SPECTROSCOPIC AND KINETIC STUDIES OF BOVINE XANTHINE OXIDASE
AND RHODOBACTER CAPSULATUS XANTHINE DEHYDROGENASE

DISSERTATION

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By

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* * * * *

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ABSTRACT

Xanthine oxidase (XO) and xanthine dehydrogenase (XDH) are both molybdenum containing enzymes that catalyze the last two steps in purine metabolism, conversion of hypoxanthine to xanthine and finally xanthine to uric acid. Both forms of the enzyme contain two 2Fe/2S centers and a FAD in addition to the molybdopterin. Research has indicated similar structure and mechanism, thus study of both enzyme forms mentioned above prove useful in understanding mechanism. Study of vibrational modes within in the metal center by resonance Raman (rR) allow enzymatic mechanism to be inferred. RR studies of reduced enzyme in complex with violapterin, representing an intermediate in the reaction with lumazine, have been examined. Upon laser excitation at 647 nm, multiple bands from the Mo-coordination sphere vibrations arise. Upon comparison of calculated shifts using the lowest energy computational model of this complex, Mo-O-R linkage to violapterin was supported by the observed shifts to lower energy in the $^{18}$O versus $^{16}$O experiments. The current proposed mechanism suggests a two-electron reduction from Mo (VI) to Mo (IV) in the first step. An alternate mechanism suggests two one-electron steps to reach the Mo (IV) species. To experimentally test this alternate mechanism, the one-electron reduction potentials of several substrates were determined and compared to kinetic parameters for each of these substrates. In the case of two subsequent one-electron steps, a correlation would be expected between the one-electron
reduction potential and the kinetic parameters. No such correlation was observed ruling out the possibility of the one-electron mechanism and providing further evidence for two-electron reduction step. Recombinant RcXDH serves as a tool to study specific residues involved in catalysis. Both wild-type enzyme and various mutants have been kinetically and spectroscopically characterized. Steady-state and pre-steady state kinetics for the E232A mutant show slower reaction rates. This result is true for both the physiological substrate and the slower substrate hydroxymethylpurine (HMP) known to form an EPR active Mo (V) intermediate in the bovine enzyme. EPR studies of this mutant with HMP give a signal similar to the very rapid signal seen with the bovine XO with small modifications and remain unchanged in D$_2$O. Kinetic studies on the E730 mutants indicate substantial rate decreases as this residue is proposed to act as the active site base initiating catalysis. Wild-type EPR experiments with HMP have also been performed and a signal such as the rapid type I seen with the bovine XO is observed. The signal indicates two inequivalent protons that disappear upon reacting in D$_2$O. In conclusion, resonance Raman studies have supported the lowest energy computational model of the enzyme-reduced product complex predicted based on our proposed mechanism. Examination of the first step in the reductive half-reaction indicates a single two-electron reduction rather than two subsequent one-electron steps. Mutational studies of the active site residues of XDH identify E730 (E1261 in bovine XO) as an essential residue, possibly acting as the active site base initiating catalysis.
DEDICATION

To my parents; my brother Jeff; and my husband, Dennis.
ACKNOWLEDGEMENTS

First I would like to thank my parents and my brother, Dr. Jeff Nielson, who have helped me to appreciate education and given me the motivation to keep going. I would like to thank Dennis, my husband, who has put up with several years of much stress. You are truly the best part of my life – I love you very much.

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<td>Description</td>
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<tr>
<td>AFR</td>
<td>activity to flavin ratio</td>
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<td>AO</td>
<td>arsenite oxidase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>CTC</td>
<td>charge-transfer complex</td>
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<tr>
<td>DMS</td>
<td>dimethylsulfide</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>DMSOR</td>
<td>dimethylsulfoxide reductase</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENDOR</td>
<td>electron double resonance spectroscopy</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>E_{red}-P</td>
<td>reduced enzyme-product complex</td>
</tr>
<tr>
<td>ESEEM</td>
<td>electron spin-echo envelope modulation</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>GMP</td>
<td>guanine mononucleotide</td>
</tr>
<tr>
<td>HMP</td>
<td>2-hydroxy-6-methylpurine</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani media</td>
</tr>
<tr>
<td>MPT</td>
<td>molybdopterin</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NHE</td>
<td>normal hydrogen electrode</td>
</tr>
<tr>
<td>OLIS</td>
<td>On-Line Instrument Systems, Inc.</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
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<tr>
<td>Re</td>
<td><em>Rhodobacter capsulatus</em></td>
</tr>
<tr>
<td>RHR</td>
<td>rapid half-reaction</td>
</tr>
<tr>
<td>rR</td>
<td>resonance Raman</td>
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<tr>
<td>SO</td>
<td>sulfite oxidase</td>
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<tr>
<td>UV</td>
<td>ultra-violet</td>
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<td>XAS</td>
<td>X-ray absorption spectroscopy</td>
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<td>XDH</td>
<td>xanthine dehydrogenase</td>
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<td>XO</td>
<td>xanthine oxidase</td>
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CHAPTER 1

INTRODUCTION

1.1 General background

Scientists have long investigated the importance of metals in biology. One of the more interesting of these metals is molybdenum due to its relative scarcity in the earth’s crust. Molybdenum is readily available to biological systems relative to other transition metals due to the solubility of its high valent oxides such as $[\text{MoO}_4]^{2-}$; it is the most abundant transitional metal in sea water at a concentration of 10 µg/L (Henderson, 1986). Its availability has evolutionarily driven its use in biological systems such as the active site of over fifty enzymes (Hille, 2002). One of the most prominent areas is in mo"lybdoenzymes. The ability for molybdenum to exist in several oxidation states makes it suitable for biological electron transfer reactions coupled to the oxygen atom or proton transfer (Steifel, 1973; Steifel et al., 1977). Molybdenum exists in oxidoreductase enzymes either in a multinuclear center as in nitrogenase (which is a molybdenum-iron-sulfur containing enzyme that catalyzes nitrogen fixation) or more often as a mononuclear center associated with at least one pterin cofactor (as observed in enzymes such as xanthine oxidase, aldehyde oxidase, dimethylsulfoxide reductase, aresenite
oxidase, and sulfite oxidase) (Howard and Rees, 1996; Hille, 1996). The structure of this common cofactor is shown in Figure 1.1.

1.1.1 The mononuclear molybdenum enzymes

Mononuclear molybdenum enzymes can be grouped into two different categories based on the reactions catalyzed. The first group catalyzes the oxidative hydroxylation of aldehydes and aromatic heterocycles through a mechanism requiring breakage of a C-H bond. This group includes enzymes such as xanthine oxidoreductase and aldehyde oxidase. Group one contains exceptions, such as CO dehydrogenase, that catalyze a reaction that does not involve a C-H bond cleavage (Hille, 2002). The second group catalyzes oxygen atom transfer to or from an electron lone pair on the substrate. DMSO reductase, sulfite oxidase, and arsenite oxidase are included in this group. These oxotransfer enzymes can further be divided into two subgroups based on the structure of the molybdenum active site.

On the basis of the above, mononuclear enzymes are divided into three families based on the structure of their molybdenum active site and the reaction catalyzed (Figure 1.2). The first is referred to as the xanthine oxidase family. This family includes those enzymes that catalyze oxidative hydroxylation reactions as mentioned above in group one. Members of this family include the enzyme for which it is named, xanthine oxidase, as well as xanthine dehydrogenase, aldehyde oxidase and CO dehydrogenase. The structure of these enzymes usually involves a pterin cofactor attached to the molybdenum via a dithiolene linkage, an oxo group (Mo=O), either a Mo-OH or Mo-OH$_2$, and either a Mo=S or Mo=O. The second family, the sulfite oxidase family, includes enzymes from
both prokaryotic and eukaryotic sources that catalyze oxygen transfer either to or from a lone pair on the substrate. The assimilatory nitrate reductases from yeast and higher plants are also a member of the sulfite oxidase family. Structurally the sulfite oxidase family contains the molybdopterin (again coordinated to the metal via the dithiolene side chain), two terminal molybdo-oxo groups, either a Mo-OH or Mo-OH$_2$, and a sulfur linkage through an active site cysteine. The third family, known as the DMSO reductase family, consists of, as its name suggests, dimethylsulfoxide reductase as well as arsenite oxidase and the dissimaltory nitrate reductases. Like those enzymes in the sulfite oxidase family, members in this group also usually catalyze proper oxygen atom transfer. Examination of active site structure clearly makes this family stand out as there are two equivalents of the pterin cofactor attached to the molybdenum. The two remaining coordinate positions are filled by an oxo, sulfido, or seleno group and an O-ser, S-cys, or Se-cys (Hille, 1996).

### 1.1.2 The molybdopterin cofactor

The mononuclear molybdenum enzymes contain a pterin cofactor often termed the molybdopterin; although, the same cofactor has also been observed in tungsten enzymes and is therefore also referred to as the pyranopterin. The molybdopterin (Figure 1) cofactor is a three ring structure composed of a pteridine nucleus fused to a pyran ring and attached to the molybdenum through a dithiolene linkage (Romão et al., 1995). Eukaryotic enzymes typically contain the molybdopterin cofactor as shown, whereas those from prokaryotic sources contain a dinucleotide of guanine, cytosine, adenine, or hypoxanthine (Hille, 1996).
The molybdopterin cofactor is not directly involved in catalysis, but does have extensive hydrogen bonding to the polypeptide. Crystallographic data of various molybdenum hydroxylases indicate hydrogen bonding of the distal amino group of the molybdopterin cofactor to a cysteine residue of the iron-sulfur center, placing it in line for electron transfer out of the metal center (Hille, 2002).

The oxotransfer enzymes such as DMSO reductase contain two equivalents of the molybdopterin cofactor. Each pterin in this case is type-cast as either P or Q based on their electronic structure and orientation within the polypeptide. The Q-type pterin has a dithiolate ligand, while the P-type pterin exhibits a p-delocalized dithiolene ligand. The two types of cofactor are distinguishable using resonance Raman spectroscopy and will be discussed in more detail later. In those enzymes possessing the Fe/S centers, the Q pterin is located nearer the iron-sulfur (Hille, 2002). In contrast to the hydroxylase enzymes, the Q pterin in the oxotransfer enzyme lies near the surface of the protein and is thought to be the site of electron entry into the molybdenum center.

The oxotransferase enzymes in the sulfite oxidase family also contain the molybdopterin cofactor, however, only one equivalent. In the specific case of sulfite oxidase the molybdenum center is located some distance from the enzyme’s heme domain, which lies in a separate domain. It is thought that in solution the heme domain undergoes a conformational change bringing it closer to the distal end of the pterin (Hille, 2002).

The pterin cofactor may also play a role in modulating the reduction potential of the molybdenum center. The Mo-S bonds tend to lower the effective charge of the metal and decrease the reduction potential. This change in covalency from protein to protein
may change the reduction potentials of the Mo-center as well as other properties in various molybdoenzymes (Hille, 2002).

Specific mutations in the genes involved in biosynthesis of the pterin cofactor are pleiotropic and render all enzymes utilizing the cofactor inactive. The pathway has been studied in several organisms and involves the products of at least twelve genes (Rajagopalan, 1991; Rajagopalan and Johnson, 1992; Shanmugan et al., 1992; Plunkett et al., 1993; Palmer et al., 1994; Palmer et al., 1996; Johnson et al., 1991; Miller et al., 1987; Rech, et al., 1996; Maupin-Furlow et al., 1995; Rech, et al., 1996; Luque et al., 1993; Wang et al., 1993; Rivers et al., 1993; Hoff et al., 1995; Kamdar et al., 1994; Lee et al., 1990; Aguilar et al., 1992; Pitterle and Rajagopalan, 1993; Pitterle et al., 1993; Wuebbens and Rajagopalan, 993; Johnson et al., 1989; Johnson and Rajagopalan, 1987).

It is currently believed that the gene operons moa-mog encode the proteins needed in the biosynthesis of the molybdopterin cofactor in prokaryotes (Shanmugan et al., 1992). Synthesis of the mononucleotide form of the pterin is controlled by the two operons entitled moa and moe. In E. coli the moa operon encodes five proteins labeled MoaA-E. The moaA-C gene products are involved in the production of an intermediate known as precursor Z, which lacks the required sulfur atoms for coordination to the molybdenum, in the early stages of the biosynthesis pathway. Within these gene products at least two specific binding domains are present, a guanine binding domain, found in the MoaA product, and a folate binding domain, located in MoaB (Rivers et al., 1993; Kamdar et al., 1994; Hoff et al., 1995). The MoaD-E gene products function as MPT synthases and are involved in adding the sulfur atoms of the mature cofactor (sulfur charging is catalyzed by MoeB) to precursor Z (Rajagopalan and Johnson, 1992;
Evidence suggests the product of the gene molR, most likely a regulatory element, is required for moa operon function (Lee et al., 1990).

The mob gene locus encodes both MobA and MobB. MobA is a pterin guanine dinucleotide synthase involved in the addition of GMP to the molybdenum cofactor complex, and MobB is also thought to produce a guanine dinucleotide synthase, and is essential to the production of the cofactor (Plunkett et al., 1993; Palmer et al., 1994; Johnson et al., 1991). The mobA/mobB genes are deleted from an E. coli strain TP1000 that is used for recombinant protein expression of enzymes utilizing the mononucleotide form of molybdopterin, since naturally occurring E. coli inherently contains the genes for this dinucleotide molybdopterin production (Temple et al., 2000). The final step of cofactor biosynthesis involves the insertion of the molybdenum and incorporation into the apo-enzyme. The gene products MoeA and MogA are responsible for the sulfur charging of the molybdate (in the case of those enzymes that possess a Mo=S group) and the molybdate insertion into the cofactor, respectively (Pitterle et al., 1993; Wuebbens and Rajagopalan, 1993; Johnson et al., 1989; Johnson and Rajagopalan, 1987). Lastly the products of the modA-E genes are involved in a variety of tasks that include ATP hydrolysis, transcriptional regulation, binding of periplasmic molybdate, and transmembrane channel formation (Figure 1.3, Table 1.1) (Maupin-Furlow et al., 1995; Rech et al., 1996; Luque et al., 1993; Wang et al., 1993).
1.2 Mononuclear enzymes and the environment

Mononuclear molybdenum enzymes are widespread in nature. Assimilatory nitrate reductases, for example, are ubiquitous in higher plants and catalyze the reduction of nitrate to nitrite in the first step of reduction to ammonia (Hille, 1996). This enzyme has received much interest lately in the environmental field, with the identification of fertilizer pollution in high farming areas. In addition, environmental biologists have also observed thriving bacterial organisms in lakes with arsenate concentrations well above the previously known tolerance limit, sparking an interest in arsenite oxidase which catalyzes the conversion of highly toxic arsenite to a less toxic (albeit still dangerous) arsenate (Hille, 1996). Dimethylsulfoxide reductase catalyzes the reductive deoxygenation of DMSO to DMS. DMS in combination with the atmosphere is thought to be prevalent in cloud formation (Hille, 1996; Lynn, 1998).

1.3 Mononuclear enzymes in physiology

Health organizations have shown interest in enzymes such as xanthine oxidase, aldehyde oxidase, and sulfite oxidase due to the adverse effects of molybdenum deficiency diseases as well as their suggested roles in ischemia reperfusion injury, oxidative stress, and tissue damage. It has also been suggested that these enzymes, due to their wide substrate specificity, may play a role in detoxification. Xanthine oxidase, for example, may detoxify potentially oncogenic and dangerous compounds, such as Purine N-oxides by oxidizing them to a less dangerous form (Krenitsky et al., 1972, 1974; Rajagopalan, 1980). It has also been suggested that uric acid may function as an anti-
oxidant and radical compounds by scavenging singlet oxygen and radicals (Ames et al., 1981).

To date, there are three types of molybdenum deficiency diseases. Type I xanthinuria, a condition that is due to the loss of xanthine oxidase activity due to mutations in the gene encoding xanthine oxidoreductase. Type II xanthinuria results from loss of both xanthine oxidase and aldehyde oxidase activities and results from loss of a sulfurase enzyme responsible for adding sulfur necessary for active enzymes of this type. Patients with either type I or II xanthinuria are rare and usually asymptomatic. Combined molybdenum deficiency syndrome is more serious. This condition is due to the inability to synthesize the unique pterin cofactor chelated to the molybdenum, and results in loss of activity of all mononuclear molybdenum enzymes discussed thus far, most importantly sulfite oxidase. Symptoms most likely occur as a result of the loss of sulfite oxidase activity rather than xanthine or aldehyde oxidase activities. These symptoms, indistinguishable from those of sulfite oxidase deficient patients, are prominent and severe, including mental retardation, neurological abnormalities, severe convulsions, dysmorphic facial features and eventually death. Pathology of the two disorders are similar, often displaying neuronal loss and demyelination in white matter. Table 1.2 outlines the causes and symptoms of such molybdenum deficiency diseases (Johnson and Duran, 2001; Raivio et al., 2001).

In the oxidative half of its reaction, xanthine oxidase produces hydrogen peroxide, superoxide, and oxygen radicals. These products ascribe to xanthine oxidase a role in ischemia reperfusion and oxidative stress (Repine, 1991). Although in the xanthine dehydrogenase form, NAD$^+$ is used as the electron acceptor, the oxidase form of the
enzyme is only able to utilize molecular oxygen *in vivo* as the ultimate electron acceptor, therefore providing unfortunately efficient formation of reactive oxygen species. During ischemia, ATP is metabolized to hypoxanthine and xanthine, and the dehydrogenase form of the enzyme is converted to the oxidase form. As oxygen is reintroduced during reperfusion, the enzyme converts the excess substrates to uric acid, hydrogen peroxide and superoxide, thereby resulting in cellular damage (McCord, 1985; Granger, 1988; Saugstad and Aasen, 1980; Hassoun et al., 1992). The superoxide produced by xanthine oxidase may also have a positive effect on humans, since it has anti-microbial properties and has been implicated in defense against *Salmonella* and influenza virus (Umezawa et al., 1997; Akaie et al., 1990).

Allopurinol (Figure 1.4) is one of the most commonly prescribed drugs in the United States. It is an inhibitor of the enzyme xanthine oxidase, which in mammals catalyzes the last two steps of purine catabolism, hypoxanthine to xanthine and finally xanthine to uric acid. This drug is used in the treatment of gout, a disease characterized by the buildup of uric acid crystals in the joints, commonly in the big toe, and acts as a tight binding irreversible inhibitor of the enzyme by rebinding in a dead-end orientation after its initial hydroxylation. Inhibition of the enzyme stops further substrate turnover, thereby decreasing the amount of uric acid production. The drug has been shown to have minimal, if any, side effects.

1.4 The xanthine oxidase family of molybdenum enzymes

The present research focuses on the xanthine oxidase family, commonly referred to as molybdenum hydroxylases, which catalyze the hydroxylation of a large variety of
substrates ranging from aldehydes to aromatic heterocycles (Hille, 1992). Members of this enzyme family have the same basic constitution of redox-active centers: a molybdenum center, two 2Fe/2S centers of the plant ferridoxin variety, and one flavin adenine dinucleotide. The redox-active centers of this enzyme are organized in a linear fashion ideal for electron transfer (Figure 1.5) (Enroth et al., 2000). Electrons are passed into the molybdenum center from the substrate and then through FeS I, FeS II, and finally into the flavin center in the course of the reductive half reaction.

These enzymes have the unique characteristic of utilizing water as the ultimate source of oxygen incorporated into the hydroxyl group of the reaction product rather than molecular oxygen as many of the heme- or flavin-containing monooxygenase systems. The molybdenum hydroxylases also generate reducing equivalents during the course of the reaction, and thus require a physiological electron acceptor. The oxidase form of the xanthine-utilizing enzyme uses O$_2$ as its final electron acceptor, whereas the dehydrogenase form primarily uses NAD$^+$ (although it is also capable of utilizing O$_2$). The presence of a NAD$^+$ binding site constitutes the primary difference between the dehydrogenase and the oxidase. A more descriptive difference of the oxidase and dehydrogenase forms of the enzyme will be discussed later.

1.5 Xanthine oxidase from *Bos taurus*

*The structure of bovine xanthine oxidase and xanthine dehydrogenase*

The crystal structure of xanthine oxidase from bovine milk was determined to 2.5 Å resolution in 2000 by Enroth et al. (Figure 1.6). The structure indicates a homodimer of molecular mass 290 kDa, and has overall dimensions for the asymmetric unit of 155 Å
X 90 Å X 70 Å. The crystals were formed from purified enzyme following the standard purification method with a few modified changes. Both the XO and the XDH form of the enzyme were crystallized independently in the presence of 1 mM salicylate, which is not known to bind the enzyme, but rather sits in the active site blocking the binding of others. The addition of salicylate is known to stabilize the enzyme thereby keeping a larger percentage in the active and sulfurated form and is therefore commonly used during purification and storage. In the crystal structure, the salicylate molecule is located 6.5 Å from the metal and is held in place by several hydrogen bonds and electrostatic interactions. The dimer interface is located on the smaller side of the subunits giving the molecule a distinctive butterfly shape. Each subunit contains the following redox-active centers: one molybdenum center, two 2Fe/2S centers, and one FAD. The monomer is comprised of three different domains housing each of the four redox-active centers. The N-terminal domain is made up of residues 1 to 165 and contains both of the Fe/S center subdomains. Residues 226-531 make up the FAD domain and are connected to the iron sulfur domain by residues 166-225, although in the region containing residues 166-191 no electron density could be observed. A partially disordered segment made up of residues 532-589 link the second domain to the third domain housing the molybdopterin (residues 590-1332). The molybdopterin lies near the Fe/S and FAD domain interface. In the crystal structure, residues 1310-1331 on the C-terminal end make contact with another enzyme molecule in the crystal lattice (Enroth et al., 2000).

The first iron sulfur center (Fe/S I) is located on the C-terminal subdomain while Fe/S II lies in the N-terminal subdomain. Fe/S I is coordinated to the four cysteine residues, 113, 116, 148, 150 located close to the flavin ring. A four helix bundle in the
second domain coordinates to the iron cluster via cysteine residues 43, 48, 51, and 73 forming Fe/S II. The two Fe/S centers are distinguishable by EPR as well as their individual reduction potentials. A distance of 7.8 Å exists between the $7\alpha$-methyl carbon on the FAD ring and nearest iron on Fe/S II, which is 12.4 Å from the closest iron atom of the Fe/S I cluster. Approximately 14 Å exists between the molybdenum and the closest iron atom of Fe/S I (Enroth et al., 2000).

The FAD domain shows a hydrogen bonding pattern around the pyrimidine portion of the isoalloxazane ring. It is noted that the FAD is bound in an extended conformation in a rather deep cleft with its $si$-face of the isoalloxazane ring accessible to solvent. In the dehydrogenase form of the enzyme enough room exists for NAD$^+$ to position itself parallel to the accessible isoalloxazane ring. However, disruption of tyrosine 419 in the chicken XDH (corresponding to tyrosine 393 in the bovine enzyme) by labeling disrupts the binding of the NAD molecule. The opposite side of the isoalloxazane ring takes part in $\pi-\pi$ interactions a phenylalanine (Enroth et al., 2000).

Since the presented research involves spectroscopic and kinetic studies of both oxidase and dehydrogenase forms of the hydroxylating enzyme, it is necessary to briefly discuss the differences between the two structures. The overall folds of the two forms are identical, with no significant changes around the molybdenum center or the iron-sulfur clusters and thus far no differences in binding or catalysis of xanthine. The bovine form of XDH can be reversibly converted to the XO form by modification of cysteines 535 and 992, which are 15.7 Å apart which would hypothetically require a conformational change in order to form a disulfide bond. Bovine XDH can be irreversibly converted to the oxidase form by proteolyzing after lysine 551 with trypsin and cleaving after leucine 219.
and lysine 569 (Kuwabara et al., 2003). These amino acids remain visible in the crystal structure of XDH, but are disordered in the oxidase form of the enzyme. The most prominent difference between the two forms exists in the FAD domain. During XDH to XO conversion, the side chain of the aspartate 429 is moved from its contact to the C6 atom of the flavin and replaced with the side chain of arginine 426, changing the electrostatics of the flavin. This electrostatic change results in shifting of several residues on the si-side of the isoalloxazane ring by as many as 20 Å, while the residue positions on the re-side are essentially unchanged during the XDH to XO conversion. This conformational change near the FAD cofactor is the primary difference in the two structures, accounting for XDH’s ability to utilize NAD as an electron acceptor, while XO cannot. Also it is important to note that this small conformational change does not in any way affect the other redox-active centers or the course of the reductive half reaction, only the NAD binding access to the FAD. It is evident that NAD access to FAD is blocked after the glutamine 423 - lysine 433 loop has undergone conformational change (Enroth et al., 2000).

1.6 Spectroscopic studies of bovine xanthine oxidase

Since its purification from cow’s milk in 1924, xanthine oxidase remains one of the most extensively studied molybdenum enzymes (Dixon and Thurlow, 1924). The standard purification involves treatment of approximately 60 liters of fresh unpasturized cow’s milk with butanol, followed by two ammonium sulfate fractionations, a hydroxyapatite column, occasional treatment with carboxymethyl cellulose, and finally a sepharose 200 gel filtration step. Typical yields from a preparation of this size are 2.5 to
3.0 grams of purified enzyme with activities of approximately 60 to 80 percent based on activity to flavin ratios. The active form of the enzyme contains a Mo=S ligand. Replacement of the sulfur with oxygen yields a catalytically inactive form of the enzyme. The sulfur may be lost spontaneously or be removed experimentally by treatment in cyanide, which removes the sulfur as thiocyanate (Massey and Edmondson, 1970). Lack of molybdenum in the enzyme also results in an inactive form. The demolybdo form of the enzyme resulting from milk purification is typically very low but may increase if the cows have nutritional deficiency in molybdenum (Hille and Massey, 1985).

1.6.1 Absorbance spectroscopy

The UV/visible absorbance spectrum of xanthine oxidase provides a unique experimental handle on the enzyme. Figure 1.8 shows the spectra for both xanthine oxidase from cow’s milk and xanthine dehydrogenase from *Rhodobacter capsulatus*. The absorbance maximum at 450 nm (465 nm for the *R. capsulatus* xanthine dehydrogenase) is due both to the iron-sulfur centers and the flavin with an extinction coefficient of $37,800 \text{ M}^{-1} \text{ cm}^{-1}$, while the absorbance at 550 nm is due exclusively to the iron-sulfur centers. The absorbance from the molybdenum center is small and difficult to observe on the spectrum due to the large absorption of the flavin and iron-sulfur centers. Reduction of the enzyme leads to bleaching of these absorption maxima. The enzyme is rather quickly reoxidized in air, returning to it oxidized spectra. As mentioned above, this enzyme requires an active site sulfur in order to function catalytically. Removal of this sulfur can also result in a slight spectral change as seen upon incubation with potassium cyanide (Figure 1.9) (Massey and Edmondson, 1970).
Due to xanthine oxidase’s wide substrate specificity, a variety of substrates for the enzyme have been examined using absorption spectroscopy as well as several other methods of spectroscopy. The absorbance spectrum from the enzyme while reacting with these various substrates can give a broad range of information from relative amount of active enzyme to steady state and pre-steady state kinetics, such as the rates of reduction of xanthine oxidase when reacted with the following substrates: 2-hydroxy-6-methylpurine (McWhirter and Hille, 1991); lumazine (Kim et al., 1996; more); 2,5-dihydroxybenzaldehyde (Xia et al., 1999) and numerous others which will be discussed in the dissertation research.

1.6.2 Electron paramagnetic resonance studies

Although absorption spectroscopy is perhaps the most straightforward, a variety of other spectroscopic methods are available. One that has proven most useful historically is electron paramagnetic resonance (EPR) due to the paramagnetic properties of reduced states of each of the redox-active cofactors. Figure 1.10 shows the characteristic EPR signals of each of the redox centers: FADH, reduced Fe/S I and Fe/S II, and Mo (V) (Hille, 1996).

The blue neutral form of the flavin semiquinone exhibits a characteristic isotropic EPR signal with a single g-value of 2.0006, a g-value very near that of the free electron and a line width of 19.4 G (Hille, 1992). The Fe/S I signal, observable at up to 40 K, is very similar to that of the spinach ferridoxin. The Fe/S II signal differs by irregular features, higher g-values, and temperature dependence of Fe/S II is only observable
below 22 K (Hille et al., 1985; Barber et al., 1982; Lowe et al., 1978; Calderia et al., 2000).

Perhaps the most useful application of EPR are studies of the molybdenum center. A multitude of signal types arise from this Mo (V) oxidation state. These signals are referred to as the “very rapid,” “rapid type I,” rapid type II,” “slow,” and “desulfo inhibited.” As the names suggest, these signals, with the exception of the desulfo inhibited signal, appear and disappear on different time scales in the course of enzyme reaction with substrate (Bray, 1988). The “slow” signal is formed with the desulfo form of the enzyme. The “rapid type I and II” signals are easily generated. They are formed by reacting substrate with xanthine oxidase that has been partially reduced. The enzyme can be reduced by either adding sodium dithionite or excess substrate. The signals are indicative of substrate in contact with enzyme that is already the Mo (V) oxidation state. Although these signals are not representative of an actual catalytic intermediates since the molybdenum is already in the paramagnetic state when it encounters the substrate, they are of considerable mechanistic importance since the species can be thought of as a Michaelis complex analog (Hille, 1996). Bray and coworkers (1969) have observed that the “rapid type I” signal observed from dithionite reduced enzyme is only slightly affected by the addition of xanthine, suggesting only a minor change in the coordination sphere of the metal on binding substrate, which implies that the molybdenum is not directly coordinated to the substrate (Hille, 1996).

The “very rapid” Mo (V) signal is the most catalytically important EPR signal. Kinetic studies of xanthine oxidase with 2-hydroxy-6-methyl purine (HMP) have identified this compound as an effective, yet slow, substrate that generates large amounts
of the “very rapid” signal (McWhirter and Hille, 1991). At pH 10.0, the signal exhibits g-values as follows: $g_1 = 2.0252$, $g_2 = 1.9550$, and $g_3 = 1.9494$. The “very rapid” signal differs from the “rapid” signals because it lacks the proton hyperfine structure seen in the “rapid” species (Bray and Vänngård, 1969; Bray and George, 1985). The signal forms to such great extent due to the slow rate of decay for the signal giving species ($0.11 \text{s}^{-1}$) compared to the rate of its formation ($1.3 \text{s}^{-1}$) (McWhirter and Hille, 1991). The reaction shows maximal signal intensity after approximately 40 seconds at $4^\circ\text{C}$ (as compared 10ms when reacted with xanthine) and can be reproducibly prepared at pH 10.0 under aerobic conditions, which consumes excess reducing equivalents thereby avoiding a build up of electrons in the iron-sulfur and flavin centers. (McWhirter and Hille, 1991).

Several isotopic substitutions have been used to gain a good understanding of the “very rapid” species. The species has been produced using xanthine labeled in the C-8 position with a $^{13}\text{C}$ in order to examine if the substrate nucleus is involved in the signal-giving species. A distinct isotropic splitting, resulting from the substituted carbon has established that bound substrate is part of the signal giving species (Tanner et al., 1978). Some of the first evidence for the catalytic importance of the sulfur group on the molybdenum came from observation of anisotropic coupling in the EPR “very rapid” signal when generated with $^{33}\text{S}$ labeled enzyme (Wahl and Rajagopalan, 1982; Malthouse and Bray, 1980; Malthouse et al., 1981). A strongly coupled oxygen was identified by reacting in $^{17}\text{O}$ labeled water, demonstrating the involvement of the Mo-OR species (Greenwood et al., 1993; Dowerah et al., 1987). The question of whether the oxygen was from the Mo-OH or the Mo=O interaction was addressed by examining EPR signals generated from model compounds with known crystal structures. The results showed that
the $^{17}$O was more strongly coupled when in the form of Mo-$^{17}$OH rather than as the oxo group (Greenwood et al., 1993; Dowerah, et al., 1987).

1.6.3 ENDOR and ESEEM studies of the “very rapid” species

The “very rapid” species has also been examined by other EPR-related techniques including electron spin echo and electron double resonance spectroscopy (ENDOR). Examination of the electron spin-echo envelope modulation (ESEEM) of the “very rapid” signal, generated with HMP, showed strong coupling to two nitrogen nuclei presumably from the substrate as well as the protons upon comparison to the desulfo inhibited signal formed from reduced cyanide inactivated enzyme with ethylene glycol (Howes et al., 1996; Lorigan et al., 1994). ENDOR studies have demonstrated some weak coupling to solvent exchangeable protons in the “very rapid” signal that was not identifiable in the EPR spectra (Howe et al., 1990). ENDOR has also been used to examine the distance between the molybdenum atom on the enzyme and the carbon (C8 in xanthine) on the substrate in the “very rapid” species. Based on these studies, a distance of 2.8 Å was determined, thereby suggesting a bridging atom (presumably oxygen) between the molybdenum and carbon rather than direct Mo-C bonding.

1.6.4 X-Ray absorption spectroscopic studies of the molybdenum coordination sphere

X-ray absorption spectroscopy (XAS) is a useful tool for study of the molybdenum environment in xanthine oxidase. Figure 1.11 shows the XAS data for the enzyme xanthine oxidase in oxidized, reduced, reduced bound to violapterin, and alloxanthine inhibited. The ligands to the oxidized molybdenum are assigned as a Mo=S
group, a Mo=S group, a singly bonded oxygen atom (either Mo-OH or Mo-OH$_2$, and the sulfur atoms contributed by the pterin cofactor (Enroth et al., 2000; Tullius et al., 1979; Bordas et al., 1980; Cramer and Hille, 1985; Hille et al., 1989; Turner et al., 1989). The XAS structure of the oxidized enzyme estimate a bond distance of 2.15 Å for the sulfo group, 1.68 Å for the oxo group, and 1.98 Å for the oxygen atom in the oxidized form of the enzyme. In the reduced form the distances for the sulfo, oxo, and oxygen are 2.39 Å, 1.67 Å, and 2.24 Å respectively (Tullius et al., 1979; Bordas et al., 1980; Cramer and Hille, 1985; Hille et al., 1989; Turner et al., 1989). As addressed in the Figure legend, at the time of these results, it was believed that the sulfide held the apical position; however, it has recently been convincingly shown that the oxo group is in apical position (Okamoto, 2004). Despite this geometrical difference, the XAS data has been extremely useful in identifying the metal ligands in the coordination sphere, as well as their bond distances.

1.6.5 Resonance Raman studies of xanthine oxidase

Although extensive studies involving the xanthine oxidase mechanism have not been examined using resonance Raman, a few studies have been undertaken. In 1990 Oertling and Hille used resonance Raman to examine the charge-transfer complex formed between reduced xanthine oxidase and violapterin, the product of the reaction of oxidized enzyme with lumazine (Figure 1.12). The charge-transfer complex has a long wavelength absorption near 650nm and has been identified as a true catalytic intermediate which represents the reduced enzyme product complex (Davis et al., 1982). Initial Raman studies of this complex exhibited a high background fluorescence, yet still
yielded support for the formation of a Mo-O-R species (Oertling and Hille, 1990). A more detailed explanation of the resonance Raman findings from the enzyme reduced – violapterin complex will be presented in Chapter 2 of this dissertation research.

Molybdenum-ligand vibrations have been examined by Raman spectroscopy for both the active and inactive forms of the enzyme. The Mo (VI) =S stretch is observed at 474 and 462 cm\(^{-1}\) upon sulfur isotopic substitution. The Mo=O stretch is commonly very weak and difficult to observe, however a weak intensity at 899 cm\(^{-1}\) has been assigned to the stretch in the active or sulfurated form of the enzyme. The dioxo or inactive form of the enzyme exhibited still weak but shifted intensity at 892 cm\(^{-1}\) (Maiti et al., 2003).

The pterin cofactor of xanthine oxidase has been characterized as a P-type pterin based on comparative Raman studies with DMSO reductase, an enzyme containing two equivalents of the pterin cofactor, one P-type and one Q-type (Figure 1.13) (Johnson et al., 1984). As mentioned above, each pterin classified as either P, containing a \(\pi\)-delocalized dithiolene, or Q, containing a dithiolate ligand. The \(\pi\) – delocalization in the P-type pterin lowers the energy of \(\nu(C=C)\), observed at 1525 cm\(^{-1}\) in DMSOR, and increases the \(\nu(C-S)\) energy, located at 858 cm\(^{-1}\) in DMSOR, relative to the expected energies. Whereas in the Q-type pterin the expected frequencies of 1575 cm\(^{-1}\) and 764 cm\(^{-1}\) for the \(\nu(C=C)\) and \(\nu(C-S)\), respectively are observed (Table 1.3) (Garton et al., 1997).

1.7 Mechanistic and kinetic studies of xanthine oxidase

As described above, xanthine oxidase catalyzes the oxidative hydroxylation of hypoxanthine to xanthine followed by that of xanthine to uric acid, the last two steps of
purine catabolism in mammals. The overall reaction also results in generation of superoxide and hydrogen peroxide. Steady state studies produce Lineweaver-Burk plots with parallel lines when plotting $1/V_{\text{max}}$ versus $1/[\text{xanthine}]$ at varying oxygen concentrations (Olson et al., 1974; Greenlee and Handler, 1964), indicating that the reaction can be divided into two half-reactions (Figure 1.14). The first of these, known as the reductive half-reaction, occurs at the molybdenum center and involves the hydroxylation itself. The oxidative half-reaction occurs at the flavin center (Bray et al., 1964; Komai et al., 1969).

1.7.1 The oxidative half-reaction

As indicated above, the oxidative half-reaction of the enzyme occurs at the flavin center and utilizes molecular oxygen as the final electron accepter, producing hydrogen peroxide and superoxide. The kinetics of the enzyme reduction with $O_2$ are clearly biphasic with a fast phase exhibiting hyperbolic dependence on oxygen concentration. The kinetic parameters for the fast phase are dependent on pH and have been determined as follows: $k_{\text{lim}}$ increases from $13 \text{ s}^{-1}$ at pH 7 to $350 \text{ s}^{-1}$ at pH 10. $K_d$ values also increase from $80 \mu \text{m}$ at pH 7 to $1.4 \text{ mM}$ at pH 10, however the increases in both parameters are such that the $k_{\text{lim}}/K_d$ is essentially unchanged over this pH range (Olson et al., 1974). The slower phase of the reaction exhibits a linear dependence on oxygen and has an observed rate constant of $1.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6 and $7.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ at pH 10. It has been speculated that the slow phase represents the reoxidation of one electron reduced xanthine oxidase to the fully oxidized form of the enzyme (Olson et al., 1974). Further studies utilizing cytochrome c and cytochrome c peroxidase have been used to scavenge
superoxide and hydrogen peroxide, respectively (Hille and Massey, 1981; Porras, 1981). The results of these studies indicate a reoxidation cycle as seen in Figure 1.15 where the six electron reduced enzyme is oxidized to four electron reduced enzyme in the first step, followed by another two electron oxidation. The last two steps involve one electron oxidation. Hydrogen peroxide is produced in the first two steps, while the byproduct of the last two oxidation steps is \( \text{O}_2^- \).

1.7.2 The reductive half-Reaction

The reductive half of the overall reaction occurs at the molybdenum center as it cycles from Mo(VI) to Mo(IV) during the conversion of xanthine to uric acid. It is this half of the reaction that is the principal focus of this dissertation research. Both steady-state and pre-steady state kinetic studies have been undertaken for a number of substrates including xanthine, 1-methylxanthine, and HMP (Edmondson, et al., 1973; Palmer et al., 1964; Davis et al., 1982; Kim and Hille, 1993; Olson, et al., 1974; McWhirter and Hille, 1991). Comparison of the steady-state and pre-steady state kinetics for a given substrate shows small changes in rates (e.g., for xanthine \( k_{\text{cat}} = 15 \text{ s}^{-1}, K_m(\text{xanthine}) = 9 \text{ mM} \) and \( k_{\text{lim}} = 17.5 \text{ s}^{-1}, K_d(\text{xanthine}) = 13 \text{ mM} \) at pH 8.5), indicating that the limiting reaction occurs in the reductive half-reaction. Studies of 8-deuteroxanthine demonstrate a kinetic isotope effect of 1.7 on the \( k_{\text{cat}}/K_m \) (Edmondson, et al., 1973). The kinetic isotope effect of the deuteron located on the carbon in the 8 position implicate the C8-H/D bond breakage is the specific rate limiting step within the reductive half reaction. Because xanthine oxidase can accept six electrons (two on the molybdenum center, one in each of the two iron sulfur centers, and two on the flavin center) it thus requires three substrate molecules to
reach full reduction (Hille and Massey, 1982). Experiments involving reaction with limiting substrate ensure single turnover conditions and indicate that the reductive half-reaction occurs in a three-step sequence involving free enzyme plus free substrate going to a transition state intermediate forming enzyme plus product as shown here: \[ E+S \rightarrow ES^+ \rightarrow E+P. \]

Xanthine oxidase is unique from a number of other hydroxylating enzymes in that in the ultimate source of oxygen comes from water rather than molecular oxygen (Hille, 1992). Under multiple turnover conditions, the reaction of unlabeled xanthine oxidase with \(^{18}\)O-labeled water resulted in labeled product (Murray et al., 1969). However, under single turnover conditions, it has been demonstrated that the oxygen is incorporated from a labile site on the enzyme. In 1987, Hille and Sprecher reacted unlabeled enzyme with limiting substrate in labeled water and found no labeled product. The reaction was repeated with labeled enzyme and unlabeled water, and labeled product was obtained. EPR spectroscopy has also been used to examine oxygen atom transfer in the reductive half-reaction. Comparative EPR studies using \(^{17}\)O labeled and unlabeled water have shown stronger coupling to the hydroxyl rather than the oxo-group of the molybdenum center, suggesting the hydroxyl oxygen in labile (Xia, et al., 1999).

In addition to the identification of the labile oxygen, the pH dependence of the reductive half-reaction has also been examined. Steady-state and pre-steady state kinetics of the reaction of xanthine with xanthine oxidase was completed at a pH range from 5 to 10 within 0.5 pH unit intervals (Figure 1.16) (Kim et al., 1996; Olson et al., 1974). The bell-shaped curve obtained from a pH versus \( k_{\text{lim}}/K_d \) plot identifies two \( pK_a \)'s of 7.4 and 6.6. The \( pK_a \) of 7.4 agrees well with that of the first ionization of the substrate xanthine.
indicating that the enzyme prefers to act on the neutral form of the substrate. By definition of \( k_{\text{lim}}/K_d \), the data represents the reaction of free enzyme and free substrate; therefore, the second pK\(_a\) of 6.6 must represent the enzyme, indicating base assisted catalysis. The residue proposed to be responsible for this ionization is glutamate 1261 of the bovine form of the enzyme, which is positioned just below the molybdenum center active site (Kim et al., 1996).

Computational studies have also been extremely helpful in the understanding of the mechanism. Relative energies of each of the three relevant tautomeric form of the neutral form of the substrate have been calculated and the 1H, 3H, 7H tautomer shown to be favored. Upon reaction, the enzyme substrate intermediate switches from sp\(^2\) to sp\(^3\) hybridization at the C8 carbon, which results in a cross-over in energy, with the 1H, 7H, 9H tautomer as most energetically favorable (Figure 1.17). A tautomerization involving proton shift from N3 to N9 thus appears to be required during catalysis (Ilich and Hille, 1997).

A mechanism for the reaction of enzyme with formamide, a known substrate for xanthine oxidase, has been computationally derived. The yield generated a plausible mechanism for this reaction (Figure 1.18). The mechanism supports sp\(^3\) hybridization of the intermediate formed between enzyme and substrate. Negative charge accumulation on the hydrogen of the substrate is also demonstrated suggesting hydride transfer. The effectiveness of this hydride transfer is dramatically affected by substitution of Mo=O for the Mo=S, illustrating the importance of the catalytically essential sulfide group (Ilich and Hille, 2002; Ilich and Hille, 1997).
1.7.3 *Electron transfer in xanthine oxidase*

As described above the oxidative and reductive half-reactions occur at different sites on the enzyme. It is therefore necessary to consider the electron transfer between these two sites. Based on the structure described above, the Fe/S centers are expected to mediate the electron transfer between the molybdenum and the flavin. The structure also shows the pterin portion of the molybdenum cofactor pointed out towards the first Fe/S center. Reductive titrations of xanthine oxidase with sodium dithionite have suggested a simple distribution of reducing equivalents among the given redox-active centers based on their reduction potentials (Olson et al., 1974). The reduction potentials were measured using potentiometry by following formations of the characteristic EPR signals of the two reduced Fe/S centers, the FADH\(^\ast\), and the Mo (V) oxidation state as a function of poised system potential (Cammack et al., 1976). A variety of modified flavins with midpoint potential ranging from -126 mV to -280 mV were substituted into xanthine oxidase and reacted with substrate. It was found that in enzymes substituted with a high potential flavin, the FAD was reduced before the iron-sulfur centers (Hille and Massey, 1991).

The pH dependence for the reduction potentials has also been examined. All of the reduction potentials for the enzyme exhibit pH dependence suggesting electron-transfer is linked to protonic equilibria. The flavin is found to exist predominately in the hydroquinone form at physiological pH; its quinine/semiquinone and semiquinone/hydroquinone potentials both decrease over the pH range 7 to 10. Both of the potentials from the molybdenum (Mo(VI/V) and Mo(V/IV)) also decrease over the pH range 6 to 10. The pH effect on the Fe/S centers is smaller than that seen with the other cofactor, but still decreases from pH 6 to 10 (Porras and Palmer, 1982).
The rates of electron transfer between these redox-active centers have been examined using flash photolysis, pulse radiolysis, and pH jump techniques. Electron transfer between the FAD and Fe/S II has been observed at approximately 100 s\(^{-1}\) (Bhattacharyya et al., 1983; Walker et al., 1991). The pH jump technique involves rapid mixing partially reduced xanthine oxidase in a dilute buffer at either pH 6 or 10 (depending on the direction of pH jump) with a concentrated buffer of a different pH. These experiments were conducted in both H\(_2\)O and D\(_2\)O. (Hille and Massey, 1986; Hille, 1991). The spectral change observed is due to the electron transfer between Fe/S I and the FAD and increases from 155 s\(^{-1}\) at pH 6 to 330 s\(^{-1}\) at pH 10. The spectral change observed upon mixing differs from the spectral change followed kinetically (representing the electron transfer between Fe/S I and FAD), and was expected to represent electron transfer from Fe/S I to the molybdenum. This second portion of the spectral change occurred within the dead time of the stopped-flow apparatus (approximately 2 ms). These results indicate that the slowest electron transfer (from FAD to Fe/S I) at 100 s\(^{-1}\) is at least 10 times faster than \(k_{cat}\) of the reaction at any pH (Hille and Massey, 1986; Hille, 1991). Protonation and deprotonation events were examined by the comparison of the D\(_2\)O and H\(_2\)O experiments. A solvent kinetic isotope effect of seven was observed for the electron transfer step between the FAD and Fe/S I. Single exponential kinetic transients indicate the effect mentioned above is due to a single proton (Hille, 1991).

Two kinetic phases with rate constants of 8500 s\(^{-1}\) and 125 s\(^{-1}\) at pH 6.0 were observed using pulse radiolysis. These rate constants represent intramolecular process, such as electron transfer, since they are independent of enzyme concentration. The slower phase represents the electron transfer from FAD to Fe/S I and agrees well with the
pH jump results. The electron transfer from the Fe/S I to the molybdenum is represented by the faster phase (Anderson et al., 1986; Anderson and Hille, 1991; George, 1985).

1.7.4 Proposed mechanism for the reductive half-reaction of xanthine oxidase

The current proposed mechanism (Figure 1.19) will be discussed in greater detail throughout this dissertation; but a brief introduction at this point is appropriate. It is currently believed that the glutamate 1261 residue acts as an active site base and initiates catalysis by abstracting the proton off the Mo-OH group. This enables the enzyme to nucleophilically attack the C8 position of the substrate, which is followed by hydride transfer from the C8 proton to the sulfur on the molybdenum forming a Mo-SH and bringing about formal reduction of the molybdenum. The step is proposed to occur in one individual two-electron step, forming a reduced enzyme-product complex with Mo (IV). One electron oxidation to form Mo (V), along with deprotonation of the Mo-SH to regenerate the Mo=S species (with product still bound) yields the “very rapid” species observed by EPR. The product is then released via the rate limiting step, and the enzyme is oxidized and prepared for a second catalytic cycle (Xia et al., 1999; Kim et al., 1996; Kim and Hille, 1993; Hille, 1997; Ilich and Hille, 1997; Ilich and Hille, 2002; McWhirter and Hille, 1991; Hille, 1996).
Figure 1.1  The structure of the molybdopterin cofactor. The molybdenum is attached via a dithiolene linkage. In enzymes from eukaryotic sources the pterin cofactor exists as shown above. Enzymes from prokaryotic sources, however, are found as a dinucleotide of guanine, cytosine, adenine, or hypoxanthine. The figure was adapted from Hille, R. (1996) *Chem. Rev.* 96, 2757-2816.
The molybdenum active site of the three families of mononuclear molybdenum enzymes. The xanthine oxidase family with one molybdopterin equivalent, and Mo=O and a Mo=S. The sulfite oxidase family with one molybdopterin equivalent and to Mo=O groups. The DMSO reductase family has two molybdopterin equivalents.

Figure 1.2
**Figure 1.3** The biosynthesis of the molybdenum cofactor. Precursor Z is first formed, followed by MPT synthesis, and finally molybdate insertion. The Figure was taken from Hille, R. (1996) *Chem. Rev.* 96, 2757-2816.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene product function</th>
</tr>
</thead>
<tbody>
<tr>
<td>moaA</td>
<td>early steps in the formation of precursor Z</td>
</tr>
<tr>
<td>moaB</td>
<td>Possible carrier protein – precursor Z formation</td>
</tr>
<tr>
<td>moaC</td>
<td>Early steps of precursor Z formation</td>
</tr>
<tr>
<td>moaD</td>
<td>MPT synthase α</td>
</tr>
<tr>
<td>moaE</td>
<td>MPT synthase β</td>
</tr>
<tr>
<td>mobA</td>
<td>Formation of dinucleotide form the pterin</td>
</tr>
<tr>
<td>mobB</td>
<td>Formation of dinucleotide form of the pterin</td>
</tr>
<tr>
<td>moeA</td>
<td>Charging the molybdate with sulfur for insertion into the cofactor</td>
</tr>
<tr>
<td>moeB</td>
<td>Charging of the MPT synthase with sulfur</td>
</tr>
<tr>
<td>mogA</td>
<td>Insertion of molybdate into the cofactor</td>
</tr>
<tr>
<td>modA</td>
<td>Molybdate binding protein in the periplasim</td>
</tr>
<tr>
<td>modB</td>
<td>Formation of transmembrane channel</td>
</tr>
<tr>
<td>modC</td>
<td>ATPase</td>
</tr>
<tr>
<td>modD</td>
<td>Presently not known</td>
</tr>
<tr>
<td>modE</td>
<td>Molybdate sensitive transcription regulator</td>
</tr>
</tbody>
</table>

**Table 1.1  Genes involved in the biosynthesis of the molybdenum cofactor.**
<table>
<thead>
<tr>
<th>Type</th>
<th>Cause</th>
<th>Results/Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 Xanthinuria</td>
<td>Lesions on xanthine oxidase gene</td>
<td>Loss of xanthine oxidase activity – usually asymptomatic</td>
</tr>
<tr>
<td>Type 2 Xanthinuria</td>
<td>Loss of sulfurase activity needed for producing active enzyme</td>
<td>Loss of xanthine oxidase and aldehyde oxidase activity – usually asymptomatic</td>
</tr>
<tr>
<td>Combined molybdenum cofactor deficiency</td>
<td>Inability to synthesize unique pterin cofactor</td>
<td>Loss of all molybdopterin containing enzyme function including sulfite oxidase – severe physiological abnormalities, mental retardation, dislocation of ocular lenses, death</td>
</tr>
</tbody>
</table>

Figure 1.4 The drug allopurinol is used in the treatment of gout, a disease characterized by the formation of uric acid in the joints. In the course of reaction allopurinol is converted to alloxanthine, tight binding inhibitor of the subsequent reaction of xanthine oxidase to hypoxanthine and finally uric acid. The Figure was taken from the web site http://www.chemsoc.org/exemplarchem/enteries/2001/sinnot/gout.htm.
Figure 1.5  Linear arrangement of the redox centers of bovine xanthine oxidase. The molybdenum is shown in grey with the pterin cofactor attached via a dithiolene linkage. Fe/S I is next in line to the molybdopterin, followed by Fe/S II. The FAD positioned last furthest to the right. The bottom portion of the picture is oriented 90 degrees rotation about the horizontal axis. It is important to briefly note that there is still some ambiguity regarding the geometrical position of sulfido group. Originally assigned by the crystal structure work, albeit inconclusive at that resolution, the sulfur was equatorial. However, recent evidence supports an apical sulfur as would be chemically expected. The picture was modified from Hille, R. (2002) *Met. Ions. Biol. Sys.* **39**, 187-226.
Figure 1.6

The crystal structure of bovine xanthine oxidoreductase. The molybdenum domain is shown in red, iron-sulfur domains shown in blue and green, and the FAD domain shown in grey. The crystal structure was solved by Enroth and coworkers and published in the *Proceedings of the National Academy of Science* in 2000.
Figure 1.7

Figure 1.7 The stereo representation of the conformational change in the active site loop containing Gln 423-Lys 433 upon conversion from the dehydrogenase to the oxidase form of the enzyme. The conformation of the loop in the dehydrogenase form is shown in green and that of the oxidase form shown in red. The Figure was adapted from Enroth et al. (2000) *PNAS* 97, 10723-10728.
The Absorbance spectra of bovine xanthine oxidase and *Rhodobacter capsulatus* xanthine dehydrogenase shown in both oxidized and reduced. The dehydrogenase form of the enzyme had an absorbance maxima from the Fe/S and flavin at 465nm shifted from 450nm in the oxidase form.
Figure 1.9

Figure 1.9 The spectral change observed upon incubation of xanthine oxidase with cyanide. The Figure was adapted from Massey, V. and Edmondson, D. (1970) *J. Biol. Chem.* **245**, 6595-6598.
Figure 1.10 The characteristic EPR signals seen with xanthine oxidase.
Various structures molybdenum coordination sphere in xanthine oxidase derived from XAS data and comparisons to the crystal structure data of similar enzyme aldehyde oxidoreductase from \textit{D. gigas}. The Figure was adapted from Hille, R. (1996) \textit{Chem. Rev.} \textbf{96}, 2757-2816. The figure represents a summary of data from the following sources: Tullis et. al. (1979) \textit{J. Amer. Chem. Soc.} \textbf{101}, 2776-2779; Bordas et. al. (1980) \textit{Biochem. J.} \textbf{191}, 499-508; Cramer and Hille (1985) \textit{J. Amer. Chem. Soc.} \textbf{107}, 8164-8169; Hille et. al.(1989) \textit{Inorg. Chem.} \textbf{28}, 4018-4022; and Turner et. al. (1989) \textit{Biochem. J.} \textbf{260}, 563-571. As previously mentioned, at the time of publication the sulfido was thought to be in the equatorial position, however, much evidence for the apical sulfide now exist.
Figure 1.12

The resonance Raman spectra of the reduced enzyme – violapterin complex. The charge-transfer complex has an absorbance at 650 nm, and was excited at 676.4 nm. Spectrum (a) represents free violapterin, (b) the CTC complex, (c) 18-O labeled VO in the CTC.
The Resonance Raman spectra of xanthine oxidase in its deflavo form (top), xanthine oxidase with the flavin intact (middle), and flavin alone in free solution (bottom). The bands at 1002, 1156, and 1523 cm$^{-1}$ characterize the pterin as a P-type pterin.
Table 1.3  Vibrational Energies of pterin modes in various enzymes  The vibrational energies of the P-type pterin modes for various enzymes: dimethysulfoxide reductase, sulfite oxidase, aldehyde oxidase, and xanthine oxidase.  The Table data was compiled from the following references:
Figure 1.14 The overall reaction of xanthine oxidase. The reductive half-reaction includes the reaction at the molybdenum center as it cycles from Mo(IV) to Mo(IV). Electrons are passed through the Fe/S centers and finally to the flavin, where the oxidative half-reaction occurs.
Figure 1.15  The oxidative half-reaction of xanthine oxidase. In the course of reoxidation of the enzyme ranging from 6 electron reduced XO to fully oxidized XO, hydrogen peroxide is produced in the first two oxidation steps, while oxygen radicals are produced in the last two steps.
Figure 1.16 The pH dependence $k_{cat}/K_m$ or $k_{red}/K_d$ of xanthine oxidase with xanthine. The dark squares represent steady state data collected by Kim et al., 1999 and the open squares represent data collected by Olson et al., 1974. Filled circles represent the pre-steady state data. The triangles show the rapid reaction data in D$_2$O. The pK$_a$ of 7.4 agrees well with the first ionization of the substrate. The pK$_a$ of 6.6 is proposed to be the ionization of the glutamate 1261 residue. The Figure was adapted from Kim et al. (1999) *J. Biol. Chem.* **271**, 6771-6780.
Figure 1.17

The relative free energy calculations for relevant tautomeric forms of the xanthine. The lowest energy free substrate is the 1H, 3H, 7H form. However, upon binding to the enzyme and conversion to sp$^3$ hybridization, the 1H, 7H, 9H tautomer is favored. The Figure is adapted from Ilich and Hille (1997) Inorg. Chim. Acta 263, 87-94.
Figure 1.18 The resulting mechanism proposed based on computational studies of the substrate formamide with xanthine oxidase. The results indicate a partial sp$^3$ hybridization on the carbon, negative charge accumulation on the hydrogen, and less effective hydride transfer upon substitution of Mo=O for Mo=S. Note that at the time of publication, the sulfur was believed to be in the apical position, although this is not thought to be the case currently. The Figure was adapted from the following references: Ilich and Hille (2002) *J. Am. Chem. Soc* **124**, 6796-6797; Ilich *et al.* (1997) *J. Phys. Chem. B* **101**, 10923-10932.
Figure 1.19 The proposed mechanism of the reductive half-reaction of xanthine oxidase. Involving base assisted nucleophilic attack on the C8 position of the substrate. The Figure was adapted from Hille, R. (1996) Chem. Rev. 96, 2757-2816.
CHAPTER 2

RESONANCE RAMAN STUDIES OF THE CHARGE-TRANSFER COMPLEX
FORMED BETWEEN BOVINE XANTHINE OXIDASE AND VIOLAPTERIN

2.1 Introduction

Vibrational energies of specific chemical bonds provide biochemists with information regarding electron distribution and structural aspects of proteins. Both resonance Raman (rR) and infrared (IR) spectroscopy are useful in observing the vibrational nature of specific bonds within a compound. The frequency of each vibration can be directly correlated to a specific chemical bond, and will shift upon isotopic substitution. Shifts in vibrational energies are also observed upon formation of a complex of multiple compounds, and can give information about electron distribution upon binding of two compounds. Due to the spectroscopic nature of the amide bonds of a protein backbone as well as the aqueous environment of such macromolecules, rR has proven a much more useful tool than IR due to the selection rules that forbid observation of amide bond vibration in rR spectra, but exist very prominently in IR.

Although molybdenum enzymes such as sulfite oxidase and DMSO reductase have been studied using rR, xanthine oxidase has not been extensively studied using this method (Garton, et al., 1997; Garton et al., 1997; Johnson et al., 1997). Xanthine oxidase
was first examined by rR in 1985 by Willis and Loehr, who examined the flavin and iron sulfur centers, but did not observe resonance enhancement of the molybdenum center. This observation was not surprising considering the extremely small absorbance attributable to the metal center in comparison to the other cofactors.

In 1982 Davis, Olson, and Palmer observed a long wavelength absorbance increase at 650nm upon reduction of XO with lumazine (2,4-dihydroxypteridine). It was demonstrated that this long wavelength absorbance represents the charge-transfer complex of a catalytically relevant complex of reduced enzyme with product bound at the molybdenum center (Figure 2.1). There is no evidence of charge-transfer complex formation with the cyanide-treated desulfo enzyme. Addition of allopurinol, a potent inhibitor of xanthine oxidase, which acts at the molybdenum center, also hinders complex formation indicating that the reaction is indeed occurring at the molybdenum (Davis, et al., 1982). The product bound in the charge-transfer complex is violapterin (2,4,7-trihydroxypteridine) and the complex has been examined by XAS (Figure 2.1). Although it is possible to produce this complex by reaction of oxidized xanthine oxidase with lumazine, reduction of the enzyme with dithionite in the presence of the product (violapterin) quantitatively generates the same charge-transfer complex. In 1990, Oertling and Hille successfully conducted rR studies of the reduced enzyme product complex at the molybdenum center proving resonance Raman’s usefulness for the mechanistic study of the enzyme. Figure 1.11 shows the spectra obtained from this study. The reaction was repeated with 18-O labeled violpaterin. The results were interpreted as indicating a Mo-O-R species at the molybdenum center with product
coordinated to the molybdenum via the catalytically introduced hydroxyl group (Oertling and Hille, 1990).

Although this study provided useful information concerning the nature of the intermediate species, spectra with greater signal-to-noise are required for detailed analysis of the rR spectrum. The present work uses the normal mode analysis previously completed in the laboratory by Craig Hemann and Dr. Predrag Ilich along with computational evaluation of model compounds to assign frequencies to specific modes of the charge-transfer complex. The current work has allowed the assignment of the C7-O stretch in the bridging oxygen and evaluation of electron distribution in the charge-transfer complex. As mentioned above, this charge-transfer complex represents the first intermediate in the reductive half-reaction. Information about the mechanism and product release is inferred from the rR study of this complex. The FAD center in xanthine oxidase is highly fluorescent upon laser excitation. In order to remove some of the fluorescence background, the charge-transfer complex was formed both with flavo and deflavo enzyme and examined by laser excitation at 647nm. The reductive half-reaction occurs at the molybdenum center and therefore would not be affected by CaCl₂ removal of the non-covalently attached flavin. Flavo and deflavo enzyme spectra gave identical results, however the observed intensity was greater for the deflavo enzyme.

The rR spectra and normal mode analysis for violapterin free in solution is also required for a comparison with the enzyme-bound data. The mode analysis for the rR spectrum has been completed using computational models and predicted hydrogen deuterium shifts by Craig Hemann and Dr. Predrag Ilich in the laboratory (Figure 2.2). The rR spectra from free violapterin were used in addition to computational studies to
assign bands in the charge-transfer complex to vibrational modes. The comparisons of these spectra are discussed further in the results and discussion section.

The detailed mode analysis of the complex has permitted assignments of the C7-O bond vibration in the violaptenin. It is this oxygen which is involved in the bridge from product to reduced enzyme in the charge-transfer complex (the C7 carbon on the violaptenin is analogous to the C8 carbon on xanthine). The rR work presented here provides information about the first intermediate in the reductive half-reaction where the enzyme has been reduced by two electrons and product is formed, but not yet released. Information about electron distribution and product release, as well as mechanistic possibilities can be inferred from such a study.

2.2 Materials and methods

2.2.1 Purification of bovine xanthine oxidase

Xanthine oxidase was purified from cow’s milk using the procedure described by Massey et. al. (1969) with minor modifications. Sixty liters of unpasturized milk was obtained from The Ohio State University Dairy Farm and was utilized for the prep within two days of milking. Each of the 25 liter carboys containing 20 liters of milk was treated with 50 ml 0.3 M EDTA pH 7.0, 20 ml of 1.0 M sodium salicylate pH 7.0, 6.0 g of cysteine hydrochloride, 20 ml of 0.1 M PMSF dissolved in ethanol, and 316 g sodium bicarbonate (added slowly). After each carboy was stirred for 5 minutes 31.6 g of pancreatin was added. The milk solution was stirred for 20 to 25 minutes and allowed to sit overnight at 5 °C. The following morning the milk solution was taken to 20% ammonium sulfate and stirred for one hour. After the hour long stir, pre-chilled butanol
(-20°C) was added to take the solution to 10% (v/v) butanol. The solution was stirred vigorously for 5 minutes, then slowly for 30 minutes. The butanol treated milk was allowed to settle overnight. The next morning the lower brown aqueous layer was drained out of the carboy into an open top 25 liter carboy and stirred while slowly adding ammonium sulfate to 50%. It was important to take care that the upper lipid-containing layer from the carboy was not mixed into the aqueous layer at this stage in order to prevent chromatography problems caused by lipid contamination. The precipitate from the 50% ammonium sulfate cut was allowed to coagulate for one hour during which it floated to the top of the solution. The lower greenish aqueous phase was discarded and the brown enzyme containing layer from the second carboy was added directly to the open top carboy containing the precipitated protein from the first carboy. This method was repeated for the third carboy and the crude xanthine oxidase was transferred to centrifuge bottles using nitrile gloves, a skimming spoon, and a screen followed by centrifugation at 17,700 x g for 30 minutes. The centrifuge bottle contained three layers, a bottom aqueous layer, a middle precipitated protein layer, and a top butanol layer. The liquid butanol and aqueous layers were decanted and the solid precipitate layer was removed from the bottles and resuspended in 0.1 M phosphate buffer at pH 6.0 containing 0.3 mM EDTA, 10 µM PMSF, and 1 mM salicylate. The protein was then dialyzed in 45 mm dialysis tubing against 20 liters of the resuspension buffer with five changes over 2 days. The dialyzed protein was centrifuged at 17,700 x g for 30 minutes to remove any precipitated protein. The supernatant was filtered through glass wool and concentrated to about half of its dialyzed volume (400 ml). Half of the concentrated crude enzyme was loaded onto the hydroxyapatite column equilibrated with
dialysis/resuspension buffer. The column was washed extensively with the same buffer and eluted in 3 ml fractions using a Gilson fraction collector with a linear pH gradient consisting of 400 ml 0.1 M phosphate buffer at pH 6.0, 0.3 mM EDTA, 1 mM salicylate, 10 µM PMSF to 400 ml 0.1 M pyrophosphate buffer at pH 8.5, 0.3 mM EDTA. The fractions were then examined for contaminating lactoperoxidase as evidenced by a characteristic shoulder at 410 nm using the spectrophotometer. The uncontaminated fractions were combined, concentrated to approximately 500 µM and run on a large S-200 column equilibrated with 0.1 M pyrophosphate, 0.3 mM EDTA pH 8.5 in 5-10 ml increments. The eluent was collected in 100 ml fractions using a Gilson fraction collector. The eluted fractions from the S-200 column were diluted to an OD of about 0.2 at 450 nm and the A_{276} to A_{450} ratio was calculated. Enzyme with a ratio less than six was considered pure, concentrated to approximately 600-1000 µM, and frozen in 2-4 ml aliquots using liquid nitrogen. Depending on the length of storage, 1.0 mM of salicylate was added to some enzyme batches prior to storage. Enzyme with ratios higher than 6.0 was concentrated and loaded onto the S-200 for a second run. Typical yields of the xanthine oxidase from 60 liters of milk were 2 - 2.5 grams of protein usually 60-80% active.

2.2.2 Chemicals

Lumazine (2,4-dihydroxypteridine) was purchased from Aldrich and used without further purification. Violapterin (2,4,7-trihydroxypteridine), prepared by Dr. Eun-Young Choi in the laboratory, was generated by reaction of xanthine oxidase with lumazine. Approximately 1 µM of oxidized xanthine oxidase and catalase (purchased from Sigma)
was mixed with 2 mM lumazine in 10 mM ammonium acetate buffer at pH 8.5. The solution was incubated at room temperature until no further spectrum indicative of lumazine was observed. The solution was concentrated in an Amicon concentrator with a 50,000 molecular weight cut off filter. The filtrate was evaporated to dryness using a rotary evaporator. Thin layer chromatography was used to examine the purity. If further purification was needed the solution was purified using HPLC under the following conditions: 0-100% acetonitrile, 50 mM tricine pH 8.1, 1.0 ml/min, octadecylsilane prep column. The product came off the HPLC in three peaks: the first small peak exhibited no spectral properties indicative of violapterin or lumazine; the second peak was lumazine (based of the absorbance spectra); and the third, represented pure violapterin.

\(^{18}\text{O}\) labeled water was purchased from Isotec, Inc. and was 85% enriched. The \(^{18}\text{O}\) violapterin was prepared by lyophilizing approximately 0.5 ml of xanthine oxidase (50 µM) and bringing it up in \(^{18}\text{O}\) labeled water. This yielded \(^{18}\text{O}\) labeled enzyme which was then reacted with excess lumazine as described above. The labeled enzyme hydroxylated the lumazine in the C7 position yielding \(^{18}\text{O}\) labeled violapterin. \(\text{D}_2\text{O}, \text{NaOD}, \text{and DCl were purchased form Cambridge Isotopes, Inc.}\)

2.2.3 Sample preparation

Room temperature samples were prepared by making a 1.5 ml solution of 60 µM active xanthine oxidase (in 20 mM pyrophosphate buffer with a two-fold excess violapterin) anaerobic. The absorbance spectrum was then followed as the enzyme was slowly titrated to full reduction with dithionite solution (prepared by bubbling buffer with argon gas and adding solid dithionite). The reaction was followed spectrophotometrically
until maximal absorbance at 650 nm was observed and the solution had turned a distinct
green color, indicating formation of the charge-transfer complex. The solution was then
transferred using an anaerobic syringe to a previously argon flushed Raman cell sealed
with a rubber septum.

Cryogenic samples were prepared by concentrating enzyme in 20 mM Bicine
buffer at pH 8.1 in an Amicon Microcon (100,000 molecular weight cut off) until the
enzyme was viscous to the point that further concentration was impossible. The enzyme
centration was estimated at approximately 2 mM based on the absorbance reading at
450 nm of a 1 in 100 dilution. The concentrated enzyme solution was transferred to a 1.5
ml eppendorf® tube by centrifugation and 2-3 grains of solid violapterin was dissolved in
the enzyme solution (the solid rather than aqueous violapterin was added in order to
avoid enzyme dilution). The concentration of violapterin was estimated at 5-10 mM.
Argon gas was blown over the enzyme solution for approximately 1.5 hours. The
eppendorf® tube was then transferred to an anaerobic glove bag which had been flushed
with dry argon for approximately 2 hours. The glove bag was flushed for an additional
hour after the sample was inserted. Approximately 10-20 grains of solid dithionite was
added to the anaerobic mixture from inside the glove bag. The solution was stirred gently
with a pipet tip, taking care not to create any bubbles, for approximately 5 minutes as the
color turned dark green/black. The eppendorf® was then transferred to an anaerobic
environment surrounding the cryo-finger of a closed-cycle liquid helium refrigerator
which had also been flushed with dry argon for approximately one hour. The dry argon
was used in order to minimize condensation forming on the cryo-finger during sample
freezing. The sample was then carefully transferred to the pre-chilled cryo-finger via a pipetmen and frozen.

Deuterium-substituted samples were prepared by running the enzyme through a G-25 column (Sigma) equilibrated with the D$_2$O buffer, which was adjusted to proper pD by addition of NaOD and DCl. The pD readings were estimated by use of the following simple relationship: pD = pH +0.4. The deuterium-substituted samples were then prepared as described above. $^{18}$O labeled product samples were prepared by reaction of fresh enzyme, prepared as described above, with $^{18}$-O labeled violapterin prepared as described in materials and methods.

2.2.4 Data collection

Raman spectra for the various samples examined in the present study were collected via the procedure described by Hemann et. al. (2003). Excitation utilized a Coherent 599 standing wave dye laser with DCM dye, pumped by a Coherent INNOVA 307 continuous-wave argon ion laser. The laser was first passed through a Pellin-Broca prism to remove undesired laser lines. A Chromex 500IS 0.5 meter single stage spectrograph was used. Backscattered photons were collected 60 degrees relative to the beam and Raleigh-scattered photons were rejected by a spectrographic slit in a holographic notch filter. The detector was a LN/CCD-1024TKB from Princeton Instruments, Inc. interfaced to a Gateway 2000 486/33C computer with CCD Spectrometric Multi-channel Analysis software version 2.2a. Band frequencies in the Raman spectrum were calculated using Peak-Fit from Jandel Scientific. Calibration standards were as follows: indene for cryogenic samples, ethylacetate and a 1:1 mixture
of benzene and carbon tetrachloride for the room temperature samples. Room temperature samples were placed in an anaerobic 5mm quartz Raman cell and spun using a standard NMR tube spinner. Cryogenic samples were placed on the cryo-finger and frozen under dry anaerobic air.

2.2.5 Computational

Geometrical optimization and vibrational frequencies were calculated using density functional theory implemented in the Gaussian 98 software package. The three parameter exchange functional of Becke combined with Lee, Yang, and Parr correlational functional was used (Becke, 1988; Lee et al., 1988). All calculations utilized Stuttgart/Dresden effective core potentials for molybdenum and the Dunning/Huzinaga valence double-zeta basis for the all other atoms (referred to as SDD in the Gaussian 98 user’s manual) (Dunning and Hay, 1976; Frisch and Frisch, 1994-1999). Gaussian 98 installed on a Silicon Graphics Octane 2 computer was used for calculations. Other calculations were completed on a Digital Equipment Corporation VAX 4000/90 workstation and an Aspen Systems, Inc. Durango 500 alpha workstation (Ilich, unpublished FORTRAN software).

2.3 Results and discussion

2.3.1 Resonance Raman of free violapterin

Figures 2.2 and 2.3 show the resonance Raman spectra of free violapterin at pH/D 7.5. At this pH/D, violapterin exists as a monoanionic form based on its pK_a values of 3.4 and 9.8 (Hemann and Choi, unpublished; Bergman et al., 1979). The specific
tautomeric forms for each ionization state, however, were ambiguous (Figure 2.4). The spectra collected by Hemann, Choi, and Hille were compared to calculated vibrational modes for each of the anionic forms. The data best fits the calculations for the monoanion protonated at positions 1 and 3, deprotonated at position 8. These calculations were also used to predict mode shifts with hydrogen/deuterium substitutions. Analyses of this type have also been done on uracil and lumazine (Ilich et al., 1997; Hemann et al., 2003).

One of the most mechanistically interesting vibrational modes of the violapterin, is the C7 oxygen stretch, this being that band which acts putatively as the bridge between the molybdenum and violapterin in the charge-transfer complex. In previous studies, the C7 stretching modes have also been predicted utilizing the calculations as described above, but could not be assigned to specific experimentally observed bands due to the large amount of peak overlap in the 1510 cm\(^{-1}\) to 1590 cm\(^{-1}\) region. In order to aid in this assignment, the spectrum of \(^{18}\)O labeled violapterin was collected (originally at room temperature by Dr. Eun-Young Choi and repeated at cryogenic temperatures in the current study.) As seen by the comparison in Figure 2.3, the 1557 cm\(^{-1}\) peak has shifted to lower frequency by 13 cm\(^{-1}\) which agrees well with the calculated band shift. Craig Hemann and Dr. Predrag Ilich in the laboratory completed the normal mode analysis for violapterin in free solution by comparing experimental data from the isotopic substitutions and the calculated vibrational frequencies. Using these assignments as background, the charge-transfer complex was examined in the current study.
2.3.2  Computational models used in the CTC mode analysis

Models in this study were developed by Craig Hemann and Dr. Predrag Ilich in the laboratory using the published alloxanthine inhibited crystal structure data of *Rhodobacter capsulatus* xanthine dehydrogenase as a starting-point (Truglio, et al., 2002; PDB code 1JRP). The structure for calculations was developed by modeling violapterin in place of the alloxanthine present in the crystal structure, coordinated via the bridging oxygen to the C7 position on the violapterin. The model was modified by protonation at the pyrazine moiety of the pterin ring and substitution of a Mo-SH for the apical Mo=S. The described model consists of the violapterin bound to the molybdenum containing the pterin cofactor (Figure 2.7). A smaller model was also used which consisted only of the two-electron reduced molybdenum-ene-dithiolate bound to the violapterin (Figure 2.6).

The larger model containing the modified molybdopterin cofactor was optimized to the lowest energy using Gaussian 98 as described in the materials and method section. The lowest energy geometric structure showed the Mo=O group in the apical position and the Mo-SH group in the equatorial plane with the bridging oxygen and the ene-dithiolate sulfurs. This result is in contrast to previous crystallographic studies that assigned the Mo=O group in the equatorial plane leaving the Mo=S in the apical position; however, the resolution was not distinct enough to allow an unambiguous assignment. The assignment of an apical Mo=S group was made on the basis of comparison to the crystallographic data for a similar enzyme, aldehyde oxidase (Truglio et al., 2001; Enroth et al., 2000). More recent studies with a new tight binding inhibitor (FYX-051) indicate that the oxo group is in fact in the apical position, consistent with these calculations (Okamoto et al., 2004).
Based on the newly discovered apical Mo=O group, a smaller model was utilized containing only the ene-dithiolate portion of the pterin cofactor as described above. The initial positions of the oxo, sulfhydryl, and hydroxyl groups were varied, allowing Gaussian to optimize independently in each case. Figure 2.6 shows initial (lower) geometry and the optimized (upper) geometries in each case. For each, the oxo group moved into the apical position upon optimization. The optimized geometry for the molecules in panels C and D are identical to those in panels E and F, therefore leaving only four possible geometries which need to be further considered (Panels A, B, C, and D). Using Gaussian 98, these four geometries were examined with the larger model incorporating the cofactor described above. Figure 2.7 shows the optimized energies for these four geometric structures ranked with most stable on top to least stable on bottom. Structure A was the model used for calculations of vibrational frequencies for the charge-transfer complex in the current studies.

2.3.3 Resonance Raman spectra of the charge-transfer complex

With the above as background, the xanthine oxidase charge-transfer complex was prepared as described in Materials and Methods in either H₂O or D₂O and excited at 647 nm. Figure 2.8 shows the resonance Raman spectra of the charge-transfer complex for the room temperature sample. The D₂O sample was prepared both to gain H/D shift information and shift the broad OH feature out of the carbonyl stretching region. The lines between the pH and the pD spectra indicate the H/D shifts. The largest shift observed upon deuterium isotope substitution is 60 cm⁻¹ seen by the 1169 cm⁻¹ band, although the 1678 cm⁻¹ to 1621 cm⁻¹ shift is also quite large. These shifts were then
compared to the calculated vibrational frequency shifts and assigned to a particular vibrational mode. The relevant vibrational modes for the charge-transfer complex are shown in Table 2.1 and named by specific “Q” designations. (The modes illustrated in Table 2.1 are named based on the computational results using the model described above and do not correspond to the names based on free violapterin calculations.) A comparison of the charge-transfer complex and free violapterin in H₂O is shown in Figure 2.9. Several bands in the 350 to 1750 cm⁻¹ region are presumably due to product binding. Band shifts are observed upon substitution into D₂O and ¹⁸O- labeled product. Violapterin vibrational modes in the 1400 to 1600 cm⁻¹ region are enhanced with excitation at 647 nm in the spectra of the CTC. Bands in the 250 to 1100 cm⁻¹ are most likely due to molybdenum – ligand vibrations. In the H₂O/¹⁶O spectra (figure 2.2), five bands at 1563, 1467, 852, 774, and 516 cm⁻¹ shift upon ¹⁸O substitution. It is important to note that complete mode assignments were not possible by direct comparison to the free violapterin, thus the basis for using the more complicated model described above. Although the hydrogen/deuterium substitutions enabled tentative mode assignments for several of the relevant vibrational modes, further study was required to eliminate some peak overlap as well as assign the most interesting vibration from the C7-O bridge.

The C7-O vibrational energy was examined by substitution of ¹⁸O for the naturally-occurring ¹⁶O violapterin. The sample spectra containing either labeled or unlabeled product bound to either labeled or unlabeled enzyme are shown in Figure 2.5. Relatively few band shifts were identifiable in this sample spectrum. The 412 cm⁻¹ band shifts down by 26 wave numbers from the Mo=¹⁶O/¹⁶O-VO to the corresponding Mo=¹⁸O/¹⁸O-VO spectra, however it is predicted to shift by only 5 cm⁻¹. Similarly, the
1467 cm\(^{-1}\) band downshifts by 12 cm\(^{-1}\), although no shift was expected. No shift was observed at the 1583 cm\(^{-1}\) peak despite the 13 cm\(^{-1}\) downshift predicted. The 1467 cm\(^{-1}\) and 1583 cm\(^{-1}\) bands are certainly in the carbonyl stretching region, but do not shift as expected based on the comparison to free violapterin. The large amount of peak overlap in this region also makes confident mode assignments difficult.

These difficulties involved two things, which were addressed by the current study. The first was to duplicate the experiment with higher purity violapterin and more concentrated enzyme. The violapterin was further purified by HPLC chromatography as described in Materials and Methods and the enzyme was concentrated to approximately 2 mM. The experiment was completed by using a very high enzyme concentration, followed by addition of solid purified violapterin. The total volume of 30-40 µl was frozen on a cold finger and the data were collected cryogenically. Each of the described samples was duplicated and examined in comparison to the free violapterin. The most significant difference can be seen by examining the spectra collected for the \(^{18}\text{O}\) substituted violapterin in complex with reduced xanthine oxidase (Figure 1.10). The bands are much more intense than those seen with the room temperature data and are better resolved. The enlarged region represents the carbonyl stretching region containing bands at 1554 cm\(^{-1}\), 1569 cm\(^{-1}\), or 1587 cm\(^{-1}\). One or more of these bands are expected to represent the C7-O stretch. Unfortunately, despite the improved intensity, there are still overlapping peaks in this region preventing the confident assignment of the C7-O stretch.

The second part of the solution addressed in the current study was a computational approach. As described above, the geometrically optimized and lowest energy structure determined by Gaussian 98 is shown in Figure 2.7. This model was used to calculate
frequencies and their predicted isotopic shifts. This method proved to be much more accurate for mode assignments than direct comparison to free violapterin. Figure 2.2 shows the final resonance Raman spectra of the charge-transfer complex in H₂O, D₂O, and ¹⁸O-C7 with mode assignments based on computational studies. The “Q” designations on the spectra correspond directly to the mode plots shown in Table 2.1. Table 2.2 summarizes calculated and observed frequencies for each mode in the H₂O, D₂O, or ¹⁸O substitutions. A total of 26 modes shifts are calculated in energy region represented by the spectrum. The expected shifts upon ¹⁶O/¹⁸O substitution are presented in the center column. These shifts are typically quite small, with only six modes having a calculated shift of 5 cm⁻¹ or more. These small shifts may be surprising considering this is a bridging atom: One might expect that this atom would have a rather large vibrational profile due to the two rather large substituents attached at each end. On the other hand, the small shift can be rationalized for the same reason; two large and undoubtedly heavy substituents are positioned on either side of the bridging oxygen, so one could speculate the isotope substitution may not be as significantly noticed as it would in a typical carbonyl group.

In the carbonyl stretching region, two bands are expected to shift. The first corresponds to mode Q23, with calculated bands at 1556 cm⁻¹ shifting to 1546 cm⁻¹. This calculation agrees well with the experimental 1563 cm⁻¹ and 1548 cm⁻¹ bands. The Q23 mode represents strong C7-O stretching as well as C-6-H and N-1-H bending vibrations. The second calculated band is at 1471 cm⁻¹, corresponding to mode Q28, is expected to shift to 1463 cm⁻¹ agreeing with the observed 1467 cm⁻¹ and 1455 cm⁻¹ bands. The Q28 mode consists of N-1-H bending, ring distortion, and a small amount of C7-O stretching.
The 900 cm\(^{-1}\) to 1400 cm\(^{-1}\) region contains no predicted shifts of greater than 5 cm\(^{-1}\), which is also observed experimentally. The four calculated significant shifts are expected in the low-frequency region, including modes which primarily involve ring distortions that necessarily torque the bridging atom. Only three of these four shifts are observed in the experimental data: The Q63/Q64 shift which is calculated to shift 9 cm\(^{-1}\), but actually shifts 5 cm\(^{-1}\); the Q68/Q68 shift predicted and observed to shift 5 cm\(^{-1}\); and the Q91/Q91 with an expected shift of 6 cm\(^{-1}\), and observed shift of 4 cm\(^{-1}\). The predicted shift of 5 cm\(^{-1}\) for Q95/Q95 is not observed experimentally.

In addition to the modes mentioned above, others were also observed and assigned. C2=O stretching motions and N-1-H/N-3-H bends dominate the Q14 mode in the H\(_2\)O spectra. In the D\(_2\)O spectra, Q16 is made up of an in-phase C2=O/C4=O stretch. The predicted frequencies for these modes are 1690 cm\(^{-1}\) and 1663 cm\(^{-1}\), respectively. These agree well with the observed bands at 1707 cm\(^{-1}\) and 1675 cm\(^{-1}\), respectively, reflecting in a 32 cm\(^{-1}\) downshift. C4=O stretching coupled to N-3-H (N-3-D) bending comprise the Q17 modes. The protonated model predicted a frequency of 1662 cm\(^{-1}\), which is observed at 1678 cm\(^{-1}\). In comparison, the predicted band in the deuterated model is 1643 cm\(^{-1}\), observed at 1621 cm\(^{-1}\). The predicted shift is only 19 cm\(^{-1}\), while the experimentally observed shift is much larger, 57 cm\(^{-1}\). Stretching and displacement of the C7-O/C7 position as well as N-H/C-H bending are evident in mode pairs Q22/Q21, Q23/Q22, Q26/Q24, and Q28/Q25. These mode pairs have predicted shifts of 6 cm\(^{-1}\), 11 cm\(^{-1}\), 8 cm\(^{-1}\), 6 cm\(^{-1}\), and observed shifts of 13 cm\(^{-1}\), 27 cm\(^{-1}\), 11 cm\(^{-1}\), and 11 cm\(^{-1}\), respectively. The mode pairs Q43/Q41, Q46/Q44, Q63/Q63, and Q68/Q69 all contain
N-D bending. They are expected (observed) to downshift by 52 (15) cm\(^{-1}\), 31 (60) cm\(^{-1}\), 25 (17) cm\(^{-1}\), and 31 (23) cm\(^{-1}\), respectively.

In conclusion, the agreement between the predicted and observed shifts for both the H/D or \(^{16}\)O/\(^{18}\)O substitutions overall is quite good when comparing against the calculations based on the larger model. The C7-O stretch, represented by modes Q23/Q22, Q28/Q25, Q63/Q64, and Q91/Q91, are assigned to frequencies at 1563 cm\(^{-1}\), 1467 cm\(^{-1}\), 852 cm\(^{-1}\), and 516 cm\(^{-1}\), respectively. The generally good agreement supports the lowest energy model which was computational derived and justifies its use in the further understanding of the mechanism of xanthine oxidase. Our model also provides evidence for the apical oxo group, rather than apical sulfhydryl. The described study has provided a conceptually clear understanding of the vibrational motions and electronic distribution of the reduced enzyme-product complex, a true catalytic intermediate.

Discrepancies in the predicted versus observed shifts are most prominent in the H/D comparisons. This can be explained by the interactions with the protein. Several residues have been suggested to interact with the enzyme-product complex including glutamic acids 802 and 1261 in the bovine enzyme (232 and 730 in the \textit{Rhodobacter capsulatus} enzyme) (Truglio et al., 2002). These residues will be discussed in further detail in Chapter 4 of this dissertation.


**Figure 2.1** The XAS structure of the reduced enzyme-product complex formed with reduced xanthine oxidase and violapterin. The complex has an absorbance at 650 nm and is a true catalytic intermediate. The E-red-P structure also forms a charge-transfer complex. The Figure was adapted from Hille, R. (1996) *Chem. Rev.* **96**, 2757-2816.
Figure 2.2

Figure 2.2 The final resonance Raman spectra of the charge-transfer complex collected at room temperature. The “Q” number present mode assignments as seen by the mode plots shown in Table 2.1. The sample in D$_2$O is shown on top, $^{18}$O substitution at bridging carbon C7 in the middle, and the H$_2$O spectrum on bottom.
Figure 2.3 The resonance Raman spectra of violapterin in free solution at pH 7.5 (bottom) and pD 7.5 (top). H/D shifts are indicated by the lines and numbers between the two spectra.
Figure 2.4

Figure 2.4 The resonance Raman spectra collected at 677 nm (room temperature) of the charge-transfer complex formed between reduced enzyme and violapterin at pH 8.3 (bottom) and pH 8.1 (top). H/D shifts are represented by numbers and lines between the two spectra.
Figure 2.5

Figure 2.5  The resonance Raman spectra (room temperature) of the charge-transfer complex formed between reduced xanthine oxidase and violapterin. These spectra represent the various combinations of unlabeled/labeled enzyme and/or violapterin. The large amount of peak overlap in the 1500 cm$^{-1}$ to 1600 cm$^{-1}$ make assignment of the C7-O stretch difficult.
Figure 2.6 The smaller model used in this study. The model consisted of only the enedithiolate portion of the pterin cofactor. Molybdenum is shown in cyan, sulfur in yellow, oxygen in red, carbon in green, and hydrogen in white. The molecule in the lower half of each panel represents the starting geometry. The upper half of each panel contains the optimized geometry. Molekel 4.0 was used to produce the Figures.
Figure 2.6
**Figure 2.7** The four different geometries obtained from the calculations of the smaller model. The energies were then calculated using the full molybdopterin model. Energy differences from molecule A are shown below B-D. Molybdenum is in green, sulfur in yellow, oxygen in red, carbon in grey, nitrogen in blue, and hydrogen in white. The Figures were produced using Molekel 4.0 and Rasmol V2.7.2.1.
Figure 2.7

A

B

\[ \Delta E = 0.3 \text{ kcal} \]

C

\[ \Delta E = 0.9 \text{ kcal} \]

D

\[ \Delta E = 1.1 \text{ kcal} \]
Figure 2.8

Figure 2.8 The resonance Raman spectra of the XO/VO charge-transfer complex at pH 8.3 (bottom) and pD 8.1 (top). The tentative mode assignments here apply to comparisons made with free violapeterin rather than with computational work and do not correspond to the modes shown in Table 2.1.
Figure 2.9

The resonance Raman spectra comparison of violapterin in complex with reduced xanthine oxidase (top) and free in solution (bottom). The data was collected at 677 nm and at room temperature. Band which arise on the top half, not observed on the bottom half are presumably due to complex formation.
Continued

Table 2.1 Mode Plots for the Charge-transfer Complex
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Table 2.2 Predicted and observed frequency shifts for isotopic substitutions in the CTC. The calculated and observed frequency shifts for H/D or $^{16}$O/$^{18}$O isotopic substitutions in the charge-transfer complex.
**Figure 2.10**

**Figure 2.10** The cryogenic sample data for the $^{16}$O/$^{18}$O product complex with reduced enzyme, or the charge-transfer complex. The enlarged region, representing the carbonyl stretching region, is of particular interest due to the C7-O-Mo bridge formed by this complex.
CHAPTER 3

KINETIC EXAMINATION OF THE FIRST STEP IN THE REDUCTIVE HALF
REACTION OF BOVINE XANTHINE OXIDASE

3.1 Introduction

The mechanism of the reductive half-reaction of xanthine oxidase has been of interest for many years. Recent data has provided the most definitive evidence for specific stages in the reaction. As indicated in Chapter 1, kinetic studies of the pH dependence of enzyme action on its substrate xanthine identify two pKₐs in the pH dependence of kₐ/K₈. This parameter follows the interaction of free enzyme and free substrate, and the second pKₐ of 7.4 agrees with the first ionization of the substrate xanthine, indicating that the neutral form of the substrate is preferred by the enzyme. The first pKₐ of 6.6 must be due to an ionizable group on the enzyme, suggesting base catalysis (Kim et al., 1996). Oxygen atom transfer has also been addressed using EPR and ENDOR to identify the source of a labile oxygen on the enzyme. Upon $^{17}$O substitution, stronger coupling is observed for the Mo-OH than for the Mo=O, suggesting the labile oxygen site is the hydroxyl group. Evidence for base catalysis and oxygen transfer are not the only interesting conclusions derived from recent data about the
reductive half-reaction of xanthine oxidase. McWhriter and Hille (1991) have identified two intermediates in the course of hydroxylation of the slow substrate hydroxymethylpurine, which were concluded to arise from the Mo (IV) and Mo (V) redox states, the latter species giving rise to the so called “very rapid” signal (named on the basis of its rate of appearance and disappearance observed by EPR). This “very rapid” species consists of the Mo (V) redox state of the product bound to metal.

Resonance Raman and XAS studies have identified and examined in detail the preceding Mo (IV)-Product complex formed between reduced xanthine oxidase and violapterin, the product of the enzyme hydroxylation of lumazine. This complex has a long wavelength absorbance at 650 nm and represents a true catalytic intermediate (Oertling and Hille, 1990; Hille et al., 1989).

More recently, there has been debate as to how the enzyme was bridged to the product. Understanding this bridging was essential to differentiating between alternate mechanisms that had been proposed. Hille and coworkers suggested a Mo-O-R species, while Bray and coworkers involved a direct Mo-R attachment (Xia et al., 1999; Howes et al., 1996). Previous Raman studies supported the Mo-O-R species, (Oertling and Hille, 1990) however further studies were required to completely rule out the direct Mo-R bonding. In contrast, however, Howes et. al. (1994) estimated (by dipolar coupling calculations) the distance between the molybdenum and the carbon using ENDOR spectroscopy of $^{13}$C substituted samples. The discrepancy was addressed again in an examination of the Mo (V) “very rapid” as well as other Mo (V) species. In 1996, Howe and coworkers revisited the $^{13}$C ENDOR samples of several of the Mo (V) species. From calculations based on the $^{13}$C hyperfine interactions in comparison to observed
anisotropy, a general estimate of less than or equal to 2.0 Å was reported, suggesting a Mo-C bond. Manikandan et al. (2001) conducted a similar ENDOR study using $^{13}$C-hydroxymethylpurine, which forms to a large degree with improved signal to noise the “very rapid” species. The results for this study identified 2.8 Å distance between the molybdenum and the C8 position of the product (Figure 3.2). This study discussed the ENDOR spectrum in the context of several parameters including anisotropic hyperfine and the effect of $^{13}$C nucleus spin density and the effect of orbital hybridization on the carbon, eliminating the possibility of a Mo-C bond. Current crystallographic studies of the bovine enzyme, as well as the similar xanthine dehydrogenase enzyme from *Rhodobacter capsulatus*, also support the Mo-O-R interpretation.

The above studies then suggest product bound to molybdenum of two different oxidation states via bridging oxygen. It is now established that the “very rapid” Mo (V) species contains a Mo-O-R bridge, however, the chemistry by which the species is formed was still undetermined. It was possible to arrive at both of these product bound intermediates via two different mechanisms. The first involved Mo (VI) gaining two electrons in a single step, forming the Mo (IV) state. The molybdenum (V) redox state was then expected to occur via a one-electron oxidative event after formation of the Mo(IV)-Product species. This mechanism involves a base catalyzed (presumably by glutamate 1261) abstraction of a proton off the Mo-OH group. The second involved a two-step process where the Mo (VI) is reduced one electron at a time through Mo (V) and Mo (IV) oxidation states via a xanthinyl radical species as illustrated in Figure 3.1. Either of these mechanisms could account for Mo (V) species formation seen by kinetic,
EPR and ENDOR studies, however, ENDOR studies also suggest an end-on binding of
the product in both the Mo (V) and Mo (IV) species (13-C ENDOR).

There is much indirect evidence consistent with the one-step mechanism. The
kinetic studies of hydroxylmethylpurine suggest that the Mo (IV) intermediate occurs
before the Mo (V) intermediate based on the rates of formation for each of the observed
intermediates, for example, the formations of the Mo (V) EPR-active species (the “very
rapid” species) results from an oxidative event rather than a reductive event as evidenced
by the free reducing equivalent released during this step of the reaction (McWhirter and
Hille, 1991). The one-step mechanism is also consistent with base catalysis as described
in Figure 1.18 and is supported by the source of the oxygen atom incorporated into the
product being Mo-OH rather than Mo=O (Hille and Sprecher, 1987; Howes et al, 1994,
1996; Xia et al., 1999). Further evidence for the base catalyzed mechanism was
identified earlier by Bray et. al. (1978) with the observation that the C8 proton of the
substrate is transferred to the molybdenum center during the course of reaction. Despite
this support for this mechanism, more evidence was needed in order to rule out the
possibility of a recently proposed mechanism involving two sequential one-electron
reduction steps as described above (Figure 3.1). In this mechanism, the substrate is
oxidized by one electron via direct-electron transfer to the molybdenum center of the
enzyme. The newly formed Mo (V) - S•⁺ species breaks down by the next one-electron
reductive event. Incorporation of oxygen occurs with the second one-electron reductive
event (Page et al., 1999).

In the current study, we have taken advantage of the wide substrate specificity of
xanthine oxidase for twelve commercially available purine substrates (Figure 3.3) in
order to differentiate between these two mechanisms. If the reduction of the molybdenum center is occurring one electron at a time, then the kinetic rate parameters should correlate linearly with the one-electron reduction potentials of each of the substrates. Therefore, the reductive half-reaction rate parameters $k_{\text{red}}$ and $k_{\text{red}}/K_d$ were determined for each of these substrates (if the substrates were effective enough to monitor kinetic activity) and compared to their one electron reduction potentials determined using pulse-radiolysis (Stockert et al., 2002).

3.2 Materials and methods

3.2.1 Purification of bovine xanthine oxidase

The enzyme was prepared as described in Chapter 2 and stored in liquid nitrogen until used.

3.2.2 Chemicals

The substrates used in the experiments were all obtained from Sigma/Aldrich and used without further purification. A stock solution of each of the substrate was prepared by dissolving the solid in 1 ml of KOH and then bringing the volume up to 10 ml with deionized $\text{H}_2\text{O}$. These solutions were stored at room temperature until use. Buffers were purchased from Sigma.

3.2.3 Reductive half-reaction kinetics

Kinetic parameters were determined by anaerobic reaction of approximately 10 $\mu$M xanthine oxidase (substrates in 0.1 M MES buffer, 0.1 N KCl pH 7.0) with varying
concentrations (pseudo-first order excess) of each of the 12 substrates at 25°C. The reaction was monitored by following the reduction of flavin at 450 nm. Substrates with suitable activity were monitored on either a Kinetic Instruments stopped-flow apparatus equipped with On-Line Instrument Systems, Inc. (OLIS) data collection software or an Applied Photophysics SX-18MV kinetic spectrophotometer. Observed rate constants from stopped-flow experiments were obtained by single exponential fits to observed kinetic transients using the stopped-flow system software. Observed rate constants were plotted in a double reciprocal plot of $1/k_{\text{obs}}$ versus $1/\text{[substrate]}$, and the parameters $k_{\text{red}}$ and $K_d$ obtained from the y-intercept and slope, respectively. Substrates with slower kinetics were monitored spectrophotometrically with a Hewlett-Packard 5352 diode array spectrophotometer. Rate constants for these substrates were obtained by linear fits to a semi logarithmic plot of $\ln (\Delta A_t/\Delta A_8)$ versus time. In some cases substrate reduction of the enzyme was so slow that the reaction could not be monitored as described above. In these cases, the enzyme was reacted with the substrate under anaerobic conditions and monitored in the course of an overnight incubation. The limiting rate was then estimated based on the approximated half-life of the reaction. Substrates for which the reaction showed no substrate concentration dependence were reacted under anaerobic conditions of pseudo-first order excess enzyme rather than substrate for reasons elaborated upon in the Results and Discussion section.

3.2.4 *Determination of one-electron reduction potentials*

The one-electron reduction potentials for each of the 12 substrates were determined by pulse-radiolysis. Using a computer-controlled 4 MeV Dynaray linear accelerator
equipped with a Supracil optics fitted custom detection system at the University of Auckland, New Zealand, the strong oxidants $\text{SO}_4^{2-}$ and $\text{N}_3^-$ were generated. These two strongly oxidizing species then rapidly remove an electron from a reference compound (in 20 mM phosphate, pH 7.0). The reference, after having lost an electron, established oxidation-reduction equilibrium with the substrate (in 20 mM phosphate, pH 7.0), which could be followed spectrally at specific wavelengths as shown in Table 3.1. The one-electron reduction potential for each of the substrates was then determined either from kinetics of the reversible reaction or from the final equilibrium position using the Nernst equation giving below.

$$E = E^o - \frac{RT}{nF} \ln \frac{[C]^n[D]^d}{[A]^a[B]^b}$$

### 3.3 Results and discussion

#### 3.3.1 Determination of the one-electron reduction potentials

In order to determine if a mechanism such as the one described in Figure 3.1 could be occurring, the one-electron reduction potentials were determined for 12 purine substrates (Figure 3.3) using pulse radiolysis. The substrates were chosen on the following criteria: 1) they were commercially available, 2) substituents on the rings were expected to result in a wide range of one-electron reduction potentials for the $\text{S}^\bullet/\text{S}$ couple, 3) the steric effects of the substituents were expected to be small, and 4) positions 2 and 6 on the substrates both contained substituents so that hydroxylation could occur only at position 8. The one-electron reduction potentials for each of the purine substrates
The potentials range from +1.39 V to +1.03 V in comparison to the NHE. In the potentiometric measurements, all substrates with the exception of 2-thioxanthine and 6-thioxanthine used SO$_4^{2-}$ as an oxidant; (2- and 6-thioxanthine used N$_3^−$. Reference compounds used included 1,2,4-trimethoxybenzene or 1,4-dimethoxybenzene and are indicated in Table 3.1 (Stockert et al., 2002).

3.3.2 The determination of kinetic parameters for each of the purine substrates

The kinetic parameters determined for each of the substrates are summarized in Table 3.2. The rate of reduction, $k_{red}$, is the first-order rate constant and represents the breakdown of enzyme-substrate complex in the high substrate concentration regime. The second-order rate constant, $k_{red}/K_d$, represents the reaction of free enzyme and free substrate in the low substrate concentration regime. The disassociation constant, $K_d$, is also presented in Table 3.2. The $k_{red}$ values ranged from 0.0001 s$^{-1}$ to 13.9 s$^{-1}$ for reaction with each of the 12 substrates. The $k_{red}/K_d$ values ranged from 1.7 M$^{-1}$s$^{-1}$ to 1.3 x 10$^7$ M$^{-1}$s$^{-1}$ (Stockert et al., 2002). The slower substrates, 2,6-diaminopurine, guanine, and 1,7-dimethylxanthine, were too slow to measure accurate rates by stopped-flow kinetics. In these cases, the enzyme was made anaerobic in an anaerobic cuvette, anaerobic substrate was added to the vessel via a gas tight syringe and the reaction monitored by a spectrophotometer set up to measure the absorbance at 450 nm every 10 minutes for 7 hours. The $k_{red, observed}$ was determined by plotting $\ln (\Delta A_t/\Delta A_8)$ versus time and obtaining the rate from the linear fit. The reaction was then repeated with varying substrate concentrations. The resulting rate was plotted in a double-reciprocal plot, with 1/$k_{red}$
observed on the y-axis and 1/substrate concentration on the x-axis. The relevant kinetic parameters, $k_{\text{red}}$, $k_{\text{red}}/K_d$, and $K_d$ were abstracted from the plot. The reciprocal of the y-intercept yields the $k_{\text{red}}$ and $K_d$ is obtained from the slope of the line. Experiments with faster substrates were preformed on the stopped-flow apparatus and $k_{\text{observed}}$ was obtained from exponential fits of the kinetic transient observed at each substrate concentration. The obtained $k_{\text{observed}}$ values were then plotted double-reciprocal plot as described above (Figures 3.4, 3.5, 3.6). In the cases of theophylline and 3-methylxanthine, the reaction was too slow to follow using either of the two methods described above and only the upper limit of the reaction rate could be estimated based on their absorbance change during 5-18 hour incubations under anaerobic conditions.

3.3.3 Comparison of the kinetic parameters and one-electron reduction potentials

If the mechanism involving two sequential one-electron steps is correct, one would expect the limiting rate to decrease as the one-electron reduction potential increases, pushing the equilibrium for the 1st electron transfer back to the left. Upon plotting the log of either $k_{\text{red}}$ or $k_{\text{red}}/K_d$ versus the change in one-electron reduction potential, no linear correlation is observed, however, as would be expected for a reaction mechanism involving two sequential one-electron reduction steps (Figure 3.7) (Stockert et al., 2002). Four of the substrates with the highest one-electron reduction potentials [7-methylxanthine ($\Delta E^0 = 1.27$ V), 1,7-dimethylxanthine ($\Delta E^0 = 1.26$ V), 3-methylxanthine ($\Delta E^0 = 1.28$ V), and theophylline ($\Delta E^0 = 1.29$ V)] are indeed poor substrates as one would expect if the one-electron reduction mechanism were occurring, and the three purines with some of the lowest one-electron reduction potentials include 2-thioxanthine ($\Delta E^0 =$
1.05 V), xanthine (ΔE° = 1.08 V), and 1-methylxanthine (ΔE° = 1.07 V), prove to be good substrates as determined by their reactions rates. Still, the three poorest substrates, guanine (ΔE° = 1.09 V), 2,6-diaminopurine (ΔE° = 1.10 V), and 2-amino-6-chloropurine (ΔE° = 1.10 V), also have low one-electron reduction potentials (Figure 3.4).

The lack of a correlation eliminates the possibility that the reaction mechanism involves two sequential one-electron steps as suggested by the proposed mechanism shown in Figure 3.1. Furthermore, the above data supports the base catalyzed mechanism as described in Figure 1.19 based on the proposed negative charge accumulation on the transferred hydrogen (Ilich and Hille, 1999).

As described above, Ilich and Hille (1997) have proposed, based on computational studies of the substrate tautomers, that a proton shift is required from the N3 position on the substrate to the N9 position upon binding to the molybdenum in the base catalyzed mechanism. One would expect that substrates containing a methyl group in this N3 position would be ineffective due to their inability to vacate the N3 position. The substrates used in this study containing a methyl substituent at the N3 position (3-methylxanthine and theophylline) were indeed uniformly poor substrates as indicated by their extremely slow reaction rates (which made confident determination of any of the studied kinetic parameters impossible).
Figure 3.1

Figure 3.1 A radical-based proposed mechanism for the reductive half reaction xanthine oxidase. The mechanism suggests two sequential one-electron reduction steps rather than one two-electron step. The mechanism was adapted from Page et. al. (1999) *Nature* **402**, 47-52.
Table 3.1 One-electron reduction potentials of various purines. The one-electron reduction potentials of various purines obtained by pulse radiolysis at the University of Auckland, Auckland, New Zealand (described in materials and methods). The Table was taken from Stockert et al., 2002.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Oxidant (R)</th>
<th>Reference (R)</th>
<th>λ (nm)</th>
<th>$10^6 K_0$ (L mol$^{-1}$ s$^{-1}$)</th>
<th>$10^8 K_0$ (L mol$^{-1}$ s$^{-1}$)</th>
<th>$K_{ox}$</th>
<th>$K_{red}$</th>
<th>E (compound$^1$/compound) / V$^2$</th>
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<tr>
<td>xanthine</td>
<td>SO$_4^-$</td>
<td>1.2,4-TMB</td>
<td>450</td>
<td>7.78±0.29</td>
<td>0.66±0.02</td>
<td>11.49±0.36</td>
<td>5.13±0.37</td>
<td>1.08±0.01</td>
</tr>
<tr>
<td>1-methylxanthine</td>
<td>SO$_4^-$</td>
<td>1.2,4-TMB</td>
<td>450</td>
<td>19.0±1.8</td>
<td>1.6±0.34</td>
<td>9.90±1.41</td>
<td>24.3±4.4</td>
<td>1.07±0.02</td>
</tr>
<tr>
<td>3-methylxanthine</td>
<td>SO$_4^-$</td>
<td>1.4-DMB</td>
<td>450</td>
<td>11.8±1.06</td>
<td>13.1±1.18</td>
<td>0.90±0.08</td>
<td>1.85±0.48</td>
<td>1.28±0.01</td>
</tr>
<tr>
<td>7-methylxanthine</td>
<td>SO$_4^-$</td>
<td>1.4-DMB</td>
<td>460</td>
<td>9.80±1.88</td>
<td>10.8±0.9</td>
<td>0.85±0.12</td>
<td>2.70±0.25</td>
<td>1.27±0.01</td>
</tr>
<tr>
<td>theophylline (1,3-</td>
<td>SO$_4^-$</td>
<td>1.4-DMB</td>
<td>460</td>
<td>11.4±6.5</td>
<td>8.77±2.26</td>
<td>1.30±0.58</td>
<td>1.71±0.30</td>
<td>1.29±0.01</td>
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<td>dimethylxanthine)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1,7-dimethyl-</td>
<td>SO$_4^-$</td>
<td>1.4-DMB</td>
<td>460</td>
<td>10.4±6.3</td>
<td>2.73±1.51</td>
<td>3.81±2.21</td>
<td>8.93±4.43</td>
<td>1.28±0.02</td>
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<tr>
<td>xanthine</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>guanine</td>
<td>SO$_4^-$</td>
<td>1.2,4-TMB</td>
<td>450</td>
<td>3.45±0.82</td>
<td>0.5±0.4</td>
<td>6.9±4.0</td>
<td>5.29±0.88</td>
<td>1.09±0.01</td>
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<tr>
<td>2,6-diaminopurine</td>
<td>SO$_4^-$</td>
<td>1.2,4-TMB</td>
<td>450</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.03±1.56</td>
<td>1.10±0.02</td>
</tr>
<tr>
<td>2-amino-6-</td>
<td>SO$_4^-$</td>
<td>1.2,4-TMB</td>
<td>450</td>
<td>10.0±0.5</td>
<td>3.8±0.15</td>
<td>2.62±0.13</td>
<td>5.9±1.24</td>
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<td>chloropurine</td>
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<tr>
<td>2-hydroxy-6-</td>
<td>SO$_4^-$</td>
<td>1.2,4-TMB</td>
<td>450</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.47±0.05</td>
<td>1.15±0.01</td>
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<tr>
<td>methylpurine</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2-thioxanthine</td>
<td>N$_2^+$</td>
<td>1.2,4-TMB</td>
<td>350</td>
<td>156±8</td>
<td>4.68±0.33</td>
<td>33.3±</td>
<td>20.8±2.8</td>
<td>1.05±0.02</td>
</tr>
<tr>
<td>6-thioxanthine</td>
<td>N$_2^+$</td>
<td>1.2,4-TMB</td>
<td>460</td>
<td>165±8</td>
<td>2.47±0.24</td>
<td>67.2±8</td>
<td>43.3±7.8</td>
<td>1.03±0.01</td>
</tr>
</tbody>
</table>

$^a$oxidant conditions. SO$_4^-$: persulphate (15 mM), 2-methyl-2-propanol (0.1 M), N$_2$: saturation; N$_2^+$: sodium azide (30 mM), N$_2$O:saturation.

$^b$E($^\infty$/R) of reference compounds at pH 7.0 (0.1 M phosphate buffer), 1.2,4-dimethoxybenzene (1,2,4-TMB), 1.13±0.01 V (2); 1,4-dimethoxybenzene (1,4-DMB), 1.30±0.01 V (Ljonsso, M., Linn, J., Reitberger, T., Eriksen, T.E., Merenyi, G. (1993). J. Phys. Chem 97: 11278-11282).

$^c$average value derived from $K_{ox}$ and $K_{red}$. 
The Mo (V) “very rapid” species formed with xanthine oxidase and hydroxymethylpurine as examined by pulsed EPR and ESEEM. The figure summarizes the data in Manikandan et al., 2001.
Figure 3.3

Figure 3.3 The theoretical substrates used in this study. All compounds were chosen based on their commercial availability.
Table 3.2 The kinetic parameters $k_{\text{red}}$, $K_d$, and $k_{\text{red}}/K_d$ for the purine substrates used in the study. The Table was taken from Stockert et al., 2000.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{\text{red}}$ (s$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$k_{\text{red}}/K_d$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xanthine</td>
<td>7.0</td>
<td>0.54</td>
<td>1.3 x 10$^7$</td>
</tr>
<tr>
<td>1-methylxanthine</td>
<td>13.9</td>
<td>4.5</td>
<td>3.1 x 10$^6$</td>
</tr>
<tr>
<td>3-methylxanthine</td>
<td>(0.0005) no reaction detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-methylxanthine</td>
<td></td>
<td></td>
<td>2.0 x 10$^3$</td>
</tr>
<tr>
<td>1,3-dimethylxanthine (theophylline)</td>
<td>(0.00005) no reaction detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,7-dimethylxanthine</td>
<td>0.070</td>
<td>1200</td>
<td>56</td>
</tr>
<tr>
<td>guanine</td>
<td>0.0001</td>
<td>59</td>
<td>1.7</td>
</tr>
<tr>
<td>2,6-diaminopurine</td>
<td>0.001</td>
<td>52</td>
<td>17</td>
</tr>
<tr>
<td>2-amino-6-chloro-purine</td>
<td>0.013</td>
<td>133</td>
<td>100</td>
</tr>
<tr>
<td>2-hydroxy-6-methylxanthine</td>
<td>0.133</td>
<td>1.15</td>
<td>1.2 x 10$^5$</td>
</tr>
<tr>
<td>2-thioxanthine</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-thioxanthine</td>
<td>1.4</td>
<td>0.95</td>
<td>1.5 x 10$^8$</td>
</tr>
</tbody>
</table>

Table 3.2 Kinetic parameters of various purine compounds.
Figure 3.4 The double reciprocal plots from the reductive half reaction data for the xanthine oxidase with the following substrates: 1-methylxanthine, 7-methylxanthine, 1,7-dimethlyxanthine, 2-aminio-6-chloropurine, 2,6-diaminopurine, and hydroxymethyl purine. The kinetic parameters extracted from each of the above graphs are summarized in Table 3.3.
Figure 3.4
Figure 3.5 The double reciprocal plots of the rapid half-reaction kinetics of 2-and 6-thioxanthine, and guanine. The behavior of 2-and 6-thioxanthine indicate no substrate concentration dependence of the reaction. The negative slope of the 2-thioxanthine graph did not allow determination of the Kd.
Figure 3.6

Figure 3.6 The graphs of $\ln(\Delta A/\Delta A_0)$ versus time for 3-methylxanthine and theophylline. The limiting rates were estimated based on these time plots.
Figure 3.7 The plots of $\Delta E^0$ versus $\ln k_{\text{red}}$ and $\Delta E^0$ versus $\ln k_{\text{red}}/K_d$ of the data obtained in this study. No linear correlation is observed in either of these plots, indicating the enzyme cannot be using two sequential one-electron steps in order to reduce the enzyme to Mo (IV). The Figure was taken from Stockert et al., 2002.
Figure 3.7
CHAPTER 4

STUDIES OF THE ACTIVE SITE RESIDUES OF RHODOBACTER CAPSULATUS
XANTHINE DEHYDROGENASE

4.1 Introduction

4.1.1 The structural differences between the oxidase and dehydrogenase forms of the xanthine utilizing enzymes

The crystal structures of xanthine oxidase and xanthine dehydrogenase from cow’s milk have been determined (Enroth et al., 2000). As described in Chapter 1, the structures of these two enzymes are quite similar, with the two forms of the protein differing only by the presence or absence of the NAD$^+$ binding site in their flavin domains. The folds around the molybdenum and iron-sulfur centers remain the same in both forms of the enzyme. As described in Chapter 1, the oxidase form can only utilize molecular oxygen as its final electron acceptor. In the oxidase form of the bovine enzyme, NAD$^+$ access to the flavin cofactor is blocked by arginine 426, which replaces aspartate 429 (Figure 1.7) upon removal of phenylalanine 549 (as a result of proteolysis of the peptide chain). The rearrangement most likely occurs due to the lack of interaction between this phenylalanine and arginine 426 (Enroth et al., 2000). This proteolysis is irreversible and results from tryptic digest after lysine 551 or pancreatin cleavage after
leucine 219 and lysine 569 (Nishino, et al., 1989). Xanthine dehydrogenase from cow’s milk can also be reversibly converted to its oxidase form by modification at cysteine 535 and 992, possibly through a disulfide bond (Nishino and Nishino, 1997; Rasmussen et al., 2000).

The current studies involve a study of the closely related dehydrogenase form isolated from the phototropic purple bacterium *Rhodobacter capsulatus*, for which an recombinant expression system exists. The bacterial enzyme is an \((\alpha\beta)_2\) heterotetramer in comparison to the eukaryotic dehydrogenase which exists as a homodimer. The crystal structure of the *Rhodobacter capsulatus* dehydrogenase has been solved to 2.7 Å and folds similarly to the bovine dehydrogenase. In the bacterial enzyme, the cofactors are located on two different polypeptides, with the iron-sulfur centers and FAD bound to the XDHA subunit and the molybdenum center on the XDHB subunit. This is not the case for the eukaryotic forms of the enzyme, in which all cofactors are in the same polypeptide. In contrast to most other bacterial molybdenum enzymes, which contain the dinucleotide form of the molybdenum cofactor, the *Rhodobacter capsulatus* enzyme contains the mononucleotide form as found in molybdenum enzymes from eukaryotic sources (Truglio et al., 2001). Furthermore, the *Rhodobacter capsulatus* enzyme exists as a stable dehydrogenase and cannot be converted to the oxidase form by proteolytic cleavage (Leimkühler et al., 2003).

As described above, the dehydrogenase form of the enzyme from either source (*Rhodobacter capsulatus* or bovine milk) are similar in fold, cofactor arrangement, and molybdenum cofactor type. Despite the tetrameric structure of the bacterial enzyme, the cofactors are positioned in the same overall arrangement (Enroth et al., 2000; Truglio et
al., 2002). That in mind, the bacterial dehydrogenase and bovine oxidase differ in that only the former can utilize NAD$^+$; there are no differences in the molybdenum-binding portion of the protein (Enroth et al., 2000). For purposes of this study, which involves only reaction at the molybdenum center, the two enzyme forms bovine xanthine oxidase and *Rhodobacter capsulatus* xanthine dehydrogenase share the same conserved active site around the molybdenum center and can therefore be directly compared.

4.1.2 Expression and characterization of the xanthine dehydrogenase from *Rhodobacter capsulatus*

The *Rhodobacter capsulatus* xanthine dehydrogenase protein was expressed in *E. coli* and characterized by Leimkühler et al. in 2003. To date this system is the only successful expression system in *E. coli* for an enzyme of this type. Two genes *xdhA* and *xdhB* encode the enzyme; however an additional gene, *xdhC*, is found to be essential in the formation of active enzyme containing the molybdenum cofactor. It has been proposed that XDHC is responsible for the insertion of the molybdenum cofactor into the XDHB subunit (Leimkühler and Klipp, 1999; Leimkühler et al., 2003). The *xdhC* gene must be co-expressed in order to obtain enzyme containing the molybdenum cofactor, but is not required afterwards for activity.

The genes discussed above were isolated from the *Rhodobacter capsulatus* genome and cloned into the expression vector pTrcHis using the NdeI and Hind III restriction sites. The resulting plasmid was named pSL207 and contained the genes encoding XDHA, XDHB, and XDHC as well as a His$_6$ tag located on the N-terminus of XDHA. The proteins were transformed into TP 1000 cells (*E. coli* strain containing a
deletion of the *mobA/mobB* genes as described in Chapter 1) and expressed (Palmer et al., 1996). The protein was then purified using the following columns: Ni-NTA, Q-sepharose, and phenyl-sepharose (Leimkühler et al., 2003). A more detailed explanation of the protein purification follows in Materials and Methods.

In order to examine the properties of the bacterial dehydrogenase relative to the bovine enzyme, the expressed protein was characterized using UV/visible absorbance spectroscopy, EPR and chemical treatments. Since the bovine xanthine oxidase enzyme required the Mo=S group for activity, the recombinant enzyme was treated with cyanide (known to remove the sulfur from the Mo=S position in bovine xanthine oxidase) in tests for removable sulfurs. The cyanide-treated enzyme was inactive and was only reactivated under anaerobic conditions utilizing sodium dithionite and sodium sulfide (conditions known to add the sulfur back into the active site in xanthine oxidase) (Leimkühler et al., 2003; Wahl and Rajagopalan, 1982). These results suggested the presence and importance of the cyanolyzable sulfur as observed with the bovine enzyme.

Next, the substrate specificities of both the bovine xanthine oxidase and *Rhodobacter capsulatus* xanthine dehydrogenase were compared by Leimkühler et al.. Table 4.1 summarizes the results of these experiments. The xanthine oxidase used in this study was only 42% active, therefore the AFR (activity to flavin ratios described in Chapter 1) that is 14 times higher (shown in Table 4.1) for the dehydrogenase enzyme is actually only approximately 5 times faster than fully active xanthine oxidase after correction for the percent of functional enzyme present in the sample (Leimkühler et al., 2003). The recombinant enzyme shares the same substrate specificities of the native xanthine oxidase, although it is slightly more active.
Steady-state experiments conducted by Leimkühler et. al. (unpublished) determined the $V_{max}$ as 107.52 s$^{-1}$, $K_{m(\text{xanthine})}$ as 64.5 µM, and $K_{m(\text{NAD})}$ as 103 µM. Based on reductive titrations with xanthine and dithionite, the activity of the wild-type enzyme was approximated at 80% active (Leimkühler et al., unpublished). Therefore, the turnover number for the fully functional enzyme is estimated at 134.4 s$^{-1}$, six times higher than that of chicken liver XDH (reported at 22.5 s$^{-1}$) (Nishino et al., 1989). The faster turnover in the *Rhodobacter capsulatus* XDH is consistent with higher activity-to-flavin ratios observed when compared to those of bovine XO (Leimkühler et al., 2003). Since current evidence suggests that product release is the rate-limiting step for xanthine utilizing enzymes, the considerably faster turnover observed with the *Rhodobacter capsulatus* enzyme when compared to that of the chicken or bovine enzymes suggests that product release occurs much faster in the bacterial enzyme.

The cofactor content of the recombinant bacterial enzyme was also evaluated and found to be comparable to the bovine enzyme, with the FAD and iron content indicating full incorporation of those cofactors. The molybdopterin content, on the other hand, was found to be about 70% relative to that of the native bovine enzyme. The UV/visible absorbance spectra of both the oxidase and the dehydrogenase are shown (Figure 1.8) and as discussed in Chapter 1, the feature at 450 nm (from FAD and Fe/S centers) in the bovine xanthine oxidase is shifted slightly to 465 nm in the bacterial dehydrogenase (Leimkühler et al., 2003).

Spectroscopic examination of the recombinant protein was also used in comparison to the bovine xanthine oxidase. EPR studies were completed in order to examine the iron-sulfur centers. As described in Chapter 1, xanthine oxidase has a
distinct EPR signal for each of the iron-sulfur centers. The bacterial enzyme also exhibits these distinct EPR signals, with a minor difference in the spectrum of Fe/S I as compared to that seen with xanthine oxidase. The signal is more axial with a $g_1=1.922$ and $g_2=2.022$ rather than rhombic as seen in the bovine enzyme (with $g$-values $(1,2,3) = 1.894, 1.932, 2.022$ and has slightly narrower line widths; however, the average $g$-value ($g_{avg}=1.952$) remains similar to that of the xanthine oxidase signal. A slight difference in $g$-values is also observed for the Fe/S II signal. The $g_{1,2,3}$-values for the bacterial enzyme are $1.896, 1.971$, and $2.073$, respectively. The bovine enzyme exhibits $g_{1,2,3}=1.902, 1.991, 2.110$. These values result in slightly different $g_{avg}$ for both enzymes (1.980 for the bacterial enzyme and 2.001 for the bovine enzyme (Leimkühler et al., 2003). The bacterial iron-sulfurs centers are also reasonably similar to those in XO as judged by X-ray absorption spectroscopy (XAS).

The active site of the molybdenum center is perhaps the most important comparison between the two enzymes in terms of this dissertation research. Both the crystal structures and the XAS results for the recombinant enzyme have shown that the center is very similar to that seen with xanthine oxidase. The results indicate that the molybdenum is coordinated to oxo group at a distance of 1.71 Å, three thiolate groups (presumably two from the pterin cofactor, and one SH group) at 2.37 Å, and one oxygen (or nitrogen) at 2.01 Å (Turner et al., 1989; Leimkühler et al., 2003).

4.1.3 The active site residues in Rhodobacter capsulatus xanthine dehydrogenase

As described above, the active site at the molybdenum center is essentially identical in both xanthine dehydrogenase from Rhodobacter capsulatus and xanthine
oxidase from cow’s milk. The similarities make the recombinant enzyme an excellent system for studying the molybdenum hydroxylases by mutagenic studies of residues with proposed catalytic roles. Since the reductive half-reaction occurs at the molybdenum site, kinetic studies involving this half of the reaction are directly relevant to that of xanthine oxidase.

Figure 4.1 shows the active site of the *Rhodobacter capsulatus* enzyme. Residues colored in blue correspond to the dehydrogenase form of the enzyme, while residues coded in red represent the corresponding residues of the oxidase form. Glutamate 232 (802 in bovine xanthine oxidase) is located on the top of the substrate binding region and is proposed to be involved in facilitating substrate tautomerization as described in Chapter 1 (Ilich and Hille, 1997). Glutamine 197 (767 in bXO) sits above the active site near the substrate binding region. This residue is proposed to hydrogen bond to the proton on the –SH group in the reduced form of the enzyme. Such an interaction would modulate the reduction potential of the molybdenum center accommodating electron transfer out of the center. Glutamate 730 (1261 in bXO) is located below the active site and is proposed to act as the active site base identified through pH dependence studies that imitates catalysis by proton abstraction off the Mo-OH group. Arginine 310 (880 in bXO) is thought to be involved in substrate interaction. The current research explores steady-state and pre-steady state kinetics, EPR, resonance Raman, and UV/visible absorbance spectroscopy of wild-type as well as various mutants of the recombinant *Rhodobacter capsulatus* xanthine dehydrogenase.
4.2 Materials and methods

4.2.1 Expression and purification of the recombinant protein

The *Rhodobacter capsulatus* xanthine dehydrogenase was purified as described by Leimkühler et. al. (2003) with some modifications. All mutant proteins were cloned, expressed, and purified by our collaborator Dr. Silke Leimkühler and coworkers. The protein was expressed in TP 1000 cells carrying the plasmid pSL207 in 500 ml of LB containing 150 µg/ml ampicillin, 1mM molybdate, and 0.02 mM IPTG at 30ºC until an OD of 1.0. The 500 ml culture was used to start an eight liter culture which was grown at 30ºC for 18 to 20 hours. Cells were harvested by centrifugation at 5000 x g and resuspended in 8 volumes of 50mM sodium pyrophosphate, 300 mM NaCl at pH 8.0 and lysed by passing through a French press three times (with the exception of the E730A mutant, which was lysed using sonication). DNaseI was added and the lysate was incubated for 30 minutes. The lysate was centrifuged at 17,000 x g for 25 minutes. The lysate solution was made 10 mM imidazole and mixed gently with 16 ml of Ni²⁺-nitrilotriacetic agarose from Qiagen at 4ºC for 30 minutes. The column material was transferred to a column and washed with 2 column volumes of 10 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. Next, ten column volumes of the same buffer containing 20 mM imidazole were run through the column. The enzyme was eluted with 100 mM imidazole in the same buffer. The enzyme was dialyzed against 50 mM Tris, 1 mM EDTA, 2.5 mM dithiothreitol, pH 7.5. The dialyzed sample was loaded onto Q-sepharose column and eluted with a 0 to 250 mM NaCl linear gradient. The pooled fractions were applied to a Sepharose 4B/folate affinity column and the inactive form of the enzyme was eluted with 20 mM Tris, 0.2 mM EDTA, pH 7.8, 10% pyrophosphate.
The active form of the enzyme was eluted with the same buffer made 15% pyrophosphate and 10 mM salicylate. Table 4.1 shows the purity and activity of the enzyme at various points during the purification. The purified enzyme was concentrated and run over a PD-10 (Amersham Bioscience) or G-25 (Sigma) gel filtration column equilibrated with 20 mM Tris, 0.2 mM EDTA, pH 7.8. Enzyme was either used immediately or stored at -80°C (with addition of 2.5 mM DTT) until use. The mutants prepared in this study were generated using PCR mutagenesis by Dr. Silke Leimkühler and coworkers. These mutant preparations involved mutagenic primers designed to insert the desired mutation by PCR amplification of the entire gene contained plasmid. The mutant enzymes were expressed and purified as described above.

4.2.2 Chemicals and materials

All buffers or chemicals used were purchased from Sigma, Fisher Scientific, or Amersham Bioscience. All column materials were purchased from Amersham Bioscience, Sigma, or Qiagen.

4.2.3 Enzyme Assay and concentration

Assays were carried out at 25°C in 20 mM Tris, 0.2 mM EDTA, pH 7.8 containing 1 mM xanthine. The activity of xanthine dehydrogenase was measured by monitoring absorbance changes either in the presence of 1 mM NAD§ (following the formation of NADH at 340 nm) or saturating O₂ (following the formation of uric acid at 295 nm). The activities are presented as an activity-to-flavin ratio (AFR) calculated by dividing the change in absorption per minute at 295 nm by the absorption at 465 nm of the enzyme
used in the assay. The concentration of the enzyme was determined by Beer’s Law measuring the absorption at 465 nm and dividing by the extinction coefficient of 31.6 mM$^{-1}$ cm$^{-1}$.

4.2.4 Anaerobic reductions

Anaerobic reduction of the enzyme was done by placing 1 ml of enzyme (various concentrations used depending on the experiment) in a glass tonometer fused to a quartz cuvette. The sample was made anaerobic by repeatedly (every 10-15 minutes) flushing and equilibrating the solution with oxygen-scrubbed argon gas for approximately 1 hour. Anaerobic substrate was prepared either by placing it in a side arm of the tonometer prior to making the sample anaerobic or by bubbling a substrate solution with argon gas for approximately 15 minutes. The substrate was added either by tipping the side arm solution into the tonometer and mixing or by a gas-tight Hamilton syringe through a rubber septum on the tonometer. Enzyme reduction was followed using a spectrophotometer at 465 nm (Hewlett-Packard 8453A Diode Array).

4.2.5 Steady-state kinetics

Steady-state experiments were carried out in O$_2$ saturated 20 mM Tris, 0.2 mM EDTA, pH 7.8 at 4°C using a Kinetic Instruments stopped-flow apparatus equipped with On-Line Instrument Systems, Inc. (OLIS) data collection software. The solution contained 1 mM NAD$^+$ and varying concentrations of xanthine. Activity was monitored at 340 nm following the formation of NADH. Experiments were repeated in triplicate over a period of approximately 60-80 seconds. Steady-state kinetic parameters were
obtained by either double reciprocal plots of $1/k_{\text{observed}}$ versus $1/[\text{substrate}]$ or hyperbolic fits to plots of observed rate versus substrate concentration.

4.2.6 *Rapid reaction kinetics*

Reductive half-reaction experiments were carried out using an Applied Photophysics SX-18MV kinetic spectrophotometer. Buffer conditions were 20 mM Tris, 0.2 mM EDTA, pH 7.8. The reactions were performed done using an anaerobic glass tonometer cycled on an anaerobic train with argon gas as described above. Wild-type or mutant enzyme (4-5 µM wild-type; 30-40 µM mutant) was rapidly mixed (one-to-one volume ratio) with various concentrations of xanthine and the reaction monitored by the reduction of the enzyme at 460 nm. The reaction was followed from 0.05 to 1.0 seconds for the wild-type enzyme and 10 to 200 seconds for the mutant enzyme forms. A plot of $1/k_{\text{observed}}$ versus $1/[\text{substrate}]$ or the hyperbolic fit of an observed rate versus substrate concentration plot were used to extract the kinetic parameters $k_{\text{red}}$, $K_d$, and $k_{\text{red}}/K_d$. The E730A/D/R/Q and R310M mutants were too slow to be accurately monitored by stopped-flow. Reactions for these mutants were instead monitored spectrophotometrically over a period of 24 hours using an anaerobic cuvette; the R310M reaction was monitored for 8 hours.

4.2.7 *Removal of sulfur from the molybdenum center by inactivation with cyanide*

The absorbance spectrum of the E730A mutant enzyme in 0.1M sodium pyrophosphate, 0.3M EDTA pH 8.5 was recorded prior to aerobic incubation with 0.0033M KCN on ice for 60 minutes. Following the incubations the spectrum was
obtained for comparison to that of untreated enzyme. The spectral changes were then compared to those seen with bovine xanthine oxidase to verify removal of sulfur as SCN\(^-\) (Massey and Edmondson, 1970).

4.2.8 Analysis of sulfur content

The amount of Mo=S present in as-isolated recombinant *Rhodobacter capsulatus* xanthine dehydrogenase was determined by the method described by Westley (1981). Xanthine dehydrogenase (2 mg in 50 mM HEPES, pH 7.2) was incubated with 100 mM KCN over night at 4 °C. The protein and released SCN\(^-\) were separated using an Amicon Microcon filter with a 50,000 molecular weight cut off. SCN\(^-\) was quantified by addition of 500 µl of ferric nitrate reagent (100 g of Fe(NO\(_3\))\(_3\) \cdot 9\) H\(_2\)O and 200 ml of 65% HNO\(_3\) per 1500 ml) to 500 µl of the Microcon eluate. The iron-complexed thiocyanate was quantitated by comparing the absorption at 460 nm to a SCN\(^-\) standard curve constructed with know concentrations of thiocyanate. The amount of Mo=S in each of the mutant proteins is summarized in Table 4.2.

4.2.9 Analysis of molybdenum cofactor content

The amount of molybdenum cofactor incorporated into the recombinant protein was determined using the method described by Johnson (1984). The amount of incorporated molybdenum cofactor in each of the XDH variants is summarized in Table 4.2.
4.2.10 *Resonance Raman of recombinant xanthine dehydrogenase*

Cryogenic resonance Raman samples of the $E_{\text{reduced}}$-product complexed protein were prepared as described in Chapter 2.

4.2.11 *EPR samples of recombinant xanthine dehydrogenase*

EPR samples were prepared by placing 50 to 100 µM enzyme (in 20 mM Tris, 0.2 mM EDTA, pH 10.0) in an EPR tube, and adding 5 equivalents of hydroxymethylpurine via a long-needle Hamilton syringe. The solution was mixed and frozen in a liquid nitrogen-acetone bath within 6 to 8 seconds. After the EPR spectrum was obtained, the sample was thawed, incubated for an additional 20 seconds, and frozen rapidly in cold acetone for the a second EPR scan. Enzyme in $D_2O$ was prepared by passing the enzyme through a Sephadex G-25 (Sigma) column equilibrated with 20 mM Tris, 0.2 mM EDTA, pH 10.0. The deuterated samples were reduced and frozen as described above, and their EPR spectra recorded. The dithionite-reduced samples were prepared in the same buffer at pH 7.8 by adding anaerobic dithionite solution via a long-needle Hamilton syringe to the anaerobic enzyme. The amount of reduction was monitored spectrophotometrically using an EPR tube cuvette adapter. The EPR spectra were collected using a Brüker Instruments, Inc. ER 300 spectrophotometer equipped with an ER 035M NMR gaussmeter and a Hewlett Packard 5352B microwave frequency counter. The spectra were collected under the following parameters: a frequency of 9.4545771 GHz, modulation amplitude of 5.054 G, receiver gain of $1.00 \times 10^5$, power of 10mW, modulation frequency of 100 kHz.
4.3 Results and discussion

4.3.1 Steady-state kinetics of *Rhodobacter capsulatus* xanthine dehydrogenase

Steady-state kinetic experiments were completed for the wild-type and each of the mutants by varying the concentration of xanthine or hydroxymethylpurine under aerobic conditions. The reaction mixture also contained 1 mM NAD$^+$, used by the dehydrogenase form of the enzyme as the final electron acceptor. The *Rhodobacter capsulatus* XDH shows only 2% use of O$_2$ as its final electron acceptor, in comparison to bovine xanthine oxidase, which uses only molecular oxygen (Leimkühler et al., unpublished).

In order to obtain a direct comparison of the wild-type and mutant XDH, the steady-state kinetic experiments were repeated under identical conditions (pH 7.8 and 4°C) on the same stopped-flow instrument (Kinetic Instruments) using both xanthine and hydroxymethylpurine as substrates. The steady-state kinetic parameters for the wild-type and mutant proteins determined here are summarized in Table 4.3. The kinetic parameters determined for the xanthine reaction with the wild-type protein agree quite well with those observed previously (Leimkühler, et al., unpublished): The $k_{cat}$ (xanthine) for the wild-type enzyme is 70 s$^{-1}$ and the $K_m$ (xanthine) at 23 µM. The determined $k_{cat}$ (HMP) and $K_m$ (HMP) is 4.4 s$^{-1}$ and 2.5 µM (Figure 4.2). The activity of the wild-type protein used in the experiment was estimated to be approximately 60% based on a comparison of the extent of reduction by xanthine as compared to dithionite.

The $k_{cat}$ (xanthine) and $K_m$ (xanthine) for the E232A mutant protein are found to be 4.4 s$^{-1}$ and 163 µM, respectively. Experiments using hydroxymethylpurine with the E232A mutant yielded a $k_{cat}$ (HMP) of 0.40 s$^{-1}$ and a $K_d$ (HMP) of 0.68 µM (Figure 4.3). This result is
not as substantially large as would be expected based on the proposed role of the 232 residue. In the crystal structure of the alloxanthine-inhibited enzyme, the alloxanthine may be bound upside-down relative to the believed orientation of xanthine in the active site (Truglio et al., 2001). Based on this observation, it was considered that the glutamate 232 residue may facilitate substrate tautomerization of substrates binding in the orientation of xanthine. The slower substrate HMP may bind in the same orientation as alloxanthine, and the mutation therefore would not affect the reaction with HMP to the same extent that it affects the reaction with xanthine. The steady-state results indicate a substantial decrease (16-fold) in going from the wild-type to the mutant with xanthine and a 10-fold decrease with HMP. Further, comparison between the xanthine and HMP results with the mutant show a 10-fold difference in $k_{\text{cat}}$. The effect of the mutant is therefore evident in both xanthine and HMP reactions. These results do not support the idea that xanthine and HMP bind with different orientations, but do however indicate a substantial effect of the mutation on the overall reaction of both substrates. Based on the above data, the E232 residue could indeed be involved in substrate tautomerization; however, the proposed tautomerization requires a proton shift from N3 to N9, which in the event that the substrates bind in the same orientation as alloxanthine, is unlikely to be facilitated by this residue given its geometric orientation. Furthermore, recent crystallographic studies on xanthine oxidase with a newly designed tight-binding inhibitor suggest that substrate is oriented such that the N3 and N9 positions on the ring are pointed downwards, rather than up towards the glutamate residue (Okamoto et al., 2004). The glutamate residue is most likely involved in stabilization of electrostatic effects, which would be expected to cause the magnitude of overall rate effects that are
observed for both substrates. Unfortunately, examination of the $K_m$ does little to interpret the role of this residue. The observed increase in $K_m$ suggests by definition that more substrate is needed in order for the reaction to reach one-half of $V_{\text{max}}$. This effect could be observed for either of the two proposed roles for the residue, substrate tautomerization or electrostatic stabilization.

The Q197E mutant with xanthine showed a modest 7-fold reduction in $k_{\text{cat(xanthine)}}$ as compared to wild-type enzyme, decreasing from 70 s$^{-1}$ to 9.6 s$^{-1}$, and just over a 3-fold decrease in $K_m (\text{xanthine})$, lowering from 23 µM to 6.5 µM (Figure 4.4). The slower HMP was not used for the Q197E mutant. Although a 7-fold reduction in overall rate is significant, the effect of this mutation on the overall reaction with xanthine is quite modest. The decrease in $K_m$ could possibly indicate the residue’s role in hydrogen-bonding to the molybdenum center. The crystal structure of the inhibitor bound xanthine oxidase, shows one hydrogen bond to the Mo=O group (which is in the apical position in this structure) (Okamoto et al., 2004).

The kinetics of the R310M mutant was too slow to examine by the stopped-flow method. For this reason, the limiting rate was estimated by examining the amount of reduction with xanthine in an overnight incubation. The limiting rate was then estimated by approximating the half-life. The very slow rate observed for this mutation was greater than expected based on the location of this residue in the active site, but may be due to stabilization of a negative substrate accumulation and will be discussed in greater detail below.

The E730A, E730D, E730R, and E730Q mutants were also examined. Repeated experiments with these E730 mutants showed no activity whatsoever and therefore the
$k_{\text{cat}}$ and $K_m$ of each could not be determined for either of the substrates. The glutamate 730 residue in the dehydrogenase enzyme corresponds to the glutamate 1261 in bovine xanthine oxidase. Mutation of this residue, proposed to act as the active site base, obviously has a very large effect on turnover rate.

4.3.2 Kinetics of the reductive half-reaction of Rhodobacter capsulatus xanthine dehydrogenase

Table 4.3 summarizes the rapid-reaction kinetic parameters obtained in this study. The rapid reaction kinetic parameters $k_{\text{red (xanthine)}}$ and $K_d (\text{xanthine})$ at 4°C and pH 7.8 were determined for the recombinant wild-type and mutant proteins using a stopped-flow apparatus and following the absorption decrease at 460 nm under anaerobic conditions. The $k_{\text{red (xanthine)}}$ and $K_d (\text{xanthine})$ for the wild-type enzyme are 67 s$^{-1}$ and 33 µM, respectively (Figure 4.5), in good agreement with the steady state results reported for the wild-type protein. From the wild-type data, the small difference is seen between the $k_{\text{cat}}$ and $k_{\text{red}}$, which indicates the limiting step is the reductive half-reaction, as generally accepted.

Rapid reaction experiments with wild-type enzyme and HMP yielded a $k_{\text{red (HMP)}}$ of 21 s$^{-1}$ and a $K_d (\text{HMP})$ of 7.0 µM. We see little substrate concentration dependence with HMP for any of the XDH variants measured, which agrees well with the observed behavior of the bovine xanthine oxidase enzyme (McWhirter and Hille, 1991).

We see a decrease in rate for the E232A mutant enzyme with xanthine just as in the steady state experiments, slowing by a factor of 12 from 67 s$^{-1}$ for the wild-type enzyme to 5.5 s$^{-1}$ for the E232A mutant. The $K_d (\text{xanthine})$ for the E232A mutant is reported at 409 µM, increased from the wild-type enzyme. The $k_{\text{red (HMP)}}$ for the E232A mutant
increased slightly from 0.28 s\(^{-1}\) to 0.42 s\(^{-1}\) (Figure 4.6) when compared to wild-type enzyme. An increase of the magnitude seen with the HMP reaction is just outside the experimental error, and the rate is essentially unchanged. As discussed above, this residue was originally thought to be involved in substrate tautomerization, but in light of the present evidence, may instead have an electrostatic stabilization effect on the transition state. The steady-state results support this new proposal, while the rapid reaction results are a bit more ambiguous. The \(k_{\text{red}}\) for wild-type and E232A with xanthine differ by 12-fold, but the difference between wild-type and mutant is not as large as with HMP (0.28 versus 0.42 s\(^{-1}\)). The essentially unchanged rates for HMP as compared with xanthine are consistent with this residue’s facilitation of substrate tautomerization. However, the \(K_d\)’s could not be determined for the HMP reaction for E232A mutant, due to the lack of any dependence of \(k_{\text{obs}}\) on HMP concentration. Again, this same behavior is seen with the bovine enzyme.

The rate of the Q197E mutant with xanthine also decreases from 67 s\(^{-1}\) to 10.24 s\(^{-1}\) in the reductive half-reaction, and the dissociation constant decreases somewhat from 33\(\mu\)M to 20\(\mu\)M in the Q197E mutant (Figure 4.8). If this residue were involved in hydrogen-bonding to the molybdenum center, as suggested by the crystallographic studies with the tight-binding inhibitor FYX-051, you may expect to see a fairly significant decrease in rate, as observed, as well as only a small change in the dissociation constants (Okamoto et al., 2004). Since \(k_{\text{red}}\) reflects the breakdown of the enzyme-substrate complex, you would expect a decrease in rate with the removal of the hydrogen bond from the Mo=O of the active site, which is expected to facilitate the reactivity of the metal center by stabilizing the transition state. Such an effect could
result in increased activation energy, and therefore more difficulty in achieving the transition state and continuing on to product.

The R310M mutant was too slow follow to the reaction with xanthine by stopped-flow, and the mutant was instead incubated with 400µM xanthine under anaerobic conditions for 5 to 6 hours while recording the absorbance at 465 nm every ten minutes. The plot of ln(ΔA/ΔA₀) versus time is shown in Figure 4.9. Even using this experimental method, the rate of reduction was not reproducible enough to obtain a confident determination of \( k_{\text{red}} \). The limiting rate was estimated based on the half-life of the reaction to be \( 10^5 \) times slower than that for wild-type enzyme, a far more significant effect than one would expect based on the location of R310 in the active site. We propose, based on the large decrease in the limiting rate (\( 10^5 \) decrease from wild-type) that the R310 residue is involved in stabilization of the negative charge accumulation of the substrate in the course of the nucleophilic attack that initiates the chemistry.

According to computational studies, negative charge accumulation on substrate facilitates the hydride transfer required to obtain the lowest energy \( sp^3 \) hybridized intermediate (as shown in Figure 1.17) (Ilich and Hille, 1997; Ilich and Hille, 1999).

Numerous attempts at determination of the \( k_{\text{red}} \) for the E730 mutants have detected little to no reduction of the mutant with xanthine even after overnight incubations (Figure 4.11; Figure 4.12). The limiting rates for each of the mutants with xanthine were obtained by estimation of the half-life from an 18 hour anaerobic incubation with excess substrate (as done with the R310M reaction). The results with the E730 mutants indicate minimally a \( 10^7 \)-fold decrease in \( k_{\text{red}} \) from wild-type.
In order to ensure that the decrease in rate was due to the mutation rather than low sulfur incorporation into the mutant active site, the molybdenum cofactor and sulfur contents were examined. The molybdenum content of the various 730 mutants was determined as described in Leimkühler et. al. (2003) and summarized in Table 4.2. The molybdenum content of the E730A mutant was indeed rather low (only 33%), however, since the reaction is monitored by following the enzyme reduction, the effect is only to lower the magnitude of the absorbance change and not the observed rate constant. In the case of the E730A mutant, the enzyme concentration was estimated at only 30% active, and the concentration adjusted accordingly to compensate for the lower molybdenum cofactor content. The degree of sulfuration was first monitored by the spectral change observed upon incubation with cyanide. The spectra are shown in Figure 4.10. Based on the spectral change observed with the enzyme used in the kinetic experiments, it can qualitatively be determined that a large portion of the enzyme was indeed sulfurated. The moles of sulfur per subunit were also determined and summarized in Table 4.2, and the result is consistent with a degree of sulfuration (although not as high as in wild-type) in the mutant high enough to observe a reaction.

The large decrease in reactivity speaks clearly as to the overall importance of E730. As mentioned previously, this residue (E1261 in the bovine enzyme) is proposed to act as the active site base which abstracts a proton from the Mo-OH group, allowing nucleophilic attack on the substrate in the C8 position. Since there was no measurable activity in these mutants, a pH dependence study could not be completed. Therefore, the presented results cannot definitively prove that this glutamate is the active site base, but do demonstrate the extreme importance this specific residue. In addition to its possible
role as an active site base, recent crystallographic studies of FYX-051 inhibited xanthine oxidase by Nishino and coworkers (2004) indicate that the residue 1261 in the bovine xanthine oxidase enzyme may hydrogen bond to the product, and thus influence additional steps in the catalytic sequence.

### 4.3.3 EPR experiments with the recombinant xanthine dehydrogenase

The wild-type and mutant enzyme was also examined by EPR. Craig Hemann, a senior research associate in the laboratory, aided in the data collection of the majority of the EPR spectra presented here. The xanthine dehydrogenase mutants were each reacted with 5 equivalents of HMP at pH 10.0. The sample was prepared as described in Materials and Methods and frozen as quickly as possible (6-8 seconds) since the *Rhodobacter capsulatus* enzyme has been shown to turnover faster than the bovine enzyme (which takes approximately 40 seconds to form maximal signal intensity). However despite repeated tries, the “very rapid” signal could not be obtained, most likely due to the fast rate of reaction.

However, the “rapid type 1” signal did appear on this time scale, and was rather long lived, lasting through 5 freeze-thaw 20 second incubations. This signal has the characteristic $g_{1,2,3}$ values of 1.9654, 1.9707, and 1.9906 as well as the hyperfine splitting due to the two inequivalent protons that is observed in the bovine oxidase. The flavin semiquinone signal was also very prominent, as expected, considering the extent to which the dehydrogenase form from *Rhodobacter capsulatus* accumulates the semiquinone during reduction (Leimkühler et al., unpublished). The reaction was also repeated in D$_2$O. The bottom two spectra in Figure 4.13 show the signal obtained from
the H₂O and D₂O samples with the semiquinone signal subtracted out. In comparison to the “rapid type 1” signal from bovine xanthine oxidase, coupling to the two inequivalent protons disappear in the D₂O sample, indicating (as seen with xanthine oxidase) that these protons are solvent-exchangeable. This observation agrees with previous results with xanthine oxidase. The similarity in results for the bovine oxidase and bacterial dehydrogenase form indicate that the recombinant protein has the same geometry in the molybdenum coordination sphere as the bovine oxidase and behaves in the same way.

The “rapid type 1” EPR signal generated by the reaction bovine xanthine oxidase at pH 10 with HMP is shown third from the bottom in Figure 4.13. As mentioned in Chapter 1, the species giving rise to the “rapid type I” signal represents a complex of substrate with enzyme that has already been reduced to the level of Mo (V). With the bovine enzyme, the “rapid type 1” signal is favored over the other Mo (V) EPR active species under conditions of low pH and high substrate concentration (Bray et al., 1978). This signal exhibits superhyperfine coupling to one strongly and one weakly coupled proton and has g-values of \( g_{1,2,3} = 1.9906, 1.9797, 1.9654 \) (Bray et al., 1978). \(^{33}\)S- and \(^{17}\)O-labeled enzyme reacted with xanthine generates signals indicative of one weakly coupled sulfur and one (or more) strongly coupled oxygen (Malthouse et al., 1981; Bray and Gutteridge, 1982). The signal appears on the same time scale as the “very rapid” signal disappears and was originally considered to represent an intermediate in the reductive half-reaction; however, the signals are not observed under single-turnover conditions and cannot represent a true catalytic intermediate (McWhirter and Hille, 1991; Kim and Hille, 1993). Under excess substrate conditions (multiple turnovers) xanthine oxidase accepts a total of six electrons for three substrate molecules (Hille and Massey,
The excess substrate molecules can interact with the fully oxidized enzyme, the two-electron reduced enzyme (created from oxidation of one substrate molecule), or the four-electron reduced enzyme (generated from the electrons from two substrate molecules). Based on the above, it has been proposed that the signal-giving species is not an intermediate within the catalytic cycle, but instead represents a complex of the partially reduced enzyme with excess substrate (Pick and Bray, 1969; Gutteridge et al., 1978). Further evidence suggests that the “rapid type 1” signal represents the Michaelis complex of substrate with molybdenum in its Mo (V) paramagnetic state (Hille et al., 1993).

The “rapid type 1” signal has also previously been generated using H and D (labeled on the C8 position) forms of the substrate in D$_2$O. Upon comparison of these signals, no differences were observed indicating that the C8-H is weakly coupled to the Mo (V)-substrate complex. Once the C8 proton is transferred to the molybdenum it becomes strongly coupled (Gutteridge et al., 1978).

The E232A variant was also reacted with HMP (as described in Materials and Methods) in an attempt to generate the “very rapid” signal. The “very rapid” signal observed with xanthine oxidase is shown in Figure 1.10 and discussed in Chapter 1. No superhyperfine coupling to protons is evident in the canonical “very rapid” signal. Comparison of this signal to the signals obtained with E232A, demonstrate a similar line shape to the “very rapid” signal; however the features are closer together and have a much narrower field width. The same signal persisted throughout several freeze-thaw incubations. The signal also has an extra feature represented by a “fourth” unrealistic g-value. This feature remained when the experiment was repeated in D$_2$O, making it
unlikely to be due to proton coupling. Upon close examination of the signals, no splitting was evident. A second sample was generated with higher concentration enzyme (200 µM), but still no evidence of proton coupling. The impossible “fourth” g-value could be a contribution from a contaminating signal; however, the observed signal does not represent any combination of previously observed signals. At this point it is uncertain as to the cause of this extra feature. The generation of such a “very rapid”- like signal is very interesting since the characteristic “very rapid” signal is not observed with another dehydrogenase form of the enzyme. The difference in the bacterial dehydrogenase and bovine oxidase “very rapid” signals definitely speaks to the difference in the environment surrounding the metal center between the native xanthine oxidase and the E232A mutant of the bacterial dehydrogenase as to be expected for an amino acid substitution so close to the molybdenum active site.

4.3.4 Resonance Raman spectra of *Rhodobacter capsulatus* xanthine dehydrogenase

In order to compare the dehydrogenase form of the enzyme with the oxidase form, the enzyme was reacted with violapterin, in an attempt to generate the complex that is observed with bovine xanthine oxidase (as presented in Chapter 2). The anaerobic enzyme-violapterin mixture was reduced with dithionite solution in an anaerobic cuvette and followed spectrophotometerically looking for the absorbance increase at 650 nm as the charge-transfer complex forms. Only a slight absorbance increase was observed around 650 nm (not shown), which was most likely only due to the formation of the flavin semiquinone. In order to definitively determine whether the charge-transfer complex was formed, a cryogenic Raman sample was prepared as described in Chapter 2
and excited with a laser at 647 nm. The spectrum obtained (shown in Figure 4.14) indicated only a very small portion of the charge-transfer complex; however, In comparison to the rR of the bovine xanthine oxidase charge-transfer complex (see Chapter 2), the maximally enhanced modes that are observed line up well with the bands observed in the oxidase enzyme.

These results show that the charge-transfer complex does form with the \textit{Rhodobacter capsulatus} enzyme, but not to the extent that is observed with the bovine enzyme. A recently designed tight-binding inhibitor of xanthine oxidase (FYX-051), also forms a charge-transfer complex with xanthine oxidase. The crystal structure of this complex has been solved (Okamoto et al., 2004). Comparison of this newly solved crystal structure of the charge-transfer complex with that for the alloxanthine-inhibited structure of RcXDH suggests that an active site water in the charge-transfer complex structure that is present in the alloxanthine structure (Truglio et al., 2000; Okamoto et al., 2004). It is possible that this active site water is involved in displacing the product from the molybdenum center. Based on this proposal, the absence of the water molecule accounts for the stability of the charge-transfer complex.

Experiments with \textit{Rhodobacter capsulatus} xanthine dehydrogenase do not indicate that this new inhibitor is nearly as effective an inhibitor of the bacterial enzyme (Leimkühler, unpublished). The decreased ability of the dehydrogenase enzyme to form the charge-transfer complex with violapterin may be connected to the ineffectiveness of this compound as an inhibitor. This observation suggests a possibility of a slightly larger active site cavity, allowing the entry of a water molecule to displace product. This hypothesis is supported by the decreased substrate specificity observed with the
*Rhodobacter capsulatus* xanthine dehydrogenase. An increased active site cavity may accommodate a wider range of substrate sizes which would allow a variety of substrates into the active site. However, the greater distance between the substrate and active site residues may not allow sufficient interaction to orientate the substrate in its proper position for catalysis to proceed.
Xanthine::NAD activity was recorded at 340 nm.

Activity to flavin ratio (AFR) was obtained by dividing the change in absorbance/min at 295 nm in the presence of NAD by the absorbance at 465 nm of the enzyme used in the assay.

Table 4.1 Purification of *R. capsulatus* XDH by Sepharose 4B/folate gel affinity chromatography. The yield in mg of protein, A280/A465 ratio, A465/A450 ration, xanthine to NAD activity, and AFR after each of the chromatography steps in the purification. The purification was completed by Dr. Silke Leimkühler (Leimkühler et al., 2003; Leimkühler et al., unpublished).
XDH-variant

<table>
<thead>
<tr>
<th>wild-type</th>
<th>E232A</th>
<th>E730A</th>
<th>E730D</th>
<th>E730Q</th>
<th>E730R</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Moco content&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>108</td>
<td>33</td>
<td>92</td>
<td>109</td>
</tr>
<tr>
<td>Mole S per subunit&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76</td>
<td>0.83</td>
<td>0.22</td>
<td>0.32</td>
<td>0.48</td>
</tr>
</tbody>
</table>

<sup>a</sup> Moco was quantitated as described in [Johnson, 1984]. Moco content of native <i>R. capsulatus</i> XDH was set to 100% and the amount of Moco determined in the active-site variants was compared to that value.  
<sup>b</sup> The amount of the cyanolysable sulfur present in XDH was determined as described in [Westley, 1981].

**Table 4.2 Determination of Moco content and amount of cyanolysable sulfur present in native <i>R. capsulatus</i> XDH and active-site variants.** The percent of molybdenum content of the purified mutants and quantitation of sulfur content. The methods of data collection are described in the materials and methods section. The data was collected and compiled by Dr. Silke Leimkühler (unpublished).
<table>
<thead>
<tr>
<th></th>
<th>Steady State</th>
<th>Rapid Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ ($s^{-1}$)</td>
<td>$K_m$ (µM)</td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>70</td>
<td>23</td>
</tr>
<tr>
<td>HMP</td>
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<td>2.5</td>
</tr>
<tr>
<td>E232A</td>
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<td></td>
</tr>
<tr>
<td>xanthine</td>
<td>4.4</td>
<td>163</td>
</tr>
<tr>
<td>HMP</td>
<td>0.40</td>
<td>0.68</td>
</tr>
<tr>
<td>Q197E</td>
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<td></td>
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<tr>
<td>Xanthine</td>
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<td>6.5</td>
</tr>
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<td>ND</td>
</tr>
<tr>
<td>R310M</td>
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<td></td>
</tr>
<tr>
<td>xanthine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HMP</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E730A</td>
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<tr>
<td>xanthine</td>
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<td>ND</td>
</tr>
<tr>
<td>HMP</td>
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<tr>
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<tr>
<td>HMP</td>
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</tr>
</tbody>
</table>

Table 4.3 Steady State and pre-steady state kinetic parameters for the recombinant enzyme. Table of the steady-state and rapid reaction kinetic parameters for each of the variants used in the current study. Values that could not be accurately determined due to slow reaction rates are not determined (ND). The limiting rate of the R310M, and E730A/D/Q/R variants was estimated as described in materials and methods.
Figure 4.1 The molybdenum active site of bovine xanthine oxidase and *Rhodobacter capsulatus* xanthine dehydrogenase. The residues examined in this study are labeled with both their residue numbers for the oxidase (red) and the dehydrogenase (blue).
Figure 4.2 The steady-state kinetic plots for RcXDH wild-type with xanthine and HMP. The data was collected for the current study as described in materials and methods. The $k_{\text{cat}}$ and $K_m$ are 70 s$^{-1}$ and 23 µM for xanthine respectively (top). The double reciprocal plot for the steady state reaction with HMP. (bottom). The $k_{\text{cat}}$ and $K_m$ for HMP are 4.4 s$^{-1}$ and 2.5 µM, respectively.
Steady State Experiments with Rc XDH wt with xanthine pH 7.8 and 4°C

Figure 4.2
Figure 4.3 The steady-state kinetic plots for RcXDH E232A with xanthine and HMP. The data was collected for the current study as described in materials and methods. The $k_{cat}$ and $K_m$ are 4.4 s$^{-1}$ and 163 µM for xanthine respectively (top). The double reciprocal plot for the steady state reaction with HMP. (bottom). The $k_{cat}$ and $K_m$ for HMP are 0.40 s$^{-1}$ and 0.68 µM, respectively.
Steady State Experiments with Rc XDH E232A with xanthine pH 7.8 and 4ºC

Steady State Experiments with Rc XDH E232A with HMP pH 7.8

Figure 4.3
The double reciprocal plot of the steady-state kinetics of RcXDH Q197E with xanthine. The $k_{\text{cat}}$ is 9.6 s$^{-1}$ and the $K_m$ is 6.5 µM.
Figure 4.5

The rapid reaction kinetics of RcXDH wild-type with xanthine. The filled squares represent the first phase. The second phase is represented by the much slower second phase. The $k_{\text{red}}$ is reported at $67 \text{ s}^{-1}$ and the $K_d$ is $33 \text{ µM}$. The data was collected by Dr. Silke Leimkühler in collaboration with Dr. Takeshi Nishino at the University of Tokyo (unpublished).
Figure 4.6

Figure 4.6 The double reciprocal plot for the rapid half-reaction of RcXDH wild-type with HMP. The resulting $k_{\text{red}}$ are $0.28 \text{ s}^{-1}$ and the $K_d$ could not be determined. The data was collected for this study as described in materials and methods.
Figure 4.7 The rapid reaction kinetic plots for RcXDH E232A with xanthine and HMP. The data was collected for the current study as described in materials and methods. The $k_{\text{red}}$ and $K_d$ are $5.5 \text{ s}^{-1}$ and $409 \mu\text{M}$ for xanthine respectively (top). The double reciprocal plot for the steady state reaction with HMP. (bottom). The $k_{\text{red}}$ and $K_d$ for HMP are $0.42 \text{ s}^{-1}$ and $96 \mu\text{M}$, respectively.
Reductive Half Reaction

RcXDH E232A with xanthine pH 7.8 and 4°C

Figure 4.7
Figure 4.8

Figure 4.8 The rapid reaction kinetic plot for RcXDH Q197E with xanthine. The data was collected for the current study as described in materials and methods. The $k_{\text{red}}$ and $K_d$ are 10.24 s$^{-1}$ and 50 µM for xanthine respectively.
Figure 4.9

Figure 4.9 The rapid-half-reaction data for RcXDH R310M with 400 µM xanthine. The reaction was too slow to follow on the stopped-flow apparatus. Data was collected using an anaerobic cuvette as described in materials and methods. The limiting rate was estimated to be at least $10^5$ lower than that of wild-type enzyme.
Figure 4.10 The spectral change observed upon removal of the sulfur from Mo=S in *Rhodobacter capsulatus* xanthine dehydrogenase via incubation with cyanide as described in materials and methods.
Figure 4.11 The rapid half-reaction data for the E730A/R variants of RcXDH. Each mutant was reacted with 400 µM xanthine and incubated under anaerobic conditions overnight. The limiting rate was estimated to be a minimum of $10^7$ lower than the $k_{\text{red}}$ of wild-type enzyme for both variants.
Figure 4.11
Figure 4.12 The rapid half-reaction data for the E730D/Q variants of RcXDH. Each mutant was reacted with 400 µM xanthine and incubated under anaerobic conditions overnight. The limiting rate was estimated to be a minimum of $10^7$ lower than the $k_{\text{red}}$ of wild-type enzyme for both variants.
Reductive Half-Reaction *Rhotobacter capsulatus* xanthine dehydrogenase
E730D 20mM Tris, 0.3 EDTA, pH 7.8 with 400uM xanthine

![Absorbance spectra](image)

Reductive Half-Reaction *Rhotobacter capsulatus* xanthine dehydrogenase
E730Q 20mM Tris, 0.3 EDTA, pH 7.8 with 400uM xanthine

![Absorbance spectra](image)

**Figure 4.12**
**Figure 4.13**

The EPR spectra obtained in this study with wild-type and E232A RcXDH. The top three spectra represent E232A with HMP at pH 10.0. The third from the bottom shows the very rapid type 1 signal obtained with xanthine oxidase. The bottom two spectra represent wild-type RcXDH in H\textsubscript{2}O and D\textsubscript{2}O.
Figure 4.14

**Figure 4.14** The rR spectrum of RcXDH reduced with dithionite in the presence of violapterin. The bands at 701, 857, 1003, 1169, 1262, 1296, 1559, and 1587 cm$^{-1}$ indicate that the charge-transfer complex observed with the bovine xanthine oxidase enzyme is formed here, but to a far lesser extent.
CHAPTER 5

CONCLUSIONS

5.1 Summary

5.1.1 Xanthine oxidase and xanthine dehydrogenase

Xanthine oxidase and xanthine dehydrogenase both catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid. Both enzymes contain one FAD, two 2Fe/2S centers, and a molybdopterin. The enzymes differ structurally by the presence of an NAD$^+$ access site that is present only in the dehydrogenase form. The dehydrogenase form prefers NAD$^+$ as the final electron acceptor, while the oxidase form utilizes molecular oxygen. The overall reaction of these enzymes can be broken down into two half-reactions: the oxidative half-reaction, which occurs at the flavin center, and the reductive half-reaction, which occurs at the molybdenum center. The present study examines mechanism of the reductive half-reaction of these enzymes.

During the course of the reductive half-reaction the molybdenum cycles from Mo (VI) to Mo (IV), and uses six reducing equivalents from 3 substrate molecules to fully reduce the enzyme (2 electrons on the molybdenum, 1 electron on each of the 2 iron-sulfur centers, and 2 electrons on the FAD) (Hille and Massey, 1981). Crystal structure
data have shown that the molybdenum active sites of these two enzymes are extremely similar, as is their kinetic behavior (Truglio et al., 2002; Enroth et al., 2000).

The most current proposed mechanism for the reductive half-reaction of xanthine oxidase involves proton abstraction from the Mo-OH group in the active site by glutamate 1261 (730 in the *Rhodobacter capsulatus* enzyme), followed by nucleophilic attack on the C8 position of the substrate with a hydride transfer to the Mo=S group (Figure 1.19). The oxygen from the Mo-OH group bridges the enzyme to the substrate in a Mo$^{IV}$-O-R species. This mechanism suggests a two-electron reduction in the first step, followed by a pair of one-electron oxidation steps to produce the Mo (V) EPR active species transiently.

A variety of techniques have been used in this dissertation research to experimentally support this base-catalyzed mechanism. Resonance Raman is used to examine the vibrational characteristics of an enzyme reduced-product complex formed between bovine xanthine oxidase and violapterin, which forms a charge-transfer complex with an absorbance maximum near 650nm. A kinetic study of the reductive half-reaction with bovine xanthine oxidase with various purine substrates was completed in order to differentiate between the suggested two-electron reduction and a recently proposed radical mechanism. Finally, the *Rhodobacter capsulatus* xanthine dehydrogenase expression system was used in order to study specific mutations in residues proposed to play a role in catalysis by steady-state and pre-steady state kinetics, resonance Raman, and EPR. Each of these experimental sections are summarized below.
5.1.2 Resonance Raman studies of the charge-transfer complex formed between bovine xanthine oxidase and violapterin

Resonance Raman studies of the charge-transfer complex formed between xanthine oxidase and violapterin have allowed the assignment of bands at specific frequencies to their originating vibrational modes. The assignments of these modes have provided information about the electronic distribution in the charge-transfer complex. This complex is important catalytically because it represents the first true catalytic intermediate in the reductive half-reaction containing reduced enzyme (in the Mo (IV) oxidation state) – product (violapterin) complex through the oxygen atom transferred from the newly deprotonated Mo-OH group to the substrate carbon being hydroxylated (C8 in xanthine/uric acid, C7 lumazine/violapterin, C2 in FYX-051/2-hydroxyFYX-051). This intermediate exists immediately upstream of the Mo (V) “very rapid” species. EPR studies of the enzyme with xanthine and 2-hydroxy-6-methylpurine identify the formation of this Mo (V) “very rapid” species prior to product release; however, with violapterin (and FYX-051) the product is released directly from the Mo (IV) species examined by rR in this study. The reduction potentials of reaction intermediates from reactions with various substrates determine whether the product is released from the Mo (V) or Mo (IV) oxidations states of the enzyme. Regardless of which species is releasing product, the E\text{red}-product complex is an obligatory intermediate. This makes the rR work presented here directly applicable not only to the reaction with xanthine, but also to the recent crystallographic studies of the potent-inhibitor FYX-051, a compound which also forms a charge-transfer complex (Okamoto, 2004).
5.1.3 Kinetic examination of the first step in the reductive half-reaction of bovine xanthine oxidase

As described in Chapter 3, two mechanisms have been proposed for the reductive half-reaction of the enzyme. The first mechanism (shown in Figure 1.19) suggests a base-catalyzed mechanism, while the second (shown in Figure 3.1) occurs by two sequential one-electron steps through the formation of a radical intermediate. In order to distinguish between the two mechanisms, the $k_{\text{red}}$s and $K_d$s of the reaction of xanthine oxidase with several purine substrates ranging from physiological xanthine to the essentially inactive 3-methylxanthine were determined. These substrates were chosen based on their commercial availability and predicted large range on reduction potentials. The one-electron reduction potentials of each of these substrates were measured by pulse radiolysis in collaboration with Dr. Robert Anderson at the University of Auckland in New Zealand. These one-electron reduction potentials were plotted against the natural log kinetic parameters $k_{\text{red}}$ and $k_{\text{red}}/K_d$. If the mechanism is occurring by two sequential one-electron steps, a linear correlation would be observed between the one-electron reduction potentials and the kinetic parameters. No such correlation was observed, ruling out the possibility of the radical-based mechanism.

Substrates methylated in the N3 position were also included in this study. The rates of reaction of the 3-methyl substrates were extremely slow (in fact too slow to allow accurate measurement). As described in Chapters 1 and 3, a proton shift from N3 to N9 is thought to be required during the course of reaction of the base catalyzed mechanism. The presence of a methyl group in the N3 position would prevent this, rendering the
substrate non-reactive. The slow rates observed in this study of such substrates are consistent with the proposed base-catalyzed mechanism as shown in Figure 1.19.

5.1.4 Studies of the active site residues in xanthine dehydrogenase

Steady-state and pre-steady state kinetics for the reaction of various form of RcXDH with xanthine or HMP were completed in this dissertation research. The mutants studied are summarized on Figure 4.1 and Table 4.3. The $k_{cat(xanthine)}$ and $k_{red(xanthine)}$ decreases 16-fold and the $K_{m(xanthine)}$ and $K_{d(xanthine)}$ increases 65.2-fold in the E232A mutant as compared to wild-type. In the case of HMP, the $k_{cat(HMP)}$ decreases as well as the $K_{m(HMP)}$. The pre-steady state kinetic parameters could not be accurately compared since the $K_{d(HMP)}$ of the wild-type enzyme could not be determined, but the $k_{red(HMP)}$ increased slightly in the E232A mutant. This residue was originally considered to play a role is substrate tautomeriation, but based on its reasonably small effect in the kinetics (with the exception of the $K_{m(xanthine)}$) is most likely involved in electrostatic stabilization of the transition state instead.

The Q197E mutant showed a 7-fold reduction in $k_{cat(xanthine)}$ and $k_{red(xanthine)}$ and modest (2- to 4-fold) decreases in $K_{m(xanthine)}$ and $K_{d(xanthine)}$. The slightly larger effect seen on the rate of reaction rather than on the binding affinity supports the proposed role (modulation of the reactivity of the molybdenum) of this residue. The recent crystallographic studies of FYX-051 suggesting hydrogen bonding to the Mo=O also support these results (Okamoto et al., 2004). It is important to note that the 7-fold reduction seen in the rate of reaction is indeed rather modest itself, signifying that the residue is probably not a major contributor to the overall catalytic efficiency.
The estimated limiting rate of the R310M mutant showed at least a $10^5$ reduction in $k_{\text{red(xanthine)}}$. As mentioned in Chapter 4, this is a much larger effect than was originally predicted based on the distance of the residue from the molybdenum active center. Based on these results we propose that the arginine residue is involved in stabilization of the negative charge accumulated (as suggested by the computational studies with formamide as a substrate described in Chapter 1 (Ilich and Hille, 1997)) on the substrate during the course of reaction. Failure to stabilize this negative charge would not allow hydride transfer from the N3 to the N9 position, thereby destabilizing the transition state (by approximately 24kcal/mol calculated in vacuo) (Ilich and Hille, 1997).

The largest effect observed upon mutation was in the E730A/D/R/Q mutants, whose rates of reduction decreased by at least 7 orders of magnitude. The large effect of these mutants was expected considering the proposed role of this residue as the active site base initiating catalysis. Recently, it has been proposed that this residue is also involved in hydrogen-bonding to the product (Okamoto et al., 2004). The dual roles of active site base and hydrogen-bonding proposed for this residue are supported by the large effect on reaction rate observed in this study.

The EPR studies of the wild-type RcXDH formed the “rapid type 1” signal with two observable inequivalent protons, which disappeared when repeated in D$_2$O when reacted with 5 equivalents of HMP. These results agree with those (described in Chapter 4) observed for bovine xanthine oxidase, indicating that one proton is weakly coupled to the Mo (V)-substrate complex and that only after transferred to the molybdenium it is strongly coupled. The EPR signal obtained from reaction of HMP with E232A, was unique in that its $g$-values were closer together than those observed in the “very rapid”
signal in bovine xanthine oxidase as well as containing an unexplained “fourth” g-value which persisted in D$_2$O. Aside from these differences, the signal resembled the “very rapid” signal in lineshape.

5.2 Future work

5.2.1 Mutations in the active site of Rhodobacter capsulatus xanthine dehydrogenase

Due to the convenience of the E. coli expression system for the Rhodobacter capsulatus enzyme, it would be useful to examine more active site mutants in addition to those examined in this dissertation research. It would be really interesting to make a mutation at the 730 residue that would result in active enough protein to complete a pH dependence study as done with the bovine enzyme. Since several of the mutants made at this residue showed no activity, this may prove to be a difficult task. Although the support presented in the current study demonstrates the importance of this residue it does not prove with 100% certainty that it is in fact acting as an active site base. A pH dependence study of the mutant protein showing a disappearance of the pK$_a$ would provide such evidence. Unfortunately, all mutations that could fulfill such a role have already been examined. Additionally, a rescue experiment was attempted by Dr. Silke Leimkühler and coworkers with the E730A mutant and was not successful (personal communication).

The R310M mutant also resulted in a greater affect than predicted as described above and in Chapter 4. We propose that this residue is involved in negative charge stabilization. Given the length and the charge of the arginine residue, a mutation to
lysine would be interesting to examine. I would expect to see some affect on the reaction, but not to the extent that it was observed upon mutation to an uncharged residue. Furthermore, mutation to a negative residue, such as glutamic acid, may cause an even greater affect than observed with the methionine mutation.

The two phenylalanine residues corresponding to the 1009 and 914 in the bovine enzyme would also make interesting mutation sites. These two residues are in position to \( \pi \)-bond with aromatic substrate and position it into the active site correctly. Mutations of these residues to any non-polar, non-aromatic residue may slow the substrate binding, affecting the overall rate of catalysis. It has been shown that the \textit{Rhodobacter capsulatus} enzyme has greater substrate specificity than the bovine enzyme; mutations in these residues may allow a greater range of substrates into the active site as observed with the bovine enzyme. Also, the effectiveness of the FYX-051 inhibitor may change upon mutation of these residues.

5.2.2 Studies on the substrates 2- and 6-thioxanthine with xanthine oxidase

During the studies presented in Chapter 3, a slight increase in absorbance at 450nm was observed prior to the reduction of the flavin in the 2- and 6-thioxanthine substrates. The same was observed with HMP, which is known to form an intermediate before reduction. Experiments have been initiated to examine the possibility of intermediate formation with these substrates, but a more detailed look is necessary. The kinetics should be repeated under single-turnover conditions (using excess enzyme rather than substrate) and monitored on the stopped-flow with the photodiode array attached.
This would allow rapid collection of the absorbance changes over the entire spectrum and visualization of any absorbing intermediates within the course of the reaction.

EPR experiments involving reaction of xanthine oxidase with each of these substrates were examined to see if the “very rapid” signal was observed. Unfortunately, only the “rapid type 1” signal formed, probably because the rates of reaction with these two substrates are much faster than that of HMP and could not be captured by freezing the sample by hand in liquid nitrogen-acetone bath. The experiment should be repeated using freeze quench techniques in order to evaluate if these substrates do indeed form the “very rapid” species.

5.2.3 Experiments with Q197E

Steady-state and pre-steady state kinetic parameters should be obtained for the reaction with Q197E with lumazine. If lumazine is a successful substrate, the mutant should be reduced in the presence of violapterin and examined spectrophotometrically and with resonance Raman. The charge-transfer complex is thought to form to only a small degree because the larger substrate size takes up space in the active site and does not allow the presence of the water molecule thought to neutralize substrate as it is released. Since Q197 is proposed to modulate the reactivity of the molybdenum center and possibly mediate product release, the mutant enzyme may form the charge-transfer complex to a greater extent.
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