983) (Codd, Astashkin, Pacheco, Raitsimring, & Enemark,
These species have been further characterized by electron spin echo envelope modulation (ESEEM) and electron-nuclear double resonance spectroscopies (ENDOR) and structures have been suggested for each signal giving species based on this data and supporting evidence from extended X-ray absorption fine structure (EXAFS) spectroscopic studies (Figure 3.1) (Astashkin, Feng, Raitsimring, & Enemark, 2005) (George G. N., Garrett, Prince, & Rajagopalan, 1996) (Astashkin, Raitsimring, Feng, Johnson, Rajagopalan, & Enemark, 2002) (Astashkin, Mader, Pacheco, Enemark, & Raitsimring, 2000) (Raitsimring, Pacheco, & Enemark, ESEEM investigations of the high pH and low pH forms of chicken liver sulfite oxidase, 1998) (Pacheco, Basu, Borbat, Raitsimring, & Enemark, 1996).

![Figure 3.1. Predicted coordination of the Mo(V) centers from the sulfite oxidases.](image)

Shown are the predicted structures of the commonly obtained EPR active Mo(V) centers of the sulfite oxidizing enzymes. The conditions by which each species was obtained are listed under the structures.

These same three Mo(V) species are seen with plant sulfite oxidase but, unlike vertebrate sulfite oxidases, the method of reduction can result in differing species. Specifically, the one-electron reduction by titanium citrate results in a distinctly different signal than that seen when plant sulfite oxidase is reduced by sulfite (a two-electron reduction followed by a one-electron oxidation by potassium ferricyanide) (Codd,
Astashkin, Pacheco, Raitsimring, & Enemark, 2002). Reduction by titanium citrate resulted in a signal that showed a splitting pattern reflecting coupling to a proton, similar to that seen previously with vertebrate sulfite oxidase in low pH conditions (Bray, Gutteridge, T, & Wilkinson, 1983) (Astashkin, Raitsimring, Feng, Johnson, Rajagopalan, & Enemark, 2002). The conclusions drawn from these experiments were that the molybdenum was coordinated to a ligand containing an exchangeable proton, in this case a hydroxyl group, at the equatorial position (Mo-OH) (Figure 3.1). The nature of the sulfite reduced sample was different in that the signal obtained indicated that there were no nearby exchangeable protons (Codd, Astashkin, Pacheco, Raitsimring, & Enemark, 2002). The Mo(V) signal of this species was reported only once previously in a low pH form of chicken sulfite oxidase that had been produced in 4-morpholine-ethanesulfonic acid (MES) buffer. This species was termed an ‘atypical’ low pH form and evidence suggested that the lack of coordinated exchangeable protons was due sulfite bound at the Mo(V) center (Bray, Lamy, Gutteridge, & Wilkinson, 1982). The observation of a similar signal in plant sulfite oxidase has generated new interest in understanding this atypical species.

Initial studies of this plant sulfite oxidase signal led to the conclusion that this plant sulfite oxidase species must be of similar coordination (Figure 3.1) (Codd, Astashkin, Pacheco, Raitsimring, & Enemark, 2002) (Enemark, Astashkin, & Raitsimring, Investigation of the coordination structures of the molybdenum(v) sites of sulfite oxidizing enzymes by pulsed EPR spectroscopy, 2006). ENDOR and ESEEM studies of this species confirmed the lack of any coordinated exchangeable protons.
(Astashkin, et al., 2005) (Enemark, Astashkin, & Raitsimring, Investigation of the coordination structures of the molybdenum(v) sites of sulfite oxidizing enzymes by pulsed EPR spectroscopy, 2006) indicating a probable anion (sulfate) bound Mo(V) species

The formation of this sulfate-bound Mo(V) species has been proposed to be the result of a conformational change in the residue R374 (A. thaliana) that decreases solvent and substrate access to the active site of the protein (Fischer, Barbier, Hecht, Mendel, Campbell, & Schwarz, 2005) (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003) (Karakas, et al., 2005). The idea is that, when in the reduced state, the protein adopts a conformation with R374 projecting downward, into the active site, thereby trapping the sulfate in the active site. A subsequent one-electron reoxidation would result in a Mo(V) species but the product would still be bound to the molybdenum. The difference in conformation between the oxidized form and this so-called “blocked” form of the proteins can be seen in Figure 3.2.

Here we aim to examine this blocked form of the protein in order to determine if the sulfate is indeed coordinated to the molybdenum cofactor and, if possible, to determine the electronic nature of this coordination from the perspective of hyperfine and nuclear quadrupole coupling. To do this, we employ two pulse primary ESEEM and 2D-HYSCORE to observe the high resolution structure of this species. Since detection of $^{32}$S-sulfite is not possible by EPR, it is necessary to use an $^{33}$S-labeled sulfite to reduce the protein. This magnetically active sulfur atom has a nuclear spin of $I=3/2$ which
should result in measurable hyperfine and quadrupole interactions with the Mo(V) center if it is coordinated to the molybdenum.

Figure 3.2. Conformation change of R374 (R450) in an overlay of crystal structures from chicken and plant sulfite oxidases. Shown is an overlay of significant active site residues and the molybdenum cofactor from plant sulfite oxidase (Green) and chicken sulfite oxidase (Grey). Bound sulfate from the 1SOX structure is shown. The arginine is labeled with its appropriate plant sulfite oxidase residue number and the chicken sulfite oxidase residue number in parentheses. The plant sulfite oxidase data is from the PDB 1OGP (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003). The chicken sulfite oxidase data is taken from the PDB 1SOX (Kisker, et al., 1997).
3.2 Materials and Methods

Enzyme Preparation. Recombinant sulfite oxidase from *Arabidopsis thaliana* was purified by a modification of the procedure described by Eilers *et al* (Eilers, et al., 2001) and Hemann *et al* (Hemann, et al., 2005). *E. coli* cells (strain TP1000, ΔmobAB, possessing the pQE80-At-sox (*Arabidopsis thaliana* sulfite oxidase) plasmid; (Eilers, et al., 2001) (Temple, Graf, & Rajagopalan, 2000)) were grown aerobically, at 37°C, overnight and then diluted 1:50 into a modified 2xYT media (16 g tryptone, 10 g yeast extract, 5 g sodium chloride per liter) that was supplemented with 1.0-1.5 mM molybdic acid and 0.1 mM IPTG (isopropyl-β-thiogalactoside). Cultures were grown at 30°C for 24 hours and the cells harvested by centrifugation. The cells were then resuspended in lysis buffer (50 mM sodium phosphate, 30 mM sodium chloride, 10 mM imidazole, pH 8.0) and lysed using a French Pressure cell (Thermo Fisher). The lysate was loaded onto a Qiagen Ni-NTA column and washed with several bed volumes of wash buffer (lysis buffer with 20 mM imidazole). The protein was then eluted from the column with elution buffer (lysis buffer with 100 mM imidazole) and concentrated to approximately 1.0 mL. The protein was next loaded onto a Q-sepharose column that had been pre-equilibrated with tris-HCl (20 mM pH 8.0). The column was washed with the equilibration buffer and the eluate containing the holo-form of plant sulfite oxidase collected. The apo-protein (plant sulfite oxidase lacking the molybdenum cofactor) was retained on the column, and was subsequently eluted with the same buffer containing 500 mM sodium chloride. The holo- sulfite oxidase was concentrated to a volume less than 1.0 mL and passed through a Sephadex G-25 column equilibrated with Tris-OAc (20 mM pH 8.0 with 5 mM EDTA).
The enzyme was concentrated once more and its concentration determined using an extinction coefficient at 280 nm of 69.8 mM$^{-1}$cm$^{-1}$ (Eilers, et al., 2001).

_Prepitation of $^{33}$S-labeled sulfite_. To prepare Na$_2^{33}$SO$_3$, 5.0 mg of elemental sulfur ($^{33}$S, 99 atom %, Cambridge Isotope Laboratories) was carefully weighed into a 2.0 cm x 0.2 cm quartz tube. This was placed in a reaction vessel constructed from a quartz cylinder ($D = H = 3$ cm) with hemispherical caps. Each cap had a radius of 1.5 cm, and one cap was open to a cylindrical neck of 0.5 cm x 3.5 cm. The reaction vessel was fully evacuated and then filled with pure oxygen (0.3 atm). The neck was flame-sealed, and heated to 1100 $^\circ$C for two minutes in a preheated tube furnace, resulting in the complete combustion of the $^{33}$S and formation of $^{33}$SO$_2$ (99.9%, as confirmed by GC-MS). The reaction vessel was then removed from the furnace, allowed to cool to room temperature, and opened by cracking the neck. A rubber septum was immediately attached to the neck and a solution of sodium carbonate monohydrate (18.8 mg in 379 $\mu$L of nanopure water) was added as rapidly as possible to the reaction vessel in a single aliquot via syringe. The mixture was vortexed for 10 minutes. The resulting solution of 400 mM Na$_2^{33}$SO$_3$ was characterized using Quantofix® sulfite test strips and was stored at -30 $^\circ$C prior to use.

_EPR sample preparation_. Two samples of At-SO were prepared for EPR investigations from the same batch of protein. One was reduced by Na$_2^{33}$SO$_3$ and another by naturally abundant Na$_2^{32}$SO$_3$. These samples will be referred to as $^{33}$S-At-SO and $^{32}$S-At-SO, respectively. The procedure for sample preparation was strictly the same in both cases; the protein was reduced with a 30-fold excess of sodium sulfite and then re-
oxidized by about 1/2 equivalent of ferricyanide (per enzyme) to maximize the Mo(V) EPR signal. The samples were frozen in liquid nitrogen immediately after the addition of ferricyanide. The resulting concentration of Mo(V) estimated using CW EPR was about 0.4 mM.

**EPR methods.** Continuous wave (CW) X-band EPR measurements were performed on a Bruker ESP 300E spectrometer at 77 K. The electron spin echo envelope modulation (ESEEM) experiments were performed on a home-built K_a-band pulsed EPR spectrometer at a microwave (mw) operational frequency of about 29 GHz. Two types of ESEEM techniques were employed, two-pulse ESEEM and hyperfine sublevel correlation (HYSCORE) spectroscopy. Detailed experimental parameters are shown in the figure legends. In all pulsed EPR experiments the measurement temperature was about 20 K. The numerical simulations of the ESEEM spectra were performed using the program SimBud (see http://quiz2.chem.arizona.edu/epr for details).

### 3.3 Continuous Wave EPR

X-band CW EPR spectra were obtained for both $^{32}$S- and $^{33}$S-labled plant sulfite oxidase (Figure 3.3). The former spectrum matches the low pH, sulfite-reduced plant sulfite oxidase spectra that have been reported previously (Astashkin, et al., 2005) (Codd, Astashkin, Pacheco, Raitsimring, & Enemark, 2002) (Enemark, Astashkin, & Raitsimring, Investigation of the coordination structures of the molybdenum(v) sites of sulfite oxidizing enzymes by pulsed EPR spectroscopy, 2006). Comparing the spectrum obtained with $^{33}$S-sulfite, an obvious line-broadening is seen in $g_1$ relative to the $^{32}$S-
spectra which may be due to an unresolved $^{33}$S hyperfine coupling. The observation of a broadening, as opposed to a well-resolved splitting (in the case of a large hyperfine constant, in which case, four well resolved lines would be observed), indicates that the hyperfine coupling constant ($h_{fi}$) for $^{33}$S is small compared to the observed line widths. Unfortunately, orientation of the nuclear quadrupole interaction tensor with regards to the g-tensor can have a major effect on the extent of splitting seen in this case, meaning that quantitative calculations base on the EPR spectra are difficult and could have large errors at best.

Figure 3.3. CW EPR spectra of $lpH \ ^{32}$S-At-SO, top, and $^{33}$S-At-SO, bottom. Experimental conditions: $\nu_{mw} = 9.455$ GHz; mw power, 200 $\mu$W; modulation amplitude, 0.1 mT; temperature, 77K. The half-height linewidths at $g_1$ turning point are indicated.

Based on the CW-EPR data, the $h_{fi}$ must be less than 15MHz, consistent with estimated $h_{fi}$ constants based on known coupling information from $^{31}$P and $^{75}$As EPR studies performed on vertebrate sulfite oxidase (Astashkin A. V., et al., 2007) (Pacheco, Basu, Borbat, Raitsimring, & Enemark, 1996) (George G. N., Garrett, Graf, Prince, &
Rajagopalan, 1998). It is estimated that the isotropic \( hfi (a_{iso}) \) for \(^{33}\text{S}\)-sulfate could be 4-6 MHz and the anisotropic \( hfi (|T_{1,33S}|) \) could be 0.2-1.6 MHz.

It should be noted again, however, that orientation of the nuclear quadrupole interaction \((nqi)\) could have a great effect on these values, leading to an error of up to 50%. For this same reason, it is difficult to accurately estimate the \( hfi \) based simply on the broadening observed. Using second derivative analysis, however, it is possible to make qualitative estimates. We were able to do so by treating each turning point \((g_1, g_2, \text{ and } g_3)\) in the spectrum as a separate Gaussian line and utilizing the following equations:

\[
\Delta M = M_{^{33}\text{S}} - M_{^{32}\text{S}} \approx \frac{\Delta B_{^{33}\text{S}}^2 - \Delta B_{^{32}\text{S}}^2}{4} \approx 76 \text{MHz}^2 \quad (\text{eq i})
\]

\[
M = (5/4)A^2 \quad (\text{eq ii})
\]

\[
M = (1/2)A^2 \quad (\text{eq iii})
\]

Equation i represents change in second derivative at the low-field turning point \((g_1)\) while equations ii and iii represent the second derivative equations for an \( I=3/2 \) system with a strong and weak \( nqi \), respectively. At the low field turning point, utilizing these equations, it was estimated that the z-component \( hfi (A_z) \) could be 8-12MHz. Doing the same with the other two turning points \((g_3 \text{ and } g_2)\) gave values for \( A_x \) of 4-6MHz and \( A_y \) of 7-11MHz. From these values, the isotropic \( hfi (a_{iso}) \) can be estimated at 6-10MHz and the anisotropic at less than 4MHz. While the accuracy of these estimates is low, off by as much as 50% as stated earlier, they do confirm the existence of a non-zero \( hfi \) coupling constant whose value correlates well with estimates based on \(^{31}\text{P}\) and \(^{75}\text{As}##
data. These spectroscopic parameters can be better quantified by utilizing pulsed EPR and computer simulations as described below.

### 3.4 Two-Pulse ESEEM spectroscopy

In order to obtain more detailed information about the hfi and nqi parameters of the sulfate bound Mo(V) species, two-pulse electron spin echo envelope modulation (ESEEM) was performed on both the $^{32}\text{S}$- and $^{33}\text{S}$-sulfite reduced species. The basic idea is to use a two pulse (microwave) sequence (Figure 3.4A) to create measurable spin-packet rephasing (spin echo). To do this a $\pi/2$ microwave pulse is used to rotate the bulk magnetic vector associated with an ensemble of magnetic moments 90º off of the z-axis onto the y-axis (Figure 3.4B, first and second panel). Spin-packets of moments in identical environments will begin to relax from the bulk vector and will diffuse/de-phase in the xy-plane, due to spin-spin and spin-lattice relaxation (Figure 3.4B, second panel). After a period of time $= \tau$, a second microwave pulse ($\pi$) will rotate the y-component of these packets 180º and after a period of time equal to $\tau$ the spin-packets converge resulting in an echo (Figure 3.4B, fourth and fifth panel). By increasing the time ($\tau$) between the first and second pulse, the spin-packets are allowed to further relax resulting in decreased echo amplitude. This decrease is affected by the local magnetic environment, i.e. hyperfine coupling and quadrupole interactions, resulting in a modulation of the ESE amplitude decay. Proper treatment of these modulations, through Fourier Transforms, provides quantitative information about the hyperfine and quadrupole interaction.
Since the relative contributions of hfi and nqi depend on the magnetic field strength of the externally applied magnetic field, it is first necessary to determine the optimal field range for the electron spin echo (ESE) experiments (Figure 3.5). From this spectrum, it has been determined that the measurements for the ESE experiments should
take place at a field strength between 1045 and 1080 mT. Seven measurements were taken in this magnetic field range, including one at each of the three turning points.

A representative ESEEM spectrum is shown in Figure 3.6, which shows the normalized primary ESEEM traces for both $^{32}$S and $^{33}$S plant sulfite oxidase recorded at the low field turning point ($g_1$). A comparison of the signals obtained from the $^{32}$S (open circles) and $^{33}$S (solid line), clearly illustrates strong oscillations in the labeled sample. Similar spectra were observed at other magnetic field strengths (data not shown). Since the only difference between the two samples is the presence or absence of the $^{33}$S atom, it can be easily concluded that the difference in the spectra is due to the $hfi$ and/or $nqi$ of the $^{33}$S isotope.

![Figure 3.5. Two-pulse ESE field-sweep spectrum.](image)

**Figure 3.5. Two-pulse ESE field-sweep spectrum.** Experimental conditions: $v_{mw} = 29.458$ GHz; time interval between the mw pulses, $t = 250$ ns; mw pulses, $2\times13$ ns; temperature, 20 K.

Amplitude and cosine Fourier Transforms (FTs) of the time domain ESEEM traces from the $^{33}$S sample at each of the three turning points ($g_1$, $g_2$, $g_3$) were acquired
(Figure 3.8 and 3.9, respectively). For comparison, the signal for $^{32}$S plant sulfite oxidase is shown in Figure 3.8 (dashed line). From inspection of the amplitude traces, it is immediately evident that the presence of $^{33}$S results in strong amplitude increases in the range of 5-25 MHz. There is a persistent feature at approximately 18 MHz, which is about five times the Zeeman frequency of $^{33}$S (~3.5MHz) over the magnetic field range of this experiment. Since the nature of these experiments utilizes a field strength and microwave frequency that results in the Zeeman frequency interaction and $hfi$ essentially cancelling out, it can be concluded that these dominating amplitude features are due primarily to the $nqi$ of $^{33}$S.

![Normalized Primary ESEEM trace of $^{32}$S- and $^{33}$S-sulfite reduced plant sulfite oxidase at low field turning point ($g_1$).](image)

**Figure 3.6. Normalized Primary ESEEM trace of $^{32}$S- and $^{33}$S-sulfite reduced plant sulfite oxidase at low field turning point ($g_1$).** Open circles and the solid line show, respectively, the normalized primary ESEEM kinetics of $^{32}$S-At-SO and $^{33}$S-At-SO recorded at $g_1$. Experimental conditions: $\nu_{mw} = 29.458$ GHz; magnetic field, 1052 mT; mw pulses, 2×13 ns; temperature, 20 K.
Based on this conclusion, this 18MHz feature can be attributed to the interdoublet transitions of $^{33}$S as characterized by the following equation and is represented by the transition lines labeled ‘B’ in Figure 3.7:

$$\nu_{id} = \Delta E^Q \pm \frac{\nu^{1/2}}{2} \pm \frac{\nu^{3/2}}{2} = \Delta E^Q \pm c_{hq} \cdot (\nu, \pm A/2) \quad \text{(eq. iv)}$$

The fact that this feature exhibits little dependence upon orientation suggests that it corresponds to the electron spin manifold where the $^{33}$S Zeeman interaction is cancelled by the $hfi$. Because of this, the quadrupole coupling constant can be estimated as $e^2 Qq/h \sim 36$ MHz. Based on the low-field features of the spectral amplitudes and positions on $g_1$ and $g_3$, we were also able to estimate, albeit crudely, the $hfi$ for the Z and X components of the isotropic $hfi$ ($A_z$ and $A_x$, respectively). These estimates of 0-13 MHz for $A_z$ and 0-8MHz for $A_x$ overlap with the values estimated from the CW EPR line broadening (8-12 and 4-6 MHz, respectively). The complexity of the $g_2$ spectra makes any $nqi$ or $hfi$ estimates impossible since no separate lines can be easily distinguished.
Figure 3.7. Zeeman splitting diagram for an $M_s = 1/2 M_I = 3/2$ system (splitting not to scale). Shown are the hyperfine splitting that result due to the presence of a nuclear spin $I=3/2$ with the nuclear and electron spin manifolds indicated. 

- A. Representative intradoublet transitions. Either may occur in the 1/2 or -1/2 electron manifold.
- B. Representative interdoublet transition. Any of these can occur in either the 1/2 or -1/2 electron spin manifolds.
- C. The Zeeman splitting of the electron manifold. The dotted line represents the approximated splitting in the case of no nuclear spin.

3.5 Simulations from ESEEM data

Additional $nqi$ and $hfi$ information can be obtained from the amplitudes of the spectral lines utilizing numerical simulations. To simplify these simulations, a field-integrated ESEEM spectrum was used. This FI spectrum is a representation of the weighted sum of the ESEEM traces at several magnetic field positions. These spectra essentially simulate an isotropic g-factor, removing orientation effects, thereby
decreasing the complexity of the simulations (the Euler angles that describe the \( nqi \) of \( hfi \) tensors relative to the \( g \)-frame do not need to be factored into the simulations).

Figure 3.8. Amplitude FTs of two-pulse ESEEM traces recorded for \( lpH \) \(^{33}\)S-At-SO (solid traces) and \(^{32}\)S-At-SO (dashed traces) at the EPR turning points, as indicated by the labels “\( g_3 \)”, “\( g_2 \)” and “\( g_1 \)”. The bottom trace is the amplitude spectrum of the two-pulse FI ESEEM. Experimental conditions: \( \nu_{mw} = 29.458 \) GHz; \( mw \) pulses, 2×13 ns; time interval between the pulses, \( \tau = 250 \) ns; temperature, 20 K. The magnetic fields at \( g_1 \), \( g_2 \) and \( g_3 \) where the measurements were performed were, respectively, 1052 mT, 1067.2 mT and 1072.1 mT.

The lower traces of Figures 3.8 and 3.9 show the FI spectra for the amplitude and cosine FTs, respectively. Figures 3.10 through 3.13 compare these spectra (solid lines) with simulated spectra utilizing different sets of \( hfi \) and \( nqi \) parameters (dashed lines) as simulated by the Enemark EPR group at the University of Arizona using the program.
SimBud (http://quiz2.chem.arizona.edu/epr). The parameters used for each panel in these figures are described in tables 3.2 through 3.6 while table 3.1 serves as a legend for these parameters. It should be noted that the amplitudes of each panel within a single figure may be different to accommodate differing amplitudes of the calculated spectra. It thus becomes important to use the FI spectral intensity as a point of reference as its amplitude remains the same in all traces, only on a different scale.

Figure 3.10 shows the results of simulations representing various combinations of a weak anisotropic hfi and an axial nqi (Table 3.2 summarizes the parameters from each panel). The center panel represents the simulations that gave the best fit to the experimentally determined results. It can be concluded that changes in nqi (the third parameter) influence the position of the 18MHz spectral line, while the hfi constant (the first parameter) mostly affects the amplitude of the feature. These simulations confirm that the 18MHz line belongs to the interdoublet transitions of $^{33}\text{S}$ where the $^{33}\text{S}$ Zeeman interactions are essentially cancelled by the hfi.
Figure 3.9. Cosine FTs of two-pulse ESEEM traces recorded for \( lpH \) \(^{33}\text{S}-\text{At-SO} \) at the EPR turning points, as indicated by the labels “\( g_3 \)”, “\( g_2 \)” and “\( g_1 \)”. The bottom trace is the cosine spectrum of the two-pulse FI ESEEM. Experimental conditions: \( v_{mw} = 29.458 \) GHz; mw pulses, \( 2 \times 13 \) ns; time interval between the pulses, \( \tau = 250 \) ns; temperature, 20 K. The magnetic fields at \( g_1 \), \( g_2 \) and \( g_3 \) where the measurements were performed were, respectively, 1052 mT, 1067.2 mT and 1072.1 mT.
Table 3.1: Explanation of the numerical values given in Tables 3.2 through 3.5.

Each cell of these tables contains the spectroscopic and structural parameters in the following order. (note that $T_{33} = -(T_{11} + T_{22})$):

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_{iso}$</td>
<td>[MHz]</td>
</tr>
<tr>
<td>$hfi$</td>
<td></td>
</tr>
<tr>
<td>$(T_{11}, T_{22})$</td>
<td>[MHz]</td>
</tr>
<tr>
<td>(aniso. $hfi$)</td>
<td></td>
</tr>
<tr>
<td>$(e^2 Q q/h)$</td>
<td>[MHz], $\eta$</td>
</tr>
<tr>
<td>($nqi$ and asymmetry constant)</td>
<td></td>
</tr>
<tr>
<td>$(\theta, \varphi, \psi)$</td>
<td></td>
</tr>
<tr>
<td>(Euler angles for $nqi$)</td>
<td></td>
</tr>
</tbody>
</table>

The parameters of Figure 3.11 (summarized in Table 3.3) were designed to test the effects of the anisotropic $hfi$ and the orientation of the $nqi$ tensor (which was kept axial in this case) with respect to the $hfi$ tensor. Breaking down the effects of the parameters on each simulation led to the conclusion that an increase in the anisotropic $hfi$ results in an increase of the amplitude of the $\nu^{3/2}$ transition lines at the frequencies below 18MHz.
Figure 3.10. Simulated amplitude FT traces for anisotropic $hfi$ and an axial $nqi$. Solid trace in each panel is the amplitude FT of the two-pulse FI ESEEM reproduced from Figure 3.8. Dashed trace in each panel is the result of numerical simulations with the $hfi$ and $nqi$ parameters shown in Table 3.2. The positions of the cells in Table 3.2 correspond to positions of the panels in the Figure. The vertical scales of the panels in the Figure are generally different to accommodate both the simulated and experimental spectra.

Table 3.2: The simulation parameters for Figure 3.8

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>(-0.1, -0.1)</td>
<td>(35.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>3.6</td>
<td>(-0.1, -0.1)</td>
<td>(40.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>3.6</td>
<td>(-0.1, -0.1)</td>
<td>(45.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>4.6</td>
<td>(-0.1, -0.1)</td>
<td>(35.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>4.6</td>
<td>(-0.1, -0.1)</td>
<td>(40.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>4.6</td>
<td>(-0.1, -0.1)</td>
<td>(45.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>5.6</td>
<td>(-0.1, -0.1)</td>
<td>(35.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>5.6</td>
<td>(-0.1, -0.1)</td>
<td>(40.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>5.6</td>
<td>(-0.1, -0.1)</td>
<td>(45.5, 0)</td>
<td>(0°, 0°, 0°)</td>
</tr>
</tbody>
</table>
Figure 3.11. Simulated amplitude FT traces for varied anisotropic $h\tilde{f}$ and orientation of the $nq\bar{i}$ tensor (which was kept axial in this case) with respect to the $h\tilde{f}$ tensor. Solid trace in each panel is the amplitude FT of the two-pulse FI ESEEM reproduced from Figure 3.8. Dashed trace in each panel is the result of numerical simulations with the $h\tilde{f}$ and $nq\bar{i}$ parameters shown in Table 3.3. The positions of the cells in Table 3.3 correspond to positions of the panels in the Figure. The vertical scales of the panels in the Figure are generally different to accommodate both the simulated and experimental spectra.

Table 3.3: The simulation parameters for Figure 3.9

<table>
<thead>
<tr>
<th></th>
<th>4.8</th>
<th>4.0</th>
<th>3.6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.5, 0.5)</td>
<td>(-0.5, -0.5)</td>
<td>(-1, -1)</td>
</tr>
<tr>
<td></td>
<td>(40.5, 0)</td>
<td>(40.5, 0)</td>
<td>(40.5, 0)</td>
</tr>
<tr>
<td></td>
<td>(0°, 0°, 0°)</td>
<td>(0°, 0°, 0°)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>4.9</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>(0.5, 0.5)</td>
<td>(-0.1, -0.1)</td>
<td>(-0.5, -0.5)</td>
</tr>
<tr>
<td></td>
<td>(35.5, 0)</td>
<td>(40.5, 0)</td>
<td>(45.5, 0)</td>
</tr>
<tr>
<td></td>
<td>(90°, 0°, 0°)</td>
<td>(90°, 0°, 0°)</td>
<td>(90°, 0°, 0°)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>4.0</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>(-1, 0)</td>
<td>(-1, 0)</td>
<td>(-1.5, 0)</td>
</tr>
<tr>
<td></td>
<td>(41, 0)</td>
<td>(40.5, 0)</td>
<td>(40.0)</td>
</tr>
<tr>
<td></td>
<td>(90°, 90°, 0°)</td>
<td>(0°, 0°, 0°)</td>
<td>(0°, 0°, 0°)</td>
</tr>
</tbody>
</table>
The simulations in Figure 3.12 (parameters summarized in Table 3.4) are designed to perform essentially the same test as the previous figure except that the $nqi$ tensor is rhombic rather than axial. Similar conclusions can be drawn from the two sets of parameters, making it possible to narrow down the ranges for three of the parameters tested. The isotropic $hfi$ must be between 3.4 and 5.0 MHz, while the anisotropic $hfi$ must be between 2 and 3 MHz. The $nqi$ must be in the range of 33 to 41 MHz, although the simulations were not sensitive enough better define the $nqi$ asymmetry parameter, $\eta$. These simulations were also not sensitive enough to surmise limits on the $nqi$ or $hfi$ tensor orientations (Euler angles).

As a control, simulations were performed for the case of an uncoordinated sulfate (Figure 3.13, Table 3.5). For this case the isotropic $hfi$ was set at zero, while the molybdenum to $^{33}$S distance was assumed to be 3.1 angstroms (this scenario represents the smallest distance for an uncoordinated sulfate) which allowed for an estimation of the largest possible ESEEM amplitudes that could be achieved for an uncoordinated product. In this case, the simulations showed that, depending on the strength of the $nqi$, either the amplitudes, the locations, or both for the spectral features failed to reproduce the experimentally obtained spectra, providing further support for the conclusion that the sulfate is actually coordinated to the molybdenum.
Figure 3.12. Simulated amplitude FT traces for varied anisotropic $hfi$ and orientation of the $nqi$ tensor (which was kept rhombic in this case) with respect to the $hfi$ tensor. Solid trace in each panel is the amplitude FT of the two-pulse FI ESEEM reproduced from Figure 3.8. Dashed trace in each panel is the result of numerical simulations with the $hfi$ and $nqi$ parameters shown in Table 3.4. The positions of the cells in Table 3.4 correspond to positions of the panels in the Figure. The vertical scales of the panels in the Figure are generally different to accommodate both the simulated and experimental spectra.

Table 3.4: The simulation parameters for Figure 3.10

<table>
<thead>
<tr>
<th>4.7</th>
<th>4.1</th>
<th>4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.5, 0.5) (32, 1) (0°, 0°, 0°)</td>
<td>(-0.1, -0.1) (35, 1) (0°, 0°, 0°)</td>
<td>(-0.5, -0.5) (33, 1) (0°, 0°, 0°)</td>
</tr>
<tr>
<td>3.4</td>
<td>4.1</td>
<td>4.6</td>
</tr>
<tr>
<td>(0.5, 0.5) (35.5, 1) (90°, 0°, 0°)</td>
<td>(-0.1, -0.1) (35.5, 1) (90°, 0°, 0°)</td>
<td>(-0.5, -0.5) (35.5, 1) (90°, 0°, 0°)</td>
</tr>
<tr>
<td>3.9</td>
<td>3.9</td>
<td>5.1</td>
</tr>
<tr>
<td>(-1, 0) (35.1) (0°, 0°, 0°)</td>
<td>(-0.5, 0) (35, 1) (0°, 0°, 0°)</td>
<td>(-1, 0) (35, 1) (90°, 0°, 0°)</td>
</tr>
</tbody>
</table>
Figure 3.13. Simulated amplitude FT traces for an unbound, closely situated sulfite or sulfate. Solid trace in each panel is the amplitude FT of the two-pulse FI ESEEM reproduced from Figure 3.8. Dashed trace in each panel is the result of numerical simulations with the $hfi$ and $nqi$ parameters shown in Table 3.5. The positions of the cells in Table 3.5 correspond to positions of the panels in the Figure. The vertical scales of the panels in the Figure are generally different to accommodate both the simulated and experimental spectra.

Table 3.5: The simulation parameters for Figure 3.11

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.2, -0.2</td>
<td>-0.2, -0.2</td>
<td>-0.2, -0.2</td>
</tr>
<tr>
<td>(0, 0)</td>
<td>(5, 0)</td>
<td>(10, 0)</td>
</tr>
<tr>
<td>(0°, 0°, 0°)</td>
<td>(0°, 0°, 0°)</td>
<td>(0°, 0°, 0°)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-0.2, -0.2</td>
<td>-0.2, -0.2</td>
<td>-0.2, -0.2</td>
</tr>
<tr>
<td>(20, 0)</td>
<td>(40, 0)</td>
<td>(40, 0)</td>
</tr>
<tr>
<td>(0°, 0°, 0°)</td>
<td>(0°, 0°, 0°)</td>
<td>(90°, 0°, 0°)</td>
</tr>
</tbody>
</table>

3.6 Two-dimensional HYSCORE experiments and simulations

In order to obtain a better understanding of the relative orientations of the $hfi$ and $nqi$ tensors to each other and to the $g$-tensor, it was necessary to perform two-dimensional hyperfine sublevel correlation (2D-HYSCORE) experiments. The experiments and
simulations were performed by the Enemark EPR group at the University of Arizona.

2D-HYSCORE is based on the same principles as four-pulse ESEEM except the pulse sequence involves different pulses: $\pi/2$, $\pi/2$, $\pi$, and $\pi/2$. This technique is homologous to homonuclear correlation spectroscopy (COSY) in NMR, in that it is a correlation of nuclear spins that are being observed. The pulse sequence creates a nuclear coherence between the first two pulses and relaxation periods but then mixes the coherences between the electronic spin manifolds via the third pulse. By varying the time between the second and third pulse, as well as the third and fourth pulse, the echo modulation can be varied in two dimensions. A 2D-Fourier transform (Frequency domain) gives a plot such as that seen in Figure 3.14 where the Fourier Transform of each time dimension is on an axis. This allows for a separation of overlapping echo features in the form of cross-peaks which correlate to the different nuclear transitions between spin manifolds. The diagonal peaks represent an incomplete inversion of electron spins by the third pulse. From the position and amplitude of the cross-peaks, information can be obtained about the $hfi$ and $nqi$ for the system, although the use of simulations makes this process much easier and much more accurate.

Figure 3.14a, c, and e (involving experiments aligned with $g_1$, $g_2$, and $g_3$, respectively, from the CW-EPR spectrum) shows the frequency-domain correlation obtained with each spectrum representing the sum of three signals obtained at different intervals between the first and second mw pulse ($\tau=170, 200, \text{and } 240 \text{ ns}$). As the data for the (+ -) quadrant did not show any signal exceeding the noise amplitudes, only the (+ +) quadrant data is shown here.
There are two low-frequency cross-peaks in these spectra at each g-value: 3.5 and 10.5 MHz for \( g_1 \), 3.6 and 8.6 MHz for \( g_2 \) and 2.2 and 7.5 MHz for \( g_3 \). There is also an 18 MHz diagonal peak in all three spectra along with other low frequency diagonal lines: 9.4 MHz for \( g_1 \), 7.4 MHz for \( g_2 \) and 7.0 and 8.2 MHz for \( g_3 \). These can be assigned to intradoublet transitions and the approximate shape of the \( nqi \) tensor can be estimated based on the following equation (Figure 3.7A):

\[
\nu^{1/2,3/2} = c_{hq} \cdot (\nu, \pm A/2) \quad \text{(eq. v)}
\]

From this, the scaling factor, \( c_{hq} \), can be estimated for \( g_1 \) (1.64 MHz), \( g_2 \) (1.74 MHz) and \( g_3 \) (1.37 MHz) which indicate an approximately axial \( nqi \) tensor. Based on these values and the splitting obtained from the cross-peaks, \( hfi \) splitting can be estimated at 4.3, 2.9, and 3.9 MHz for \( g_1 \), \( g_2 \) and \( g_3 \), respectively. Since the sign of these values is unknown, it is necessary to use simulations to determine the most likely sign for each parameter. This led to four sets of parameters, as shown in Table 3.6, with isotropic and anisotropic \( hfi \) estimates based on the values found for \( A_z \), \( A_y \) and \( A_x \). The parameters in Set I most closely resemble the estimates from the ESEEM data and have therefore been used as a starting point for the simulations.
Figure 3.14. 2D-HYSCORE experiments and simulations for the $^{33}$S-sulfite reduced low pH form of plant sulfite oxidase. Panels a, c and e, the (++) quadrants of the HYSCORE spectra of $l_{pH}^{33}$S-At-SO obtained at the $g_1$, $g_2$ and $g_3$ EPR turning points, respectively ($B_0 = 1052$ mT, 1067.2 mT and 1072.1 mT, respectively). The spectra shown represent sums of the spectra obtained at time intervals between the first and second mw pulses $\tau = 170, 200$ and 240 ns. Other experimental conditions: $\nu_{mw} = 29.458$ GHz; mw pulses, 12, 12, 22 and 12 ns; temperature, 20 K. Panels b, d and f, simulated HYSCORE spectra for $g_1$, $g_2$ and $g_3$ at the EPR turning points, respectively. Simulation parameters: $a_{iso}$ = 3.3 MHz, anisotropic $hfi$ tensor in the principal axes system, $(T_{11}, T_{22}, T_{33}) = (1.3, 1.5, -2.8)$ MHz; $e^2Qq/h = 40$ MHz; $\eta = 0$; Euler angles for the orientation of the $hfi$ tensor in the g-frame: $\phi_h = 90^\circ$, $\theta_h = 90^\circ$, $\psi_h = 0^\circ$; Euler angles for the orientation of the $nqi$ tensor with respect to the g-frame: $\phi_q = 0^\circ$, $\theta_q = 75^\circ$, $\psi_q = 0^\circ$. The simulated spectra (as experimental ones) represent sums of the spectra calculated for $\tau = 170, 200$ and 240 ns.
Table 3.6: Possible sets of hfi constants $\mathbf{A} = (A_x, A_y, A_z)$ as estimated from analysis of HYSCORE spectra for use in the simulations shown in figure 3.14.

The isotropic hfi constants $a_{\text{iso}}$ and the anisotropic hfi constants $\mathbf{T} = (T_x, T_y, T_z)$ were estimated from $\mathbf{A}$. The sets differ by the choice of the relative signs of the different components of $\mathbf{A}$.

<table>
<thead>
<tr>
<th>Set No.</th>
<th>$(A_x, A_y, A_z)$ [MHz]</th>
<th>$a_{\text{iso}}$ [MHz]</th>
<th>$(T_x, T_y, T_z)$ [MHz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>±(3.9, 2.9, 4.3)</td>
<td>±3.7</td>
<td>±(0.2, -0.8, 0.6)</td>
</tr>
<tr>
<td>II</td>
<td>±(-3.9, 2.9, 4.3)</td>
<td>±1.1</td>
<td>±(-5.0, 1.8, 3.2)</td>
</tr>
<tr>
<td>III</td>
<td>±(3.9, -2.9, 4.3)</td>
<td>±1.8</td>
<td>±(2.1, -4.7, 2.5)</td>
</tr>
<tr>
<td>IV</td>
<td>±(3.9, 2.9, -4.3)</td>
<td>±0.8</td>
<td>±(3.1, 2.1, -5.13)</td>
</tr>
</tbody>
</table>

Simulations of the HYSCORE signals have been performed by the Enemark EPR group at University of Arizona (Figure 3.14 b, d, and f). These simulations showed reasonable agreement with experimental data when using $a_{\text{iso}} = 3.3$ MHz and an anisotropic hfi tensor with the principal axes system $(T_{11}, T_{22}, T_{33}) = (1.3, 1.5, -2.8)$ MHz. The quadrupole coupling constant $e^2 Q q / h = 40$ MHz agrees with the estimates made from the two-pulse ESEEM spectra, and the asymmetry parameter was found to be close to zero, $\eta < 0.3$ (near-axial $nqi$ tensor). The orientations of the hfi and $nqi$ tensors relative to the axis system for the $g$-tensor are described by the Euler angles $(\varphi_h, \theta_h, \psi_h) = (90^\circ, 90^\circ, 0^\circ)$ and $(\varphi_q, \theta_q, \psi_q) = (0^\circ, 75^\circ, 0^\circ)$, respectively. While these estimated parameters, particularly those of the hfi constants, lie slightly outside the limits predicted from the
ESEEM data, the generally good agreement of these two sets of data indicates that the HYSCORE hfi estimates are probably accurate.

3.7 Discussion

Using a previously described “blocked” form of plant sulfite oxidase, in which a conformational change in a single active site arginine traps sulfate in the active site, along with $^{33}$S labeled sulfite, we have shown here that there exists an intermediate, product-bound Mo(V) species that can be formed during catalytic turnover of the protein. The superhyperfine coupling between the $^{33}$S, with its nuclear spin of $I=3/2$, and the EPR-active Mo(V) center allows for analysis of this intermediate. Through continuous wave and pulsed EPR techniques, we are able to approximate not only the hyperfine coupling constant of this labeled sulfur, but also the $nqi$ between the two nuclei. We are also able to make general approximations of the hfi and nqi tensor orientations, relative to the g-tensor, utilizing 2D-HYSCORE spectroscopy. Finally, we are able to make use of ESEEM and HYSCORE simulations to not only corroborate our conclusions, but also to glean more information from the experimental data.

Information about the interaction between the sulfur and molybdenum nuclei has been obtained through primary ESEEM. The samples prepared with $^{33}$S labeled sulfite showed large amplitudes in the ESE oscillations, whereas only a small oscillation were obtained from samples prepared with $^{32}$S labeled sulfite. Since the only difference between the samples was the presence of the $^{33}$S sulfur, the differences between the ESE
spectra were attributed to either the hfi or nqi of the labeled sulfur in its interaction with molybdenum.

Analysis of amplitude and cosine Fourier Transforms of the ESEEM data has allowed us to see some obvious differences between the $^{33}$S and $^{32}$S prepared samples. Firstly, it became evident that the presence of $^{33}$S resulted in dramatic increases of amplitudes in the 5-25 MHz range. A recurring feature at approximately 18 MHz appears at all three turning points. Due to the nature of the experiments, the changes seen in the spectra have been attributed to the nqi of $^{33}$S with the specific 18 MHz feature attributed to the interdoublet transitions of $^{33}$S. Based on this data, an nqi of 36 MHz is estimated and values assigned for the z and x components of the hfi ($A_z$ and $A_x$ respectively). These values (0-13 and 0-8 MHz, respectively) are in good agreement with the values predicted from the CW-EPR experiments. From this we can conclude, at the least, that there is an interaction between the sulfur and molybdenum nuclei. Though this information is useful in establishing that there is an interaction between the molybdenum and the sulfur, little information has been gained on the specific nature of the interaction or the orientation of the nqi and hfi tensors relative to the g tensor.

To determine the nature of this interaction, it is necessary to perform simulations to determine exactly what type of results would be expected for various assumed structures for the signal giving species. Utilizing the nqi and hfi estimates obtained from the CW-EPR and ESEEM experiments as starting parameters, we set out to determine how the tensor orientation, nqi, hfi, and anisotropic hfi affected the signal obtained.
Looking at the simulations as a whole, we are able to conclude that the isotropic hfi must be between 3.4 and 5.0 MHz and the anisotropic hfi between 2 and 3 MHz while the nqi must be between 33 and 41 MHz. We are also able to simulate the conditions in which the sulfate is situated closely to the molybdenum, but is not coordinated, by simulating under the conditions of zero hfi. Under these conditions, it was impossible to obtain acceptable fits to the experimental spectra without forming a bond between sulfate and the molybdenum, thus allowing us to conclude that the line broadening seen in the CW-EPR and the amplitude changes in the ESEEM must be due to a product-bound Mo(V) species.

Unfortunately, these simulations were not sensitive enough to fully determine the tensor relationships of this system. 2D-HYSCORE experiments and simulations not only confirmed the conclusions drawn from the CW-EPR and ESEEM experiments, but also allowed for the estimation of some of the parameters describing the orientation of the tensors of the system including the scaling factor of the nqi $\eta < 0.3$ (near-axial nqi tensor) and the Euler angles describing the orientation of the hfi and nqi tensors respective to the g-tensor, $(\phi_h, \theta_h, \psi_h) = (90^\circ, 90^\circ, 0^\circ)$ and $(\phi_q, \theta_q, \psi_q) = (0^\circ, 75^\circ, 0^\circ)$, respectively.

Since we see similar estimates for hfi from continuous wave and pulsed EPR experiments, as well as from computational studies which confirm the accuracy of these experimental results, we can conclude that sulfate is bound to the molybdenum of this Mo(V) species. The somewhat large hfi of ~3MHz is close to the expected value base on estimates made from $^{31}$P and $^{75}$As bound forms of sulfite oxidase. This is the first time
that sulfate coordination has ever been directly observed in the sulfite oxidases and confirms previously stated theories that the blocked form of the protein represents a sulfate coordinated species (Astashkin, et al., 2005). The existence of this intermediate is also important for understanding the reaction mechanism of sulfite oxidizing enzymes in general and the information obtained allows for detailed comparison of the multiple Mo(V) signal giving species of the sulfite oxidizing proteins. We also show that $^{33}$S labeling paired with pulsed EPR techniques can be a very useful tool for examining metalloenzymes and that analysis of results is no more complicated than with other commonly used quadrupolar nuclei. This work has also paved the way for further studies, CW-EPR and ESEEM experiments of the same general setup, of several pathologically relevant sulfite oxidase point mutants.

Very recently, a Mo(V)-product complex, as reflected in low pH EPR signals in which no exchangeable protons are present, has been observed in several point mutants (Y343F (human) and R160Q (human) as well as R55Q (bacterial sulfite dehydrogenase) (homologous to the R160Q human mutant)) (Raitsimring, Astashkin, Feng, Wilson, Rajagopalan, & Enemark, 2008) (Astashkin A. V., et al., 2008) (Rapson, et al., 2010) in addition to the original observation in the chicken form (Bray, Lamy, Gutteridge, & Wilkinson, 1982). The nature of this signal suggests that these mutant forms of the protein may be in a sulfate-bound coordination similar to that which we discuss in this work.
All these mutants exhibited greatly reduced activity, if any, (Astashkin A. V., et al., 2008) (Rapson, et al., 2010) while the wild type plant protein is fully active under these conditions, with only the pH affecting the level of activity seen. It has been suggested, for both the R160Q (human) and R55Q (bacterial) mutants, that a sixth, weakly-coordinated ligand, residing in the second axial position and resulting in nearly octahedral coordination geometry may be present. The nature of this ligand has been studied by DFT, EXAFS, ESEEM and X-Ray crystallography (Astashkin, Klein, & Enemark, Toward modeling the high chloride, low pH form of sulfite oxidase: Ka-band ESEEM of equatorial chloro ligands in oxomolybdenum(V) complexes, 2007) (Astashkin A. V., et al., 2008) (Doonan C. J., et al., 2007) (Feng C. , et al., 2003) (Karakas, et al., 2005) (Klein, et al., 2009). Both the Y343F mutant (Raitsimring, Astashkin, Feng, Wilson, Rajagopalan, & Enemark, 2008) and wild type plant sulfite oxidase (this work) (Codd, Astashkin, Pacheco, Raitsimring, & Enemark, 2002) showed no evidence of altered coordination.

Recently, it has been shown that in the absence of any anion, other than substrat and product, wild-type human sulfite oxidase can generate this coordinated form as well (Rajapakshe, et al., 2010). In this work, it was found that addition of chloride leads to a loss of this signal, giving the normal low pH form with obvious proton splitting seen in the CW-EPR signals. Further testing has shown that chloride had similar effects on the R55Q bacterial mutant, the Y343F mutant and, to a much lesser extent, the wild type plant sulfite oxidase. The R160Q human mutant, however, showed no effect from chloride and remained in “blocked” form, thereby supporting previous conclusions that
this mutant is a stuck in this conformation irreversibly. This work demonstrates that under physiological conditions, where many anions would be present, it is unlikely that this sulfate-coordinated species would persist in vertebrate or bacterial forms. This also lends support to the idea that this sulfate-bound form is a catalytic dead-end for all but the plant form of sulfite oxidase. The fact that the plant form is resistant to chloride treatment indicates that this sulfate-bound species may be a normal intermediate in the catalytic mechanism.

It is interesting that plant sulfite oxidase can generate this product-bound species and remain active while the other forms of sulfite oxidase are quite literally “blocked” when in this product-bound conformation. From a structural standpoint, it is not immediately apparent what causes the plant form to so easily adopt this conformation, even in the presence of anions. It is possible that the plant form has avoided being “blocked” and is able to remain active due its ability to utilize oxygen as an electron acceptor. The nature of the electrochemical environment of the active site of the plant form is obviously different from that of the bacterial and vertebrate forms of the protein, due to its unique ability to readily utilize oxygen as a terminal electron acceptor (as well as its limited activity toward cytochromes as terminal acceptors). Because of this, it is possible that the molybdenum would still be able to pass electrons to oxygen when sulfate is bound, thereby allowing the protein to avoid this thermodynamic trap. Additionally, these same factors may allow for the native conformation of the blocking arginine to be adopted, even with sulfate still bound, thus allowing for product release and complete reoxidation of the molybdenum. It is, therefore, interesting that the same
structural and electronic differences that allow plant sulfite oxidase to utilize oxygen may also be responsible for keeping the protein from being trapped in the sulfate-bound form which it so readily forms.

The discovery of a product-bound Mo(V) intermediate is of general relevance for understanding the reaction mechanism of the sulfite oxidases. Based on this work, we can conclude that a Mo(V) sulfate-bound intermediate may be part of the normal catalytic cycle of plant sulfite oxidase since, unlike the vertebrate and bacterial forms, the plant form retains full catalytic activity. We propose that the plant form retains its activity because of its unique ability to utilize oxygen as an electron acceptor. Based on these conclusions, we propose that the oxidative half-reaction of plant sulfite oxidase may be a bifurcated reaction where product release could occur before or after the first electron transfer event.
Chapter 4: The production of superoxide during aerobic turnover of plant sulfite oxidase

4.1 Introduction

Vertebrate and plant sulfite oxidases, as well as bacterial sulfite dehydrogenases, all catalyze the oxidation of sulfite to sulfate as a part of sulfur metabolism, and are thought to do so in the same way (Feng, Tollin, & Enemark, Sulfite Oxidizing Enzymes, 2007). Vertebrate forms play an important role in the metabolism of sulfur-containing amino acids and lipids, catalyzing the final step prior to excretion of sulfate and serve a role in detoxification of potentially harmful sulfite ions. Bacterial forms are involved in sulfur assimilation under certain chemolithotrophic growth conditions (Aguey-Zinsou, Bernhardt, Kappler, & McEwan, 2003). It has been suggested that plant forms not only detoxify sulfite but also protect the plant from harmful sulfur dioxide (Eilers, et al., 2001) (Nowak, et al., 2004). While mutations resulting in a loss of sulfite oxidase activity are lethal in humans, plant knock-outs, while resulting in diminished vigor, are viable under normal growth conditions (Hansch, Lang, Rennenberg, & Mendel, 2007).

Understanding the oxidative and reductive half-reactions that make up the catalytic cycle of a redox active enzyme is essential in understanding the overall reaction of the enzyme. In the case of the sulfite oxidases, the general mechanism is shown in Figure 2.8. Catalysis has been studied in detail for both the vertebrate and bacterial forms of the protein with comparable rate constants found for each (Kappler U., et al., 2006). For the plant enzyme, it has been shown that the reductive half-reaction for the occurs much faster than that of vertebrate forms (>1000 s⁻¹ for plant sulfite oxidase and
183.9s\(^{-1}\) for chicken liver sulfite oxidase) (Hemann, et al., 2005) (Brody & Hille, The Kinetic Behavior of Chicken Liver Sulfite Oxidase, 1999). Despite the fact that the active sites of these two proteins exhibit a high degree of structural homology, it is thus evident that there are significant differences in the kinetic properties of the vertebrate and plant forms of the protein.

By contrast to the reductive half-reaction, the oxidative half-reactions of the sulfite oxidases are mechanistically quite different. Vertebrate forms have a cytochrome \(b_5\)-type heme domain which shuttles electrons to the terminal acceptor, cytochrome \(c\) (Kessler & Rajagopalan, 1972), while the bacterial forms have a cytochrome \(c_{552}\) heme-containing subunit that serves the same purpose (Kappler, et al., 2000). The plant sulfite oxidase, on the other hand, lacks any redox-active center, other than the molybdenum cofactor. Furthermore, while both vertebrate and bacterial forms have been shown to pass the reducing equivalents obtained from substrate on to the electron transport chain and are localized in the periplasm or intermembrane space of the mitochondria, plant sulfite oxidases have been shown to be localized to the peroxisomes and shows negligible reactivity toward cytochrome \(c\) as an electron acceptor (Nowak, et al., 2004) (Hemann, et al., 2005).

Recent studies have shown that plant sulfite oxidase utilizes molecular oxygen as its terminal electron acceptor (Nowak, et al., 2004) (Hansch, et al., 2006), with hydrogen peroxide being the ultimate product of the reaction. The vertebrate sulfite oxidases, by contrast, show only minimal reactivity toward oxygen (Cohen & Fridovich, 1971)
(Kessler & Rajagopalan, 1972). In vitro studies have suggested that plant sulfite oxidase may be involved in a regulatory pathway in peroxisomes in which the enzyme lowers the sulfite levels in the peroxisome, releasing catalase from inhibition by sulfite (Hansch, et al., 2006). It is also known that high levels of hydrogen peroxide are able to non-catalytically oxidize sulfite to sulfate.

Figure 4.1A shows the reaction mechanism of vertebrate enzyme starting from the reaction of fully oxidized protein with sulfite and subsequent reductions of the heme before passing electrons to the terminal acceptor, cytochrome c. This reaction, by necessity, occurs via two sequential one-electron transfer events. This general mechanism is common to the vertebrate sulfite oxidases and bacterial sulfite dehydrogenases. Figure 4.1B shows the proposed mechanism of plant sulfite oxidase leading into the present work in which the oxidized protein reacts with substrate resulting in two-electron reduced molybdenum. Following the release of sulfate, a two-electron reduction of molecular oxygen results in a reoxidized molybdenum and the production of hydrogen peroxide.

We have set out here to study the oxidative-half reaction of sulfite oxidase from A. thaliana, in detail, to gain a better understanding of the mechanism of the plant enzyme and its physiological role in the peroxisome. Pursuing preliminary results suggesting that hydrogen peroxide production may be occurring through a superoxide intermediate, we have explored this possibility through a series of rapid-reaction and
steady-state experiments, and present evidence that nearly the entirety of catalytic throughput generates superoxide, which only subsequently disproportionate to $\text{H}_2\text{O}_2$.

![Diagram of reaction mechanisms](image)

**Figure 4.1. The reaction mechanisms of vertebrate and plant sulfite oxidases.** A. The overall reaction mechanism of vertebrate sulfite oxidases (also bacterial sulfite dehydrogenases). B. The proposed reaction mechanism of *A. thaliana* sulfite oxidase.

### 4.2 Materials and Methods

*Enzyme preparation.* As described in chapter 3 (sec 3.2)
**Oxidative Half-Reaction Studies.** Rapid kinetic experiments were performed in 20 mM Tris-OAc buffer, pH 8.0. Plant sulfite oxidase (~20 μM) was placed in a tonometer fitted with a sidearm cuvette, made anaerobic by repeated cycles of evacuation and flushing with argon gas and reduced by titration with a ~1 mM sodium sulfite solution using a fitted Hamilton titration syringe, following the change in absorbance associated with reduction was monitored using a Hewlett Packard 8452A UV-visible spectrophotometer. Rapid-reaction kinetic experiments were performed using an Applied Photophysics Inc. SX-18MV stopped-flow instrument equipped with a diode array detector. The anaerobic, reduced enzyme was loaded into the system and allowed to equilibrate to 4°C. Oxygenated buffers were prepared by bubbling pure oxygen through buffer on ice for two hours to yield a saturated oxygen solution of 2.05 mM. Anaerobic buffer was mixed with oxygen-saturated buffer using a gas tight Hamilton syringe to yield solutions of known oxygen concentration from 4 μM to 2.05 mM. The spectral changes at 360 and 480 nm were used to follow the reoxidation of enzyme, with rate constants obtained by fitting the transients to single- or double-exponentials using manufacturer-supplied software (the ProData Viewer package). This experiment was performed multiple times with at least three transients obtained at each oxygen concentration. The rate constants from each transient were determined and the average values, which correspond to the observed pseudo first-order rates, $k_{\text{obs}}$, were plotted against the oxygen concentration in order to obtain the second-order rate constant, $k_{\text{ox}}$.

Rapid-reaction studies were also performed in the presence of cytochrome $c$ in order to follow superoxide generation directly via its rapid reduction of the cytochrome.
These reactions were performed by reacting 1.5 µM plant sulfite oxidase (made anaerobic and reduced as described above) with oxidized cytochrome c (~38 µM) in an aerobic solution of 82, 205 and 532 µM O₂. The reduction of cytochrome c by enzyme-generated superoxide was followed by monitoring the increase of absorbance at 550 nm and quantified using an effective extinction coefficient of $\Delta \varepsilon = 19.8 \, \text{mM}^{-1}\text{cm}^{-1}$ which was calculated by dividing the published value of $\Delta \varepsilon = 21.0 \, \text{mM}^{-1}\text{cm}^{-1}$ (Massey, 1959) by a correction factor of 1.058 to account for the spectral resolution of our stopped-flow instrument. This factor was determined empirically from a comparison of the absorbance at 550 nm of a fully reduced sample of cytochrome c in a Cary 500 spectrophotometer at a 0.04 nm spectral resolution to the absorbance of the same sample in our HP8452A spectrophotometer (with a nominal resolution of 2.0 nm). The rate constants were again determined by plotting the absorbance change against time and fitting with a double exponential equation. As a control, the reaction in the presence of cytochrome c was also performed with added superoxide dismutase (200 U/mL) in order to determine the extent of any spectral change that was not dependent on superoxide. At least three transients from each of two separate experiments were used to determine the average value as reported.

**Oxygraph Studies** Steady-state experiments following O₂ consumption directly were performed using a Hansatech Oxygraph system (Oxygraph System from Hansatech Instruments Ltd, King's Lynn, UK (www.hansatech-instruments.com)) with data analysis performed using the manufacturer’s software. All experiments were performed at 4°C
under aerobic conditions with a stir bar spinning at 100 rpm. Sodium sulfite (1.0 mM in 20 mM Tris-OAc, pH 8.0) was added to the reaction chamber and the chamber volume was adjusted so that no air bubble remained. The solution was allowed to equilibrate to 4°C, at which point enzyme was added (60 nM final concentration) to start the reaction. In order to determine the optimal amount of SOD required, SOD was added in concentrations of 35, 70, 105, 140, 175, and 210 Units/mL. Based on these results, superoxide dismutase was subsequently added to final concentration of 200 Units/mL for all experiments, this having been empirically determined to be sufficient to ensure quantitative scavenging of superoxide. A Unit here is defined as the amount of superoxide dismutase required to inhibit the reduction of cytochrome c by 50% in the reaction of xanthine oxidase with xanthine, as described by McCord and Fridovich (McCord & Fridovich, Superoxide dismutase. An enzyme function for erythrocuprein (hemocuprein), 1969) (McCord, Crapo, & Fridovich, Superoxide-dismutase assays: A review of methodology in superoxide and superoxide dismutases, 1977). The initial rate of oxygen consumption was recorded for each reaction and a plot of the decrease in rate of oxygen consumption versus superoxide dismutase concentration was made.

In separate experiments, superoxide dismutase and cytochrome c were also added, as appropriate, to final concentrations of 200 Units/mL and 94.5 µM, respectively. Reactions were initiated by addition of enzyme at a final concentration of 60 nM or 1.6 µM (initial experiments only, data not shown). To determine the amount of superoxide generated, the following reactions were performed: (1) enzyme and substrate only; (2) enzyme and substrate with superoxide dismutase; and (3) enzyme and substrate with
cytochrome c. Initial rates were determined from the initial slopes of the observed time courses. Initial and final oxygen concentrations were also recorded. For each reaction, at least three repetitions were performed and the values averaged. These experiments were also performed multiple times to verify the reproducibility of the data. As a control, experiments were performed with xanthine oxidase, a known producer of superoxide, using xanthine as substrate. The reaction conditions for these experiments were: 7.8 nM functional xanthine oxidase, 100 µM xanthine, 0.1 M pyrophosphate buffer, pH 8.5, 4°C.

**Steady-State Cytochrome c Reduction Studies.** Steady-state kinetic experiments following cytochrome c reduction by sulfite oxidase generated superoxide were performed using a Hewlett-Packard 8452A UV-visible spectrophotometer. Experiments were performed at 4°C under aerobic conditions in 20 mM Tris-OAc buffer, pH 8.0. Enzyme was added (final concentration of 20 nM) along with cytochrome c (final concentration ~20 µM) and sulfite (1.0 mM). Initial rates were determined by following the absorbance change per unit time in the initial, linear phase of the reaction. The total amount of reduced cytochrome c was also determined from the total absorbance change for the reaction, using an empirically determined effective extinction change of $\Delta \varepsilon = 19.8$ mM$^{-1}$cm$^{-1}$. This same reaction was performed in the presence of superoxide dismutase (200 U/mL) to correct for any non superoxide-dependent spectral changes and the initial rates were observed as before. As stated previously for the oxygraph data, all data presented are the average of at least three trials and the experiments were repeated several times for accuracy and reproducibility. As previously described for the oxygraph studies, controls were performed using xanthine oxidase in reaction with xanthine.
Additional controls were performed to establish that sulfite did not directly reduce cytochrome \( c \) under the present reaction conditions, and similarly that hydrogen peroxide at the concentrations ultimately generated by dismutation of superoxide did not reoxidize cytochrome \( c \). All of these controls yielded negative results.

Other experiments were performed in such a way that allowed the observation of both the reduction of cytochrome \( c \) by superoxide and the effect of superoxide dismutase in the course of a single transient a single transient. This was achieved by setting up a stirrer in the spectrophotometer so that the reactants could be added directly to the reaction mix without removing the cuvette from the light path. The reaction was started first by adding sulfite to the desired concentration. Next, cytochrome \( c \) was added followed by the enzyme in 10- to 20-second intervals. Finally, superoxide dismutase was added and data collected for another 10-20 seconds.

Additional steady-state kinetic studies were performed in order to determine the effects of pH and temperature on superoxide production in plant sulfite oxidase. Reaction conditions were the same as for the above mentioned oxygraph experiments. The pH range studied was from 6.0 to 10.0 and consisted of the buffers bis-Tris (pH 6.0), bis-Tris-propane (pH 7.0), Tris (pH 8.0), and glycine (pH 9.0 and 10.0). All buffers were at a concentration of 20 mM. Temperature dependence of the reaction was monitored over the range of 4°C to 30°C.
4.3 Rapid-reaction kinetics of reduced enzyme with oxygen

Rapid-reaction kinetic studies were performed in order to determine the rate constants for the oxidative half-reaction of plant sulfite oxidase. After titration of the enzyme to its reduced form under anaerobic conditions with sulfite, it was mixed with buffers of various oxygen concentrations. Enzyme reoxidation was followed by monitoring the increase in absorbance at 360 and 480 nm, which correspond to the enedithiolate-to-molybdenum charge transfer and the cysteinethiol-to-molybdenum charge transfer, respectively (Garton, Garrett, Rajagopalan, & Johnson, 1997) (Figure 4.2). A plot of $k_{obs}$ versus $[O_2]$ gave a straight line passing through the origin, with a slope reflecting a second-order rate constant of $5.3 \pm 0.3 \times 10^4$ M$^{-1}$s$^{-1}$ (Figure 4.3). This result indicates that there is no formation of an E$\text{red}$$\cdot$O$_2$ Michaelis complex prior to oxidation of enzyme, and also that the reaction is irreversible (as expected, given the strong thermodynamic favorability of the reaction).

In order to determine whether superoxide is generated in the course of enzyme reoxidation, the above experiment was repeated in the presence ferricytochrome $c \pm$ superoxide dismutase. Biphasic transients were seen for the reaction of reduced enzyme with 532 µM O$_2$ (Figure 4.4, upper transient), with a fast phase occurring with a rate constant of $47.7 \pm 0.4$ s$^{-1}$ followed by a slower phase with one of $7.5 \pm 0.1$ s$^{-1}$ (Figure 4.4, upper transient). Addition of superoxide dismutase abolished cytochrome $c$ reduction, indicating that all reduction is due to superoxide production (Figure 5.4, lower transient).
The total absorbance change seen at 550 nm in this experiment was 0.056, and evenly split between the faster and slower kinetic phases (0.029 and 0.027, respectively). This reflects the reduction of approximately 2.82 μM cytochrome c, and given the enzyme concentration of 1.5 μM indicates that approximately 1.0 equivalent of superoxide is generated in each of the two kinetic phases seen in the cytochrome c reduction experiment. This implies a faster reoxidation of Mo(IV) to Mo(V), followed by a slower reoxidation of Mo(V) to Mo(VI), with each step yielding one equivalent of superoxide.

Figure 4.2 Oxidized and reduced absorbance spectra of A. thaliana sulfite oxidase. Upper- Oxidized. Lower- reduced. Both spectra were obtained at an enzyme concentration of 25μM. The reduced spectrum was generated by reducing anaerobic plant sulfite oxidase with excess sulfite.

These rate constants may be compared with the observed rate constant of 27.7 ± 0.8 s⁻¹ at 4°C, following enzyme reoxidation directly (Figure 4.3). While initial single
exponential fits to these data were very good, closer examination of the transients at 360 nm suggested a double exponential equation provided a somewhat better fit to the data. The kinetic phases were not well-resolved however, presumably due insufficient spectral resolution of the presumed Mo(V) intermediate from the Mo(VI) and Mo(IV) states of the enzyme. Attempts to resolve the absorption spectrum of a Mo(V) species were not further pursued here.

Figure 4.3. The dependence of observed rate constant versus \([O_2]\) for the reoxidation of *A. thaliana* sulfite oxidase. The observed linear dependence yields a slope with \(k_{ox} = 5.3 \pm 0.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}\), with a y-axis intercept at \(y=0\). The data points are the average of at least three transients with error bars shown for each point. The reaction was performed in Tris-acetate buffer, pH 8.0 at 4ºC.
Figure 4.4. The reduction of cytochrome $c$ in the course of the oxidative half-reaction of *A. thaliana* sulfite oxidase. Reduction of cytochrome $c$ was monitored via stopped-flow, following the absorbance change at 550 nm. Representative transients for each reaction are shown. *Solid line*, the reaction of 1.5 µM plant sulfite oxidase with 532 µM $O_2$ in the presence of 38 µM cytochrome $c$. The transients were best fit as biphasic reactions with a faster rate constant of $47.7 \pm 0.4 \text{ s}^{-1}$ and a slower rate constant of $7.5 \pm 0.1 \text{ s}^{-1}$. Kinetic values reported are the average obtained from at least three transients. This plot was biphasic with an $R^2$ value of 0.9975. *Dotted line*, the same reaction in the presence of 200 U/mL of superoxide dismutase, demonstrating that there is no reduction of cytochrome $c$ in the absence of superoxide. The reaction was carried out in Tris-acetate buffer, pH 8.0 at 4°C.

4.4 Steady state studies following oxygen consumption

To further examine whether superoxide was being produced by plant sulfite oxidase, a simple superoxide dismutase concentration dependence experiment was performed. This experiment served the double purpose of determining whether superoxide is produced as well as determining the optimum superoxide dismutase
concentration necessary for other experiments where superoxide scavenging could be competitive between spontaneous dismutation, superoxide dismutase and cytochrome c. Utilizing the oxygraph to monitor the oxygen concentration of the reaction mix as it changed with time, steady-state experiments were performed at superoxide dismutase concentrations of 0, 35, 70, 105, 140, 175, and 210 Units/mL (Fig. 4.5). From in the superoxide dismutase concentration range of zero to 140 Units/mL, a linear dependence was seen in the oxygen consumption of the system, strongly suggesting that superoxide was being produced by the enzyme. Superoxide dismutase showed a maximum effect at a concentration of 140 Units/mL. At and above this concentration, the rate of oxygen consumption was decreased by ~45% suggesting that 90% of all molecular oxygen utilized by the system is being converted to superoxide. Based on these results, we performed all subsequent experiments with 200U of superoxide dismutase.

To confirm that superoxide was being produced at significant levels in the reaction of plant sulfite oxidase with O₂, the effect of superoxide dismutase and cytochrome c on O₂ consumption, by the enzyme, was examined in utilizing the same experimental setup. Superoxide dismutase catalyzes the dismutation of two equivalents of superoxide to peroxide and O₂ with a second-order rate of 1.4x10⁹ to 2.0x10⁹ M⁻¹s⁻¹ under reaction conditions (pH 8.2) similar to our own (pH 8.0), returning half of the O₂ initially reduced to the level of superoxide back into solution (Klug, Rabani, & Fridovich, 1972). A 25% reduction of O₂ consumption by superoxide dismutase, for example, would imply that 50% of the initially consumed O₂ was reduced to the level of superoxide, a 50% reduction would imply that the entirety of catalytic throughput formed
superoxide in the initial instance. Ferricytochrome \( c \) is reduced by superoxide with a second-order rate constant of \( 4.0 \times 10^5 \) to \( 1.1 \times 10^6 \, \text{M}^{-1} \text{s}^{-1} \), depending on the ionic strength and pH of the solution, (McCord & Fridovich, The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfite and oxygen, 1969) substantially faster than the uncatalyzed dismutation reaction (second-order rate constant of \( 10^5 \)–\( 10^7 \, \text{M}^{-1} \text{s}^{-1} \) depending on the pH (McCord, Crapo, & Fridovich, Superoxide-dismutase assays: A review of methodology in superoxide and superoxide dismutases, 1977) (Bolann, Henriksen, & Ulvik, 1992)

Reduction of cytochrome \( c \) returns all the \( \text{O}_2 \) initially consumed as superoxide back to solution. A 50% reduction in \( \text{O}_2 \) consumption by cytochrome \( c \) would thus imply that half of the initially consumed \( \text{O}_2 \) was reduced to the level of \( \text{O}_2 \), complete abolishment of \( \text{O}_2 \) consumption would imply that all \( \text{O}_2 \) consumption resulted initially in the formation of superoxide.

Under our standard conditions, with 60 nM enzyme and 1.0 mM sodium sulfite in 20 mM Tris-OAc, pH 8.0 at 4°C at an \( \text{O}_2 \) concentration of \( \sim 500 \, \mu \text{M} \), \( \text{O}_2 \) consumption was \( 88.9 \pm 0.2 \, \mu \text{M} / \text{min} \) (Figure 4.6, solid line). In the presence of 200 U/mL superoxide dismutase, this rate of consumption was reduced by \( \sim 45\% \), to \( 49.1 \pm 0.4 \, \mu \text{M} / \text{min} \) (Figure 4.6, dashed (short) line), implying that approximately 90% of \( \text{O}_2 \) utilized in the reoxidation of plant sulfite oxidase is converted to superoxide. Addition of cytochrome \( c \) resulted in a reduction of oxygen consumption to \( 2.0 \pm 0.1 \, \mu \text{M} / \text{min} \), a decrease of 98% (Figure 4.6, dashed (long) line). Taken together, these results demonstrate that the vast majority of \( \text{O}_2 \) consumption during turnover by plant sulfite oxidase, >90% of the \( \text{O}_2 \)
consumed, generates superoxide in the initial instance. Peroxide is presumably formed only in the subsequent dismutation of superoxide.

Figure 4.5. Superoxide dismutase concentration dependence on the reduction of oxygen consumption by the pSOx reaction monitored by oxygraph. The reactions were performed in TRIS-OAc buffer (pH 8.0) at 4°C. Sulfite was present in excess (1.0 mM).

To test the accuracy of this method, control experiments were performed utilizing xanthine oxidase in its reaction with xanthine and O₂ (Figure 4.7). Upon addition of cytochrome c, there is a decrease in the rate of oxygen consumption of 32.0%; in the presence of superoxide the rate decreased by 10.8%. These results indicate that some 22-32% of the O₂ used by xanthine oxidase is reduced to superoxide, in good agreement with previously published values of 22-30% superoxide production under our reaction
We also examined the effects of pH and temperature on this superoxide production in order to determine if any possible ionizations were required or if temperature related molecular motions could have an effect. A summary of the results for varying the pH (6.0 – 10.0) and the temperature (4°C to 30°C) is shown Tables 4.1 and 4.2, respectively. Under all conditions examined, superoxide production was at least 95% and as high as 99% of the total O₂ consumption. From the pH dependence data, we can conclude that there is no E_red·O₂ complex formed under any of our reaction conditions.

4.5 Observation of superoxide production by steady-state reduction of cytochrome c.

In addition to the above oxygraph experiments, spectrophotometric assays of superoxide generation were performed, following the superoxide dismutase-dependent reduction of cytochrome c directly, by following the spectral change at 550 nm. In these experiments, addition of superoxide dismutase will abolish any cytochrome c reduction that is due to the production of superoxide, since the SOD-catalyzed dismutation is much faster than cytochrome c reduction under our reaction conditions (which is in turn considerably faster than the spontaneous dismutation reaction).

The initial steady-state rate of reduction of cytochrome c, by 20 nM plant sulfite oxidase in the presence of 400 µM O₂, was 31.5 ± 0.5µM/min (Figure 4.8, solid line). This rate agrees well with the rate of 88.9µM/min seen in the oxygraph experiments.
above when the difference in enzyme concentrations in the two experiments is taken into account (20nM in this case compared to 60nM for the oxygraph studies). Addition of superoxide dismutase to the reaction mix abolished reduction of cytochrome c essentially completely (Figure 4.8, dashed line), indicating that cytochrome c reduction is in fact due to reaction with enzyme-generated superoxide. The steady-state rate of cytochrome c reduction reflected a $k_{obs}$ for the reaction of 26.3 $s^{-1}$. Because this experiment was performed at saturating sulfite concentrations, this value can be compared to the published values for $k_{cat}$. Our value of 26.3 $s^{-1}$ at 4°C compares very favorably with the published value of 100 $s^{-1}$ at 25°C (Hemann, et al., 2005) when the difference in temperature is taken into account.

An experiment was also performed in which the entire process was monitored in a single transient, with cytochrome c, plant sulfite oxidase and superoxide dismutase added sequentially as the spectral change associated with cytochrome c reduction was monitored. These results clearly showed that addition of superoxide dismutase, abolished cytochrome c reduction thereby reinforcing our previous results (Figure 4.9).

Due to the ability of hydrogen peroxide to reoxidize cytochrome c, it was necessary to do a control in the presence 100 U/mL of catalase, which rapidly converts two equivalents of $H_2O_2$ to $H_2O$ and $O_2$. The results showed that the initial rate of cytochrome c reduction was not affected and that, only on longer timescales, could this re-oxidation become a factor (data not shown). This suggests that the two-electron reduction of molecular oxygen to hydrogen peroxide is not the dominant pathway for
reoxidation and that essentially all the O$_2$, consumed in the reoxidation of plant sulfite oxidase, undergoes one-electron reduction to yield superoxide. Superoxide is only subsequently converted to hydrogen peroxide non-enzymatically via spontaneous dismutation.

Figure 4.6. Effect of cytochrome c and superoxide dismutase on the steady-state rate of oxygen consumption by the plant sulfite oxidase reaction as monitored oxymetrically. Representative transients for each reaction are shown. **Solid line** - the reaction of sulfite oxidase (60 nM) with sulfite (1.0 mM) and ~510 µM O$_2$. **Dashed (short) line** - the same reaction in the presence of 200 U/mL of superoxide dismutase. **Dashed (long) line** - the same reaction in the presence of 94.5 µM cytochrome c (and no superoxide dismutase). Cytochrome c was added at ~10 seconds after starting the reaction. The reaction was carried out in Tris-acetate buffer, pH 8.0 at 4°C.

As with the oxygraph experiments, a test of the accuracy of this method was performed utilizing the reaction of xanthine oxidase with xanthine and O$_2$ (Figure 4.10). In this reaction, we saw a decrease in the rate of cytochrome c of 100% upon addition of
superoxide dismutase. The rate of reduction of cytochrome c was 14.1 μM min⁻¹, a rate equivalent to those seen in the oxygraph experiments and corresponding to 25% superoxide production, which corresponds well with our previously reported values.

![Figure 4.7](image_url)

**Figure 4.7.** Effects of cytochrome c and SOD on the rate of oxygen consumption by the xanthine oxidase reaction as monitored by oxygraph. **Solid line** – Reaction mix contained XO (26nM) and xanthine (0.1mM). **Dashed line** - Reaction mix contained XO (26nM) and xanthine (0.1mM) and 200U of SOD. **Dotted line** - Reaction mix contained XO (26nM) and xanthine (0.1mM) and 100µM cytochrome c. Experiments were performed at 4°C.
Table 4.1: pH Dependence of Superoxide Production by *A. thaliana* sulfite oxidase

<table>
<thead>
<tr>
<th>pH</th>
<th>Percent Superoxide Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>97.2 +/- 1.6</td>
</tr>
<tr>
<td>7.0</td>
<td>95.3 +/- 0.9</td>
</tr>
<tr>
<td>8.0</td>
<td>98.7 +/- 1.2</td>
</tr>
<tr>
<td>9.0</td>
<td>99.0 +/- 1.4</td>
</tr>
<tr>
<td>10.0</td>
<td>96.6 +/- 0.8</td>
</tr>
</tbody>
</table>

This experiment was performed in the same manner as the oxygraph experiments. The pH of the buffer was varied from 6.0 to 10.0 (bis-Tris (pH 6.0), bis-Tris-propane (pH 7.0), Tris(pH 8.0), and glycine (pH 9.0 and 10.0)). All buffers were made to 20mM concentration. Experiments were performed at 4°C.

Table 4.2: Temperature Dependence of Superoxide Production in *A. thaliana* sulfite oxidase

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Percent Superoxide Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>98.1 +/- 2.4</td>
</tr>
<tr>
<td>10.0</td>
<td>96.8 +/- 1.1</td>
</tr>
<tr>
<td>15.0</td>
<td>98.8 +/- 0.8</td>
</tr>
<tr>
<td>20.0</td>
<td>96.9 +/- 1.0</td>
</tr>
<tr>
<td>25.0</td>
<td>99.1 +/- 1.1</td>
</tr>
<tr>
<td>30.0</td>
<td>97.3 +/- 1.6</td>
</tr>
</tbody>
</table>

This experiment was performed in the same way as the oxygraph experiments. The temperature of the reaction mix was varied from 4°C to 30°C.
Figure 4.8. Effect of superoxide dismutase on cytochrome c reduction by *A. thaliana* sulfite oxidase reaction as monitored spectrophotometrically. Representative transients for each reaction are shown. Solid line, the reaction of plant sulfite oxidase (20 nM) with sulfite (1 mM) and O$_2$ (532 µM) in the presence of 20 µM cytochrome c. Dashed line, the same reaction in the presence of 200 U/mL of superoxide dismutase. Dotted line, the initial slope of the first reaction, yielding a value of 31.5 µM/min. The experiment was performed in Tris-acetate buffer, pH 8.0 at 4°C.
Figure 4.9. Effect of superoxide dismutase on cytochrome c reduction by *A. thaliana* sulfite oxidase as monitored spectrophotometrically. The reaction was started by sequentially adding cytochrome c (~20 µM final concentration) and plant sulfite oxidase (200 nM) to a sulfite solution (1.0 mM) in a cuvette. Superoxide dismutase (200 U/mL) was added after a short time. Arrows on the spectra indicate the points of cytochrome c, plant sulfite oxidase, and superoxide dismutase additions to the reaction mix. The experiment was performed in Tris-acetate buffer, pH 8.0 at 4°C.

4.6 Discussion

Plant sulfite oxidase differs from other molybdenum-containing proteins in that its molybdenum center has significant reactivity toward O₂. The molybdenum centers of other well-characterized enzymes such as xanthine oxidase and chicken sulfite oxidase (which utilize heme, iron-sulfur or FAD prosthetic groups as electron shuttles toward their final electron acceptor) react only very sluggishly with O₂. We show here that plant sulfite oxidase not only reacts rapidly with O₂, but does so via one-electron transfer
events with the result being the near-quantitative production of superoxide. Hydrogen peroxide, as reported previously (Hansch, Lang, Rennenberg, & Mendel, 2007), is only subsequently generated in the presumably spontaneous dismutation of the superoxide that is initially generated.

![Figure 4.10](image)

**Figure 4.10. Effect of superoxide dismutase on cytochrome c reduction by xanthine oxidase reaction as monitored by UV-Visible spectrophotometer.** Solid line – Reaction mix contained xanthine oxidase (200nM) and xanthine (1mM) in the presence of 20µM cytochrome c. Dashed line - Reaction mix contained xanthine oxidase (200nM) and xanthine (1mM) in the presence of 20µM cytochrome c and 200U of superoxide dismutase.

This reaction of plant sulfite oxidase with molecular oxygen is a second-order reaction with a rate constant of $5.3 \pm 0.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ in 20 mM Tris-acetate, pH 8.0, 4°C. Steady-kinetic studies, monitoring both the reduction of cytochrome c by superoxide and the effects of cytochrome c and superoxide dismutase on oxygen consumption, show
conclusively that superoxide is being produced essentially quantitatively independent of temperature and pH. Rapid reaction kinetics following the generation of superoxide during oxidation of anaerobically reduced enzyme by the reduction of cytochrome c are biphasic, indicating that enzyme reoxidation occurs in two sequential one-electron transfer steps, with rate constants of $47.7 \pm 0.4 \text{ s}^{-1}$ and $7.5 \pm 0.1 \text{ s}^{-1}$. Given the very rapid rate of the reductive half-reaction of plant sulfite oxidase by sulfite ($>1000 \text{ s}^{-1}$; ref. (Eilers, et al., 2001)), it is evident the oxidative half-reaction is rate-limiting to catalysis.

It is, therefore, possible to make a comparison between the reported values for $k_{\text{cat}}$ and the electron transfer rates which we have determined. At 532 µM $O_2$, $k_{\text{ox}}$ of 28 s$^{-1}$ at 4°C and 116 s$^{-1}$ at 25°C. This later value agrees with our value for $k_{\text{cat}}$ at 25°C of $122 \pm 1.1 \text{ s}^{-1}$ and the published value of $\sim 100\text{s}^{-1}$ (Hemann, et al., 2005). From the temperature dependence of $k_{\text{ox}}$ over the range of 4°C to 30°C, an activation energy of $E_a = 46.1 \pm 1.2 \text{ kJmol}^{-1}$, is obtained (Figure 4.11). Based on this data, it is clear that plant sulfite oxidase generates superoxide essentially quantitatively in its reaction with molecular oxygen.

With this discovery, plant sulfite oxidase becomes one of only five peroxisomal proteins known to generate superoxide, the others being NADPH-dependent cytochrome $b_5$ reductase, monodehydroascorbate reductase (MDHAR), a protein of unidentified function that utilizes NADH as a reductant, and xanthine oxidase (Lopez-Huerts, Corpus, Gomez, & del Rio, 1999) (Lopez-Huertas, Sandalio, Gomez, & del Rio, 1997) (del Rio, Sandalio, Corpas, Lopez-Huertas, Palma, & Pastori, 1998) (Stewart, Hille, & Massey, 1985). These enzymes are each believed to be the components of a peroxisomal membrane electron transport chain, with the first three proteins found exclusively in the
membrane. With these proteins, superoxide production represents only a small amount of the total catalytic throughput, but this is not the case with the plant sulfite oxidase. It is possible that the significant levels of superoxide as well as and peroxide generated by these enzymes plays a physiological, possibly anti-pathogenic, role in plants (Bonekamp, Volkl, Fahimi, & Schrader, 2009) (Angermuller, Islinger, & Volkl, 2009).

Based on the data obtained here, we propose a modified reaction mechanism in which reoxidation of the enzyme occurs via a bifurcated reaction where product release may occur before or after the first electron oxidation (Figure 4.12). We know from our rapid-reaction kinetics that there is no Michaelis complex formed with oxygen suggesting that product displacement is not necessary for reoxidation to occur. From the rapid-reaction kinetic studies involving reduction of cytochrome c we saw a slower, electron-transfer event allowing for the possibility that product release may happen between the two electron transfer events. Furthermore, the results from the pulsed EPR studies, (chapter 3) strongly suggest the presence of a sulfate-bound Mo(V) intermediate in the catalytic cycle (Astashkin A. V., et al., 2007).

In our proposed mechanism, there are two pathways by which enzyme can be reoxidized. Starting from a product bound reduced (Mo(IV)) molybdenum, hydrolysis of the product could occur followed by two sequential electron transfers. From the same starting point, it is possible that one electron transfer event occurs before product is released, thus resulting in a sulfate-bound Mo(V) intermediate. After hydrolysis of
product from the molybdenum center, the final electron oxidation occurs resulting in a fully oxidized protein.

Figure 4.11. Temperature dependence of the overall oxidative half reaction of *A. thaliana* sulfite oxidase. \( R^2 = 0.9992, m = -5650.15, E_a = 46.1 +/- 1.2 \text{ kJ/mol} \)

In order to understand the physiological implications of such high levels of superoxide production by plant sulfite oxidase, it is important to examine several important aspects of superoxide chemistry in the cell. Firstly, it is important to consider that superoxide can react with hydrogen peroxide to form the very hydroxyl radical (HO\(^\cdot\)). The chemistry of this reaction was first described by Fenton, in 1876, when he published work concerning the reactivity of ferrous compounds with hydrogen peroxide to form hydroxyl radicals (Fenton H. H., 1876) (Fenton H. H., 1894). This chemical reaction, and those similar to it, are generally referred to as Fenton chemistry. These
reactions were further explored by Haber, Willstatter and Weiss, who proposed that hydroxyl radicals specifically may be involved. These workers went on to characterize the reaction mechanism as catalyzed by ferrous iron (Haber & Willstatter, 1931) (Haber & Weiss, 1932) (Haber & Weiss, 1934). The reactions involved, as determined in these works, are widely known as Haber-Weiss or Haber-Willstatter reactions and are summarized in the equations below:

\[ \text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \cdot\text{OH} + \text{OH}^- \quad (i) \]

\[ \text{Fe(III)} + \text{O}_2^- \rightarrow \text{Fe(II)} + \text{O}_2 \quad (ii) \]

\[ \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- \quad (iii) \]

Equation (i) is the original reaction proposed by Fenton to describe the chemistry involved in the production of hydroxyl radicals in the reaction of iron with hydrogen peroxide. Equation (ii) is the reaction proposed by Weiss and Baxendale (Weiss, 1935) (G, Baxendal, George, & Gargrave, 1951) to proceed after the Fenton reaction. Equation (ii) was derived from the original Haber, Willstatter and Weiss equations in which they set out to describe the mechanism by which superoxide and iron were involved in peroxy- and hydroxy radical formation (Haber & Willstatter, 1931) (Haber & Weiss, 1932) (Haber & Weiss, 1934). Combining equations (i) and (ii) gives equation (iii), which represents the net reaction of the iron-catalyzed Haber-Weiss reaction.

What has been learned from this work and subsequent studies is that the reaction occurs readily with small amounts of chelated ferrous compounds and that large amounts
of the highly reactive hydroxyl radical is produced in the process. This production of hydroxyl radical can result in the oxidative destruction of most organic compounds, including DNA and fatty acids. Additionally, it is known that superoxide can react with nitric oxide (a common cellular signaling molecule) to form peroxynitrite, a compound with similar oxidative capabilities to the hydroxyl radical (Beckman, Beckman, Chen, Marshall, & Freeman, 1990). Superoxide has also been shown capable of iron atom removal from iron-sulfur clusters (Gardner & Fridovich, 1991).

The close proximity of a protein that produces large quantities of superoxide, such as plant sulfite oxidase, to such a vast supply of hydrogen peroxide, such as that found in the peroxisome, means that even small amounts of free or chelated ferrous iron could result in the production of large quantities of hydroxyl radical. Although the presence of superoxide dismutase and catalase should decrease the likelihood of this reaction, multiple studies have shown that the complexity of cells and organelles leads to concentration gradients allowing for localized Fenton chemistry to occur (Winterbourn, 1995) (Wardman & Candeias, 1996). Since little work has been done to determine the role of Fenton chemistry in the plant peroxisome (or the plant cell on the whole), we can only speculate as to the effect of this high level of superoxide on the cell. It is obvious that whatever problems it may cause, plant have adapted in such a way that they can thrive, despite the presence of so many harmful reactive oxygen species.

Interestingly, neither vertebrate nor bacterial forms of sulfite oxidase react with oxygen at an appreciable rate. It is not known what determinants of the active site of
plant sulfite oxidase render it so reactive toward O$_2$. Indeed, the molybdenum center itself is virtually indistinguishable structurally from that of the vertebrate form of the enzyme, and most nearby amino acid residues, and in particular those responsible for binding substrate, are strictly conserved. Of known molybdenum centers in biology, only that of arsenite-complexed xanthine oxidase has been shown capable of direct reduction of oxygen, resulting in the production of superoxide (Stewart, Hille, & Massey, 1985).

Figure 4.12. Overall mechanism for the reaction of A. thaliana sulfite oxidase with sulfite. Shown here are the two proposed mechanism in which reoxidation can occur. The top portion shows single-electron reoxidation with a Mo(V)-P intermediate followed by another single-electron oxidation along with product dissociation. The bottom portion shows product dissociation followed by two single-electron oxidations. The axial oxygen is believed to be transferred to the product (oxygen marked with asterisk).

Here we have shown conclusively that A. thaliana sulfite oxidase produces superoxide essentially quantitatively, generating hydrogen peroxide only in a subsequent dismutase reaction. This puts plant sulfite oxidase in a small group of superoxide-
producing proteins in the plant peroxisome. Plant sulfite oxidase is also among the few proteins whose molybdenum centers are capable of readily utilizing oxygen as an electron acceptor. Our results also underscore the diversity that exists among the sulfite oxidase family of molybdenum enzymes. It is evident that plant sulfite oxidase is unique in several respects when compared to other molybdenum-containing enzymes.
Chapter 5: The reaction of plant sulfite oxidase with the substrate analog dimethylsulfite.

5.1 Introduction

The mechanism by which the sulfite oxidases catalyze the oxidation of substrate has been well studied. Although several different mechanisms have been proposed (Figure 2.9), the generally accepted mechanism involves substrate lone pair attack on the equatorial oxygen of the molybdenum center, resulting in an intermediate with product bound to reduced enzyme (Figure 2.9A) (Hille R., 1994). Density functional calculations on model systems (Pietsch & Hall, 1996) had previously concluded that a nucleophilic attack on one oxo group of a simple MoO₂-containing model by the lone pair electrons from the substrate (trimethylphosphine in this case) provides the most energetically favorable course of the reaction since reduction of the metal center allowed for a shortening and strengthening of the remaining Mo=O bond, making it a molybdenum oxygen triple bond. This strengthening recoups some of the enthalpy lost with the removal of an oxo group.

Further support for such a lone pair attack mechanism came from work investigating the properties of a synthetic compound modeling the Mo(IV) substrate bound complex that was formed upon reaction of a MoO₂ synthetic compound with trimethylphosphine. (Nemykin, Laskin, & Basu, 2004). The workers synthesized and characterized this intermediate and, by combining high-resolution crystallographic data with surface induced dissociation data obtained from Fourier Transform ion cyclotron resonance mass spectroscopy, they were able to determine bond dissociation energies of
the molybdenum-substrate and molybdenum-product bonds during the intermediate steps involved in oxygen atom transfer. Based on these dissociation energies, they were able to calculate the heats of formation for the intermediates and products and they subsequently determined that the majority of the energy required for product release was acquired in the formation of the product bound intermediate as a result of a substrate lone pair attack at an oxo group (Nemykin, Laskin, & Basu, 2004).

An argument against this mechanism has been the observation that several anions were known to inhibit vertebrate sulfite oxidases. These anions were also known to alter the EPR spectra of the partially reduced Mo(V) species, suggesting that they directly coordinated to the molybdenum (Bray, Gutteridge, T, & Wilkinson, 1983). Based on this data, the mechanism shown in Figure 2.9B was proposed in which substrate coordinates directly to the molybdenum to initiate the reaction (George G. N., Garrett, Prince, & Rajagopalan, 1996) (Astashkin, Raitsimring, Feng, Johnson, Rajagopalan, & Enemark, 2002). This mechanism was supported by a subsequent computational study that utilized that claimed to demonstrate a stabilized hexacoordinate molybdenum substrate complex. This work went on to characterize all of the transition states and intermediates of the reaction, including a heptacoordinate enzyme-substrate complex (Pal, Chaudhury, & Sarkar, 2007).

In order to address the possibility of a lone pair attack mechanism and determine how substrate interacts with the molybdenum to initiate catalysis, experiments were performed with chicken sulfite oxidase utilizing the substrate analog
dimethylsulfite, in which both oxyanionic groups are blocked by methylation (Brody & Hille, The reaction of chicken liver sulfite oxidase with dimethylsulifite, 1995). The structure of dimethylsulfite is shown in Figure 5.1. Since dimethylsulfite still contains a lone pair, the idea was that it should still be able to react if the reaction proceeded via a substrate lone pair attack. This work showed that substrate binding was affected by multiple orders of magnitude with $K_d$ increasing from $33 \mu M$ to $11 mM$ (with sulfite and dimethylsulfite, respectively) and $k_{cat}/K_m$ decreasing from $2.8 \times 10^6 M^{-1} s^{-1}$ to $1.4 \times 10^4 M^{-1} s^{-1}$. The steady state and rapid reaction kinetic constants, $k_{cat}$ (79 s$^{-1}$ and 89 s$^{-1}$) and $k_{red}$ (194 s$^{-1}$ to 170 s$^{-1}$), however, were virtually unaffected. While the difference in $K_d$ indicated that the oxyanion groups of substrate stabilize the enzyme substrate complex, since methylation of the oxygens had no affect on the reaction velocity, it could be concluded that the lone pair of substrate was sufficient for reactivity once the substrate binding site was saturated at high sulfite concentration.

![Figure 5.1. The structures of sulfite and dimethylsulfite. A. The structure of sulfite with the lone pair electrons shown. B. The structure of dimethylsulfite with the lone pair electrons shown.](image)

Because of the inherent differences that exist between the plant and vertebrate forms of sulfite oxidase, these experiments have been repeated here. The kinetics of plant sulfite oxidase have been examined and the effects of dimethylsulfite compared to the earlier work with chicken sulfite oxidase. Importantly, criticism of the previous work has
been addressed with a new control experiment demonstrating that the dimethyl substrate did not hydrolyze to a significant degree in the course of the experiment. Additionally, the energetics of the reactions with sulfite and dimethylsulfite have been quantitatively compared. To determine the effect of the ionization of active site residues on substrate binding, the dependence of pH for the steady state reaction has also been performed.

5.2 Materials and Methods

*Enzyme Preparation.* As described in Chapter 3

*Steady state kinetics assays.* Steady-state kinetic experiments following the reduction of potassium ferricyanide were performed using a Hewlett-Packard 8452A UV-visible spectrophotometer as described previously (Hemann, et al., 2005). Experiments were performed at 25°C under aerobic conditions in 20 mM Tris-OAc buffer, pH 8.0. Enzyme was added (final concentration of 20 nM) along with ferricyanide (final concentration ~40 to 80 μM) and sulfite (from 1.0 to 500 μM) or dimethylsulfite (from 1.0 to 100 mM). Initial rates were determined by following the absorbance change per unit time in the initial, linear phase of the reaction. The total amount of reduced ferricyanide was determined from the total absorbance change for the reaction, using an empirically determined effective extinction change of $\Delta \varepsilon = 1020 \text{ M}^{-1}\text{cm}^{-1}$. All curve fittings were performed using SigmaPlot software.

For temperature dependent experiments, the same conditions were used with temperature varied from 4 to 30°C. pH dependent studies were performed in the same manner, varying the buffer to accommodate the change in pH: for pH 6.0 bis-Tris was
used, for pH 7.0 bis-Tris propane, pH 8.0 Tris-acetate, pH 9.0 glycine and pH 10.0 CAPS with all buffers at a concentration of 20mM and containing 5mM EDTA. Temperature and pH dependence curves were fit using SigmaPlot software. Complete substrate dependence curves were utilized for each temperature and pH point shown. All data presented are the average of at least three trials and the experiments were repeated several times for accuracy and reproducibility. For the sake of consistency, if it was necessary to use protein from different batches of isolate, standard steady-state activity assays were performed to ensure similar kinetic constants.

*Testing for hydrolysis of dimethylsulfite in solution.* A stock solution of dimethylsulfite was prepared in aerobic, room temperature buffer (20mM Tris-acetate, pH 8.0, 5mM EDTA) and allowed to incubate. Samples were taken every fifteen minutes for use as substrate in steady state assays as performed above but with a concentration of 100 or 250μM. The relative activity of the protein was determined under these conditions and the changes were tracked with time. All data points are the average of three samples taken simultaneously from the same stock. The experiment was repeated twice with the same result.

*Rapid-reaction kinetic studies.* Rapid kinetic experiments were performed in 20 mM Tris-OAc buffer, pH 8.0 in an Applied Photophysics Inc. SX-18MV stopped-flow instrument equipped with a diode array detector. The reduction of fully oxidized, aerobic enzyme (20-40μM) with sulfite and dimethylsulfite (excess concentrations of at least ten times $K_m$) was followed by monitoring the absorbance decrease at 360 and 480nm wavelengths which are associated with reduction of *A. thaliana* sulfite oxidase.
5.3 Steady state kinetics with sulfite and dimethylsulfite

In order to determine the reactivity of plant sulfite oxidase with dimethylsulfite, steady state experiments were performed, in which the substrate dependence of the catalytic velocity was determined under aerobic conditions using the one electron acceptor potassium ferricyanide ($K_3\text{Fe(CN)}_6$) as the terminal electron acceptor. The reaction was monitored by tracking the absorption change at 420nm, corresponding to the reduction of ferricyanide. It should be noted that while oxygen (the physiological electron acceptor for plant sulfite oxidase) is present in excess, previous work (unpublished) has shown that the rate of reduction of ferricyanide by plant sulfite oxidase is unaffected by the presence of oxygen with the aerobic and anaerobic kinetics being the same.

Utilizing dimethylsulfite as substrate, Michaelis-Menten type kinetics were observed. From a hyperbolic fit to a plot of $v_{\text{obs}}$ versus substrate concentration, a $k_{\text{cat}}$ of $105.0 \pm 2.2\text{s}^{-1}$ was observed and $K_m$ of $7.1 \pm 0.8 \text{mM}$ was determined (Figure 5.2). Compared to the published values for the reaction with sulfite, no change was seen in the limiting rate of catalysis at high substrate concentration ($k_{\text{cat}} \sim 100 \text{ s}^{-1}$ with sulfite as substrate) but $K_m$ increased approximately 300 fold ($22\mu\text{M}$ with sulfite) (Hemann, et al., 2005). The experiment was repeated utilizing sulfite as substrate with results for both $k_{\text{cat}}$ ($139.4 \pm 7.8\text{s}^{-1}$) and $K_m$ ($45.6 \pm 8.8 \mu\text{M}$) in good agreement with the literature values as mentioned above (Figure 5.3) (Hemann, et al., 2005).
Figure 5.2. Dependence of *A. thaliana* sulfite oxidase activity on dimethylsulfite concentration. A hyperbolic fit (solid line) yields $k_{\text{cat}}$ of $105.0 \pm 2.2\text{s}^{-1}$ and $K_m$ of $7.1 \pm 0.8\text{mM}$. Reactions were performed in 20mM Tris-acetate, pH 8.0.

Figure 5.3. Dependence of plant sulfite oxidase activity on sulfite concentration. A hyperbolic fit (solid line) yields a $k_{\text{cat}}$ of $139.4 \pm 7.8\text{s}^{-1}$ and a $K_m$ of $45.6 \pm 8.8\text{µM}$. Reactions were performed in 20mM Tris-acetate, pH 8.0.

An additional experiment was performed in order to determine whether the observed reaction might be due to sulfite formed by hydrolysis of dimethylsulfite, rather
than dimethylsulfite per se. To do this, advantage was taken of the difference in $K_m$ for the reactions with sulfite and dimethylsulfite. The experiments involved reaction of a very low concentration of dimethylsulfite (100µM and 250µM) was used as the substrate, in which the stock solution of dimethylsulfite was allowed to sit and samples were taken every fifteen minutes to be tested for overall activity. Because the dimethylsulfite concentration is low (at least 28 times lower than its $K_m$), the reaction would be expected to proceed very slowly. Were dimethylsulfite to hydrolyze to form sulfite, it would be expected that sulfite would accumulate with time with concomitant increase in the reaction velocity, since the $K_m$ for sulfite is so much lower. If, on the other hand, accumulation is not rapid, we would see no significant changes with time. The latter result was expected since it is documented that hydrolysis of dimethylsulfite occurs at a rate of $10^{-5}$ s$^{-1}$ (Guthrie, 1978). Although none of our steady-state experiments measured more than sixty seconds of a given reaction, we nevertheless recorded activity levels every fifteen minutes for two hours for this control. The results were exactly as expected, with no significant changes in reaction velocity observed over the course of two hours (Figure 5.4). It is especially noteworthy that no changes larger than normal spectral noise could be discerned over the course of the first hour of the experiment.

5.4 Temperature dependence of the steady-state kinetics of plant sulfite oxidase

In order to further characterize the catalysis of both sulfite and dimethylsulfite, temperature-dependent steady-state assays were performed for both sulfite and dimethylsulfite and the constants $k_{cat}$ and $k_{cat}/K_m$ were obtained (Table 5.1). An
Arrhenius plot of the $k_{\text{cat}}$ values from the reaction with sulfite gave an activation energy of $E_a = 48.8 +/- 1.1$ kJ mol$^{-1}$ (11.7 +/- 0.3 kcal mol$^{-1}$) (Figure 5.5A). An Eyring plot of this data showed an enthalpy of $\Delta H^{\ddagger\ddagger} = 50.7 +/- 1.4$ kJ mol$^{-1}$ (12.1 +/- 0.3 kcal mol$^{-1}$) and an entropy of $\Delta S^{\ddagger\ddagger} = 34.3 +/- 4.9$ J K$^{-1}$ mol$^{-1}$ (8.2 +/- 1.2 cal K$^{-1}$ mol$^{-1}$) (Figure 5.5B).

Plotting the values for the reaction with dimethylsulfite made it possible to calculate an activation energy of $E_a = 50.2 +/- 2.7$ kJ mol$^{-1}$ (12.0 +/- 0.6 kcal mol$^{-1}$) (Figure 5.6A). An Eyring plot of this data showed an enthalpy of $\Delta H^{\ddagger\ddagger} = 51.8 +/- 1.9$ kJ mol$^{-1}$ (12.4 +/- 0.5 kcal mol$^{-1}$) and an entropy of $\Delta S^{\ddagger\ddagger} = 36.3 +/- 5.2$ J K$^{-1}$ mol$^{-1}$ (8.7 +/- 1.2 cal K$^{-1}$ mol$^{-1}$) (Figure 5.6B).

Figure 5.4. Hydrolysis of dimethylsulfite in aqueous solution. Steady-state assays were performed in order to determine the rate at which dimethylsulfite hydrolyzes to give sulfite. A stock solution of dimethylsulfite was prepared in aerobic, room temperature buffer (20mM Tris-acetate, pH 8.0, 5mM EDTA) and allowed to incubate. Samples were taken every fifteen minutes for use as substrate in steady state assays. Black symbols (100 $\mu$M dimethylsulfite). Red symbols (250$\mu$M dimethylsulfite). Reactions were performed in 20mM Tris-acetate, pH 8.0.
Figure 5.5. Temperature dependence of the steady-state reaction of *A. thaliana* sulfite oxidase with sulfite. **A.** Arrhenius plot for the reaction with sulfite with error bars shown. A linear fit of the data gave an activation energy of $E_a = 48.8 \pm 1.1 \text{ kJ mol}^{-1}$ ($11.7 \pm 0.3 \text{ kcal mol}^{-1}$). **B.** Eyring plot for the reaction with sulfite. A linear fit of the data gave an enthalpy of $\Delta H^{\pm} = 50.7 \pm 1.4 \text{ kJ mol}^{-1}$ ($12.1 \pm 0.3 \text{ kcal mol}^{-1}$) and an entropy of $\Delta S^{\pm} = 34.3 \pm 4.9 \text{ J K}^{-1} \text{ mol}^{-1}$ ($8.2 \pm 1.2 \text{ cal K}^{-1} \text{ mol}^{-1}$). Reactions were performed in 20mM Tris-acetate, pH 8.0. Fits were performed using Sigma Plot Software.
A. Arrhenius plot for the reaction with dimethylsulfite with error bars shown. A linear fit of the data gave an activation energy of $E_a = 50.2 \pm 2.7 \text{ kJ mol}^{-1}$ ($12.0 \pm 0.6 \text{ kcal mol}^{-1}$).

B. Eyring plot for the reaction with dimethylsulfite. A linear fit of the data gave an enthalpy of $\Delta H^\ddagger = 51.8 \pm 1.9 \text{ kJ mol}^{-1}$ ($12.4 \pm 0.5 \text{ kcal mol}^{-1}$) and an entropy of $\Delta S^\ddagger = 36.3 \pm 5.2 \text{ J K}^{-1} \text{ mol}^{-1}$ ($8.7 \pm 1.2 \text{ cal K}^{-1} \text{ mol}^{-1}$). Reactions were performed in 20mM Tris-acetate, pH 8.0. Fits were performed using Sigma Plot Software.

Figure 5.6. Temperature dependence of the steady state reaction of A. thaliana sulfite oxidase with dimethylsulfite. A. Arrhenius plot for the reaction with dimethylsulfite with error bars shown. A linear fit of the data gave an activation energy of $E_a = 50.2 \pm 2.7 \text{ kJ mol}^{-1}$ ($12.0 \pm 0.6 \text{ kcal mol}^{-1}$). B. Eyring plot for the reaction with dimethylsulfite. A linear fit of the data gave an enthalpy of $\Delta H^\ddagger = 51.8 \pm 1.9 \text{ kJ mol}^{-1}$ ($12.4 \pm 0.5 \text{ kcal mol}^{-1}$) and an entropy of $\Delta S^\ddagger = 36.3 \pm 5.2 \text{ J K}^{-1} \text{ mol}^{-1}$ ($8.7 \pm 1.2 \text{ cal K}^{-1} \text{ mol}^{-1}$). Reactions were performed in 20mM Tris-acetate, pH 8.0. Fits were performed using Sigma Plot Software.
Table 5.1: Temperature dependence of the steady-state reaction of plant sulfite oxidase with sulfite and dimethylsulfite

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Temperature (°C)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfite</td>
<td>4</td>
<td>31.5 +/- 1.1</td>
<td>23.1 +/- 1.5 µM</td>
<td>1.36 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>55.6 +/- 1.3</td>
<td>34.2 +/- 1.9 µM</td>
<td>1.63 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>113.3 +/- 1.1</td>
<td>30.9 +/- 5.5 µM</td>
<td>3.67 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>139.4 +/- 7.8</td>
<td>44.5 +/- 6.1 µM</td>
<td>3.13 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>180.4 +/- 1.5</td>
<td>27.5 +/- 5.7 µM</td>
<td>6.56 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dimethylsulfite</td>
<td>4</td>
<td>23.1 +/- 3.5</td>
<td>13.5 +/- 1.3 mM</td>
<td>1.71 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>45.7 +/- 1.9</td>
<td>11.7 +/- 0.9 mM</td>
<td>3.91 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>95.2 +/- 3.3</td>
<td>9.6 +/- 1.7 mM</td>
<td>9.92 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>105.0 +/- 2.2</td>
<td>7.1 +/- 0.8 mM</td>
<td>1.48 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>144.3 +/- 6.4</td>
<td>6.4 +/- 2.3 mM</td>
<td>2.25 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Examination of the k<sub>cat</sub>/K<sub>m</sub> values for both sulfite and dimethylsulfite showed that, while k<sub>cat</sub>/K<sub>m</sub> is relatively temperature-independent for reactions with sulfite with only a five-fold increase in going from 4°C to 29°C (due to a similar increase in k<sub>cat</sub>), k<sub>cat</sub>/K<sub>m</sub> for dimethylsulfite showed a larger temperature dependence with a thirteen-fold increase.

5.5 pH effects on catalysis in plant sulfite oxidase with sulfite and dimethylsulfite

Studies were next performed to determine how pH would affect catalysis and substrate binding. Since the generally accepted mechanism of substrate binding and orientation in the active site involves multiple interactions between active site residues and substrate, it was expected that pH would have less of an effect on k<sub>cat</sub>/K<sub>m</sub> for dimethylsulfite than sulfite but would show little to no effect on k<sub>cat</sub>. The results of these experiments are summarized in Table 5.2.
Table 5.2: pH dependence of the steady state reaction of plant sulfite oxidase with sulfite and dimethylsulfite

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfite</td>
<td>6.0</td>
<td>78.3+/−6.7</td>
<td>24.9+/−8.6</td>
<td>3.14x10$^6$</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>81.8+/−5.6</td>
<td>27.3+/−7.2</td>
<td>3.00x10$^6$</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>86.1+/−6.3</td>
<td>25.8+/−7.5</td>
<td>3.34x10$^6$</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>99.9+/−8.4</td>
<td>29.6+/−9.4</td>
<td>3.38x10$^6$</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>133.0+/−4.7</td>
<td>27.5+/−4.8</td>
<td>4.84x10$^6$</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>196.7+/−7.9</td>
<td>49.8+/−7.7</td>
<td>3.95x10$^6$</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>121.9+/−5.3</td>
<td>114.2+/−14.7</td>
<td>1.07x10$^6$</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>102.2+/−7.0</td>
<td>137.8+/−22.1</td>
<td>7.42x10$^5$</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>77.0+/−3.1</td>
<td>119.9+/−11.9</td>
<td>6.42x10$^5$</td>
</tr>
<tr>
<td>Dimethylsulfite</td>
<td>6.0</td>
<td>54.8+/−1.9</td>
<td>5.9+/−0.9</td>
<td>9.29x10$^4$</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>76.9+/−3.8</td>
<td>6.2+/−1.3</td>
<td>1.24x10$^4$</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>89.5+/−5.2</td>
<td>7.9+/−1.8</td>
<td>1.13x10$^4$</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>97.8+/−5.7</td>
<td>7.6+/−1.7</td>
<td>1.29x10$^4$</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>105.2+/−5.7</td>
<td>6.1+/−1.4</td>
<td>1.72x10$^4$</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>146.1+/−5.3</td>
<td>6.1+/−0.9</td>
<td>2.40x10$^4$</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>102.9+/−4.4</td>
<td>6.3+/−1.1</td>
<td>1.63x10$^4$</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>97.1+/−6.6</td>
<td>10.5+/−2.5</td>
<td>9.25x10$^3$</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>93.0+/−7.5</td>
<td>11.8+/−3.2</td>
<td>7.88x10$^3$</td>
</tr>
</tbody>
</table>

The pH dependence of $k_{cat}$ with sulfite as substrate has already been examined and our results were similar to those previously published for plant sulfite oxidase with pK$_a$ values of 8.1 and 8.8 (compared to 8.1 and 8.9 as reported by Hemann et al, 2005) and a maximum activity at pH 8.4 (Figure 5.7A). With dimethylsulfite, we saw that pH had a nearly identical effect on $k_{cat}$. For both substrates we saw a maximum of 2.5-fold pH effect. The pK$_a$ values seen for the reaction with dimethylsulfite, 8.2 and 8.8, are quite similar to those in the reaction with sulfite (Figure 5.7B).

Comparing the effects of pH on the $k_{cat}/K_m$ values for each substrate we saw a slightly greater effect in the reaction with sulfite (Figure 5.8A), a 6.5-fold increase,
compared to only a three-fold increase in $k_{cat}/K_m$ with dimethylsulfite as substrate (Figure 5.8B). For sulfite, we saw maximum value for $k_{cat}/K_m$ at pH 8.3 and pK$_a$ values at 7.9 and 8.6. With dimethylsulfite, we saw a maximum value at pH 8.5 and pK$_a$ values of 8.1 and 8.9.

5.6 Discussion

Comparisons of the steady-state reaction of plant sulfite oxidase with sulfite and dimethylsulfite show that methylation of the of the oxyanion groups of the substrate has very little effect on the limiting rate of catalysis but causes a 300-fold increase in $K_m$. The implication is that the oxyanion groups are involved in substrate binding and that their methylation must disrupt these binding interactions with active site residues, as previously concluded with the chicken enzyme (Brody & Hille, The reaction of chicken liver sulfite oxidase with dimethylsulifite, 1995). Given the structure of the substrate binding site, with multiple positively charged residues that interact with the negatively charged oxygens to properly orient the substrate, these results are not surprising.

In order to address the possibility that dimethylsulfite may hydrolyze to sulfite in water, a series of steady-state experiments have been performed to determine the extent to which sulfite is formed over time under experimental conditions. Our results demonstrate that very little sulfite is formed over the course of two hours with little to no accumulation over the first hour of the reaction. Based on these results, we can conclude that any reaction seen during the steady-state experiments is a direct result of a reaction with dimethylsulfite and not a hydrolysis by-product such as sulfite.
It was interesting to see that temperature had any effect associated with the substrate and, while these effects were quite modest when comparing the values for $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$, certain remarks can be made based on the trends seen in the kinetic data. The effect of temperature on $k_{\text{cat}}/K_m$ was nearly three times greater for dimethylsulfite than for sulfite suggesting that increased molecular motions in the active site are affecting substrate binding. Due to the much larger size of dimethylsulfite and the small changes seen in the kinetic constants, it seems likely that increased molecular motions of the active site residues allow for easier access of dimethylsulfite to the molybdenum cofactor. Additionally, since the ability to properly orient substrate is lost upon methylation of the oxyanions, it is also possible that these increased molecular motions help to more rapidly orient substrate in the active site.
Figure 5.7. The pH dependence of $k_{cat}$ for the reactions of plant sulfite oxidase with sulfite and dimethylsulfite.  

A. The reaction of sulfite oxidase with sulfite with error bars shown. Values of 8.1 and 8.8 were determined for the two pK$_a$s.  

B. The reaction with dimethylsulfite with error bars shown. Values of 8.2 and 8.8 were determined for the two pK$_a$s. Fits were performed using Sigma Plot software.
Figure 5.8. The pH dependence of $k_{cat}/K_m$ for the reactions of plant sulfite oxidase with sulfite and dimethylsulfite. **A.** The reaction of plant sulfite oxidase with sulfite with error bars shown. Values of 7.9 and 8.6 were determined for the two pK$_a$s. **B.** The reaction with dimethylsulfite with error bars shown. Values of 8.1 and 8.9 were determined for the two pK$_a$s. Fits were performed using Sigma Plot software.

Eyring and Arrhenius plots of the temperature dependence of $k_{cat}$ have allowed for the calculation of the activation energy and both the enthalpies and entropies of reaction
for both substrates. Very small differences are seen between the two substrates, with the reaction of dimethylsulfite having a slightly higher barrier to catalysis ($E_a = 11.7$ kcal/mol for sulfite and $E_a = 12.1$ kcal/mol for dimethylsulfite). This small change is consistent with a critical role of a lone pair of electrons in initializing the reaction and that once the substrate is properly oriented, the reaction proceeds unhindered. These values were in good agreement with the value of ~14 kcal/mol for the studies conducted by Pietsch and Hall with a MoO$_2$ model system in a reaction with trimethylphosphine (Pietsch & Hall, 1996). Furthermore, attempts were made to determine the reductive half-reaction rate constants with both substrates (data not shown). Even with dimethylsulfite, the reaction was still too fast for detection on our stopped-flow. Since the protein is fully reduced in the dead-time of the instrument, the reduction must have a $k_{\text{red}}>1500 \text{s}^{-1}$. The fact that methylation of the substrate does not hinder catalysis further indicates that the oxyanions of sulfite serve a role, essentially, in substrate binding while only the lone pair electrons are required for initiation of catalysis. The fact that catalysis is unaffected with dimethylsulfite and that the reductive half-reaction is unaffected suggests that the oxidative half-reaction is the rate-limiting step of catalysis.

Analysis of the pH dependence data further supports these conclusions. Firstly, the pH dependence of $k_{\text{cat}}$ is similar for both sulfite and dimethylsulfite. The pK$_a$s observed for the two substrates correspond well (8.1 and 8.8 for sulfite and 8.2 and 8.8 for dimethylsulfite) and indicate that two ionizations are required for catalysis. Additionally, only minor differences are seen when comparing the pH dependence of $k_{\text{cat}}/K_m$ for the two substrates. While we did see a slight shift in pH maximum, from 8.3
to 8.5 (for sulfite and dimethylsulfite, respectively), the pKₐs were quite similar (7.9 and 8.6 for sulfite and 8.1 and 8.9 for dimethylsulfite). The small pH dependence of the kinetic parameters for these substrates comes as no surprise since the electrostatic interactions between active site residues and substrate are believed to aid in binding and orientation only. The slightly smaller pH effect seen with dimethylsulfite is presumably due to the fact that it makes fewer interactions with these residues because of the methylation of the oxyanions.

It is interesting that both sulfite and dimethylsulfite show approximately the same pKₐs for kₐ (8.1 and 8.8 for sulfite and 8.2 and 8.8 for dimethylsulfite) and kₐ/Kₘ (7.9 and 8.6 for sulfite and 8.1 and 8.9 for dimethylsulfite) and while there are multiple residues in the active site only Tyrosine241, discussed previously, would have a pKₐ in this range. The pH dependence of kₐ and kₐ/Kₘ seen for these substrates lends credence to the idea that Tyrosine241 could be playing a role in substrate binding and catalysis. We know from the pH dependence of both kₐ and kₐ/Kₘ that the first pKₐ (8.1 and 8.2 for sulfite and dimethylsulfite, respectively) must be due to an ionization necessary for catalysis. The second pKₐ of approximately 9.0, exists for both substrates in both plots and may therefore indicate that there is an ionization that is important for both catalysis and substrate binding and that this interaction is involved in both the high and low substrate regimes. The experimental data for the recombinant human sulfite oxidase Y343F mutant supports this theory since the mutant showed small effects on both kₐ and kₐ/Kₘ, instead primarily affecting electron transfer out of the active site (Figure
While there are multiple interactions possible for sulfite that would allow for a favorable orientation of the lone pair toward the molybdenum center, this is less true for dimethylsulfite due to the methylation of the oxygen atoms with concomitant loss of electrostatic interaction with the active site arginines and possibly also for steric reasons. It may be possible that Tyr 241 interacts with dimethylsulfite. Based on the presumed orientation of sulfite in the active site in the Michaelis complex, with electrostatic interactions occurring between the oxygen atoms and the arginines, it would be possible for Tyr 241, which sits at the top of the substrate binding pocket with its hydroxyl group projected into the pocket and approximately parallel to the apical oxo group of the molybdenum, to be involved in a hydrogen bonding interaction with the remaining unmethylated (oxo) group of substrate, which would be situated near this tyrosine and would be oriented approximately parallel to the apical oxo.

Examination of the crystal structure of product bound chicken sulfite oxidase (1SOX) allows for both the identification of possible electrostatic interactions between product and active site residues and the measurement of distances between product and the molybdenum center (Figure 5.9) (Kisker, et al., 1997). This structure shows sulfate near enough to the active site residues to form many interactions. The shortest distance from the equatorial oxo group of the cofactor to any oxygen on the product is 2.42Å while the distance from the molybdenum to the same oxygen is 4.67Å, suggesting that if
substrate is being oriented in the active site via electrostatic interactions with active site residues, it would be too far away from the molybdenum center for an oxyanion attack. The close proximity of the product to equatorial oxo group, however, provides physical evidence supporting the idea of catalysis initiated by nucleophilic attack on the equatorial oxo group.

Figure 5.9. Electrostatic interactions and distance measurements for bound sulfate in the crystal structure of chicken sulfite oxidase (1SOX). A. Potential electrostatic interactions between sulfate and the side-chains of active site residues. All interactions are restricted to distances less than 3.0Å. B. Distance measurements between the sulfate and molybdenum center based on crystallographic data. Measurements are atom-center to atom-center (Kisker, et al., 1997)

The present data confirms that the reaction with dimethylsulfite must be occurring via a lone pair electron attack, as originally proposed (Hille R. , 1994) (Brody & Hille, The reaction of chicken liver sulfite oxidase with dimethylsulfite, 1995), and not via an oxyanion attack. We have presented here the results from pH and temperature
dependence reactions with both sulfite and dimethylsulfite where we saw only slight differences between the substrates. The lack of major pH or temperature effects on $k_{\text{cat}}$ with dimethylsulfite suggests that catalysis is unaffected by the methylation of the oxyanions. The changes in $k_{\text{cat}}/K_m$ due to temperature and pH, however small, still have trends that suggest that substrate binding is dependent largely upon electrostatic interactions which are limited when the substrate oxyanions have been methylated. Our results agree with those seen for chicken liver sulfite oxidase (Brody & Hille, The reaction of chicken liver sulfite oxidase with dimethylsulifite, 1995). Importantly, we have also extended these same conclusions with new data in which we have shown that dimethylsulfite does not hydrolyze to sulfite in the course of the reaction and, therefore, dimethylsulfite is a substrate for sulfite oxidase. Our data support the conclusion that catalysis is initiated via a lone pair electron attack at the equatorial oxo group of the molybdenum center however, without the ability to determine the rapid reaction kinetic constants of the reductive half-reaction for A. thaliana sulfite oxidase, it is difficult to say determine precisely the extent to which methylation of the oxyanion groups affects substrate binding. Further studies, including mutational analysis and x-ray crystallography will be required to determine the nature of these effects and their subsequent catalytic consequences.
Chapter 6. General summary and future direction

6.1 ESEEM studies with $^{33}\text{S}$-labeled sulfite.

The existence of a Mo(V) substrate/product-bound complex was first discussed in 1982 when a novel EPR signal was obtained from a sample of chicken sulfite oxidase (Bray, Lamy, Gutteridge, & Wilkinson, 1982). The lack of observable proton splitting led to the conclusion that excess sulfite in the preparation buffer had caused a sulfite-bound intermediate to form. Nearly two decades later this same signal, simply called “atypical” and not actually pursued, was found observed in plant sulfite oxidase. Further examination of this species took place in the form of continuous wave and pulsed EPR experiments. What made the EPR signal from the plant enzyme interesting is that it was not buffer, but the method of reduction and pH, that was the basis for the signal observed. At a low pH (<7.0) where the protein was reduced by sulfite and reoxidized to the Mo(V) state by potassium ferricyanide, a pure species was obtained in quantity.

A comparison of the crystal structure of plant sulfite oxidase (Schrader, Fischer, Theis, Mendel, Schwarz, & Kisker, 2003) with that of a product bound chicken sulfite oxidase (Kisker, et al., 1997) showed a single active site residue that had an altered conformation. It was immediately suggested that this residue, R374 in the plant protein, may be involved in holding substrate in the active-site so catalysis could occur. This form of the protein was called a “trapped” or “blocked” form due to the fact that, upon reduction by substrate, the conformational change would keep product in the active site until reoxidation occurred. This form took on more significance when it was shown that
several human sulfite oxidase mutants showed severely diminished activity and exhibited this same blocked EPR signal, thus leading researchers to believe that this sulfite/sulfate bound form was a catalytic dead-end. Differences between the plant and vertebrate forms of sulfite oxidase have led to a reconsideration of this “blocked” form that is so easily produced in the plant protein.

In the present study, we sought to determine whether the blocked species was the result of direct coordination of an anion to the molybdenum and also whether that species was a sulfite or if it was the product sulfate. The latter is an important distinction due to its mechanistic implications. In order to do this we used $^{33}$S-labeled sulfite to create this blocked species. Since $^{33}$S has a nuclear spin of $I=\frac{3}{2}$, in the case where labeled sulfite or sulfate is near the molybdenum, a hyperfine interaction should be seen.

What was seen was a slight peak broadening of $g_1$ in the CW-EPR spectrum. Although not as definitive as a true splitting, this was indicative of at least a moderate hyperfine interaction. From a theoretical analysis of this data we obtained estimates for the coupling parameters of the $^{33}$S atom which, while possibly off by as much as 50%, were close to estimates calculated from previous experiments and they gave us a baseline for comparison with ESEEM data.

We performed two-pulse primary ESEEM experiments to further examine this signal giving species. From these experiments we found that the $^{33}$S sample gave much larger ESE amplitudes than the $^{32}$S sample indicating a direct coordination of sulfite/sulfate to the molybdenum center. From this data we were able to obtain estimates
for the isotropic and anisotropic hyperfine coupling constant ($h\text{fi}$) as well as for the nuclear quadrupole interaction ($nqi$). Simulations were performed, based on these estimates, for the purpose confirming the results and expounding them. Our simulations confirmed our estimates and provided a better understanding of the features seen in the experimental time-domain spectra. More importantly, we were able to show that, under no reasonable conditions, could we obtain spectra comparable to our experimentally obtained spectra unless sulfate was coordinated to the molybdenum. Further confirmation of our results came from 2D- hyperfine sublevel correlation (2D-HYSCORE) experiments. Simulations of this data allowed for a better interpretation of the data and for the relative orientation of the tensors ($g$-, $h\text{fi}$, and $nqi$) to each other.

Based on this data we conclude that the blocked EPR signal is due to a sulfate-bound Mo(V) intermediate species. Taking into account recent findings about the nature of this species in vertebrate and plant sulfite oxidases, we conclude that this sulfate-bound species is catalytically relevant in the plant protein and a necessary intermediate of the catalytic cycle in plant sulfite oxidases. The plant enzyme appears to avoid a “thermodynamic trap” in forming such a stable species by reoxidizing with product still bound. The nature of the plant enzyme allows for product release after the first reoxidation step, thereby permitting complete reoxidation and preserving activity. It may be possible that the conformation of R374 changes in the plant enzyme upon the first reoxidation, thus allowing product release. Without further testing, however, this is purely speculative.
6.2 Studies of the oxidative half-reaction of plant sulfite oxidase

Vertebrate sulfite oxidase has been well studied for over forty years. The complete reaction has been characterized extensively through a host of biochemical and biophysical techniques. Much of the work has centered around the interaction between the molybdenum containing domain and the heme containing domain, specifically the nature of electron transfer from the molybdenum to the heme (and then on to cytochrome c, its physiological terminal electron acceptor) that must occur during the oxidative half-reaction of the protein. It was quite surprising, therefore, when a plant sulfite oxidase was found that had no heme domain. Unlike all other members of the sulfite oxidase family, plant sulfite oxidase has only a molybdenum center and has not been shown to co-purify with any other proteins (as in the case of the bacterial sulfite dehydrogenase, SorA, which is tightly associated with its electron accepting heme subunit, SorB). Furthermore, the plant enzyme showed no activity toward b- and c-type cytochromes, instead readily utilizing oxygen as an electron acceptor, both characteristics not shared by the other classes of sulfite oxidizing enzymes.

Further studies of this new protein showed that it was not only unique in its oxidative-half reaction but also in its cellular localization, it being localized to peroxisomes while vertebrate forms are in the mitochondria and bacterial forms are cytosolic proteins. Additionally, it was found that in its reaction with oxygen, plant sulfite oxidase produces hydrogen peroxide. Since the other classes of sulfite oxidizing enzymes perform one-electron transfer events during the oxidative half-reaction, studies...
were undertaken to explore the possibility that the hydrogen peroxide production was actually the result of superoxide production and subsequent dismutation. Initial results confirmed this possibility though a thorough study was not immediately pursued.

Here we sought out to further examine the plant enzyme, specifically with regard to its reactivity toward oxygen. We utilized rapid-reaction kinetics to observe the reoxidation rates of the protein in its reaction with oxygen. We found that the reaction was second-order with $k_{ox} = 5.3 \pm 0.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and the results indicated that the reaction was essentially irreversible and occurred without the formation of a Michaelis-complex between the molybdenum center and oxygen. Furthermore, by observing the reduction of cytochrome $c$ under rapid-reaction conditions, we saw that the reaction was proceeding in two steps and that that rate of the first step was faster than the second with rate constants of $47.7 \pm 0.4 \text{ s}^{-1}$ and $7.5 \pm 0.1 \text{ s}^{-1}$ at $4\degree C$, respectively. Temperature dependence rapid-reaction studies of the reoxidation yielded and activation energy of $E_a = 46.1 \pm 1.2 \text{ kJ/mol}$ which is comparable with the value we obtained for the temperature dependence of $k_{cat}$, $E_a = 48.8 \pm 1.1 \text{ kJ mol}^{-1}$. The similar rate constants for $k_{ox}$ and $k_{cat}$, along with the comparable activation energies, suggest that the rate-limiting step occurs during the oxidative half-reaction.

Utilizing an experimental setup originally used to test for superoxide production in xanthine oxidase, we utilized UV-visible spectroscopy and oxygen electrode steady-state experiments to determine the extent to which superoxide was being produced under turnover conditions by the plant enzyme. We found that the protein is producing
superoxide essentially quantitatively with nearly 100% of the oxygen consumed during
the reaction being reduced to superoxide. We conclude that the previously characterized
hydrogen peroxide production is the direct result of a dismutation reaction. We also
performed these studies under varied temperature and pH conditions to better understand
the nature of this superoxide production. We saw no temperature or pH effects on
superoxide production confirming that it is unlikely that a Michaelis complex forms with
oxygen.

We have shown here that plant sulfite oxidase is a very unique class of sulfite
oxidizing enzyme as well as showing that it is a unique among molybdenum-containing
proteins in utilizing a direct reaction with oxygen in its oxidative half-reaction. We
propose a new mechanism to explain the behavior of this protein under turnover
conditions, in which product release is not necessary prior to the first oxidation. This
proposed mechanism fits well with the ESEEM data from chapter 3.

6.3 The reaction of plant sulfite oxidase with dimethylsulfite

The generally accepted mechanism for initiation of catalysis in the sulfite
oxidases involves attack of the substrate lone pair on the equatorial oxygen of the
molybdenum center, resulting in an intermediate with product bound to reduced enzyme
(Figure 2.9A). This mechanism was first suggested by Hille in 1994 and gained strong
support based on computational work in which it was shown that the most energetically
favorable reaction required a substrate (trimethylphosphine in this work) lone pair attack
at an oxo group. Additional support for this mechanism came from studies on a MoO$_2$
synthetic compound in a complex with trimethylphosphine for which a crystal structure was obtained and the information gained was subsequently used for further computational studies in which the dissociation energies for the molybdenum-substrate and molybdenum-product bonds were determined. (Nemykin, Laskin, & Basu, 2004). The results of this work showed that the majority of energy required for product release must come from the lone pair electrons on substrate.

Despite suggestive evidence supporting this mechanism, no work had actually been done to prove this using a sulfite oxidase. To address this, Brody and Hille performed a series of steady-state and rapid-reaction kinetic studies with chicken sulfite oxidase and utilizing dimethylsulfite as a substrate. Dimethylsulfite has the same structure as sulfite except that the oxanions have been methylated. Since it still contains a lone pair, according to the proposed mechanism, catalysis should be unaffected. Additionally, due to the manner in which substrate is bound in the active site, it was thought that the methylated oxanions would affect the ability of electrostatic interaction to occur with active site residues and would therefore decrease substrate affinity. The results were exactly as predicted with only substrate affected by the oxanion methylation and catalysis relatively unaffected.

Here we have repeated these experiments with plant sulfite oxidase firstly to reproduce the results and secondly to address criticism from the first experiments. We saw nearly identical effects in plant sulfite oxidase as were seen with chicken sulfite oxidase. One major criticism of the original work had to do with the fact that
dimethylsulfite may hydrolyze in water to yield sulfite and that it was a reaction with sulfite that was being seen. To determine if this was the case, we performed steady-state experiments to determine that rate at which this hydrolysis was occurring. We found that over the course of an hour no significant amount of sulfite was produced thereby validating our results.

Additional experiments were performed in order to determine the pH and temperature effects on both the reaction with sulfite and dimethylsulfite. We saw no major differences between the two substrates. The effect of temperature on substrate binding was small for both substrates but, in the case of dimethylsulfite, was half of that seen for sulfite. Using Arrhenius plots, we were able to determine the activation energy for each reaction and found that there was only a small increase for dimethylsulfite. The pH effects, much like the temperature effects, were minimal. We did see slight differences between the two substrates but nothing that was indicative of a significant difference in the way the substrates are bound in the active site. Because the active site residues serve mainly to orient substrate in the active site, not much of an effect was expected for catalysis. It was not surprising to see a smaller effect on substrate binding for dimethylsulfite since the methylation of the oxyanions would disrupt its ability to participate in electrostatic interactions.

With this work, we have shown that a sulfite lone pair attack at the equatorial oxygen of the molybdenum cofactor is responsible for initiation of catalysis. Based on the pH and temperature dependence data, we conclude that the limited number of
orientations available to dimethylsulfite, due to its lack interaction with active site residues, likely hinders its ability to properly position its lone pair for effective attack at the oxo group, but once the substrate binding site is saturated, \( k_{\text{red}} \) is unchanged. Finally, the presence of a \( pK_a \) of \(-9.0\) for both substrate in both \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) vs. pH plots suggests a possible role for tyrosine 241 in substrate binding and catalysis. Previously, it has been shown that this residue (Y343) plays such a role in human sulfite oxidase but is mostly responsible for electron transfer out of the active site.

### 6.4 Future directions

Based on the data presented here, a series of rapid-reaction and steady-state experiments have been initiated to determine which pathway is more favored for reoxidation: an electron transfer preceding product release or both transfer events occurring after product release. Our preliminary data, from rapid-reaction studies in the presence of sulfate, indicates that the reaction may be a mix of the two, although it seems that product release likely occurs after the first electron transfer. We have seen nearly an order of magnitude difference in the rate constants for the first and second electron transfer events, making this product bound species more favorable, since the second transfer is slowed to a much greater extent than the first, indicating that product is still bound after the first transfer.

Similarly, CW-EPR is being used to determine amount of sulfate bound Mo(V) that exists in a sample prepared at physiological pH. This will be done by obtaining pure samples of the three EPR-active Mo(V) species, high pH, low pH and sulfate complexed,
and using deconvolution to determine the relative populations of each species at a given pH.

Utilizing enzyme-monitored turnover conditions, we are also attempting to determine whether the Mo(V) species has a distinct absorption spectrum such as seen with YedY from *E. coli*. EPR samples are being prepared under turnover conditions in order to quantify the Mo(V) signal and to determine the absorption spectrum of the signal giving species. The information obtained here, should allow us to determine the extent to which molybdenum is reoxidized prior to product dissociation during turnover.

The unique ability of plant sulfite oxidase to produce superoxide must have a structural basis. Tyrosine 241 has been shown to be intrinsically linked to electron transfer out of the active site (work done on human sulfite oxidase Y343F mutants) and we plant to prepare plant sulfite oxidase mutants of this residue (specifically Y241F) in order to determine its effect on superoxide production during turnover. Furthermore, an additional tyrosine, Y49, sits directly under the molybdenum cofactor and is unique to plant sulfite oxidase with all other sulfite oxidases a phenylalanine is found in this position. We propose to test a phenylalanine mutant to determine the effects on superoxide production and the ability to utilize oxygen as an electron acceptor.
Works Cited


157


Kappler, U., Bailey, S., Feng, C., Honeychurch, M. J., Hanson, G. R., Bernhardt, P. V., et al. (2006). Kinetec and structural evidence or the importance o tyr236 for the integrity o the mo active site in a bacterial sulfite dehydrogenase. *Biochemistry*, 45(32), 9696-9705.


