

NATHANIEL JOSHUA COSPER
Metals in Medicine and Nature: Function and Form
(Under the Direction of ROBERT A. SCOTT)

X-ray absorption spectroscopy (XAS) was used to investigate structure, function, and mechanistic details of enzymatic catalysis in a variety of biological systems. Most significantly, these studies have resulted in the proposal for a mechanism for radical generation in the enzyme lysine aminomutase, as well as generating understanding about the active site and inhibitor-binding mode of methionyl aminopeptidase, an enzymatic target for anti-cancer drugs. Other biological systems that were studied include urease, neuronal nitric oxide synthase, cytochrome *bo*₃, an accessory protein of the nitric oxide reductase (*nos*) gene cluster, Archaeal zinc-containing ferredoxin, heavy metal responsive regulator proteins, Archaeal transcription factor, and beta-carbonic anhydrase.

Additionally, in an effort to design a biological pathway for the degradation of environmental contaminants, particularly halogenated aromatic compounds, the substrate specificity of catechol dioxygenase was rationally designed to significantly increase its ability to cleave halogenated and substituted catechols.

INDEX WORDS: biophysical, EXAFS, function, metalloprotein,
metalloenzymes, structure, X-ray absorption spectroscopy,
XAS.

METALS IN MEDICINE AND NATURE: FUNCTION AND FORM

by

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DEDICATION

To my loving wife and wonderful family.

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As always, the quest for knowledge isn't made alone. With but a few words, I can't possibly thank all who have helped and pushed me. At best, I can recognize those whose efforts have been most recent, or most consistent throughout the years. To these people, and to all whose names don't appear, yet whose actions and words have made my quest more pleasant and fruitful, thank you. In particular, I am indebted to the following:

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CHAPTER 1
INTRODUCTION

General Introduction to Metalloenzymes

Cells are the core unit of biological material. Life can exist at many levels of complexity. Regardless of whether a cell exists as a unicellular organism or as part of a functional unit in a large creature, it can be thought of as a factory, consuming food and producing energy. Key machines in this metabolic process are proteins involved in the Krebb's cycle. Functional proteins, termed enzymes, are units designed to carry out chemical reactions catalytically. Presumably, in the primordial soup, these proteins were created randomly through the combination of common chemicals, spurred by intense local energy sources, like lightning. However, once created, the proteins had no protection from the environment and no means for replication. Thus, as a means of survival, cells have evolved several mechanisms to ensure their viability.

For protection, cells have evolved a barrier, or cell wall, which separates the internal proteins from the external environment. This wall allows the modulation of pH, salt concentrations, and other conditions that allow the cell to function efficiently. For reproduction purposes, DNA evolved as a mechanism for storing information. DNA can be considered a blueprint for how to create the machines necessary for generating energy. This blueprint is read by RNA molecules, which then combine amino acids to form proteins.

Thus, the fundamental processes of life can be considered in three steps: 1) A protective barrier is created to store all the necessary components for life. 2) DNA exists and contains information that is used to build proteins. 3) Proteins exist to convert food into energy. Numerous other functions are necessary to ensure a properly functioning cell, in support of these three principles. For example, the cell must know when to make certain proteins. This is accomplished through regulatory proteins that turn on expression of individual genes. (Genes are small sections of DNA that contain the blueprint for one protein. The compilation of all genes for a given organism is called the genome.) Other examples of ancillary cellular functions include: uptake of essential chemicals, export of

toxic compounds, transport of compounds within the cell, synthesis of DNA and protein precursors, metabolism of carbon and nitrogen sources, transport of oxygen, etc.

How do proteins work? Over the last few decades, biological research has been focused on determining how proteins are built and how the structure of a protein affects its function. Typically, proteins are built by linking amino acids together. However, many proteins contain non-amino acid cofactors that play a critical role in the function of the protein. In particular, inorganic chemists have been interested in proteins that contain metal active sites. These proteins, called metalloproteins, use metal sites for two primary purposes. First, the presence of a metal ion tends to have a stabilizing affect on the structure of the protein. Thus, when proteins are exposed to rigorous conditions, they might contain a metal for additional support. Second, metal ions can play a functional role, interacting directly with chemical transformation processes. It has been estimated that one-third of all proteins contain metals.

The presence of transition metals, belonging to groups III – XII of the periodic table, presents the opportunity to conduct the types of analyses that is typical of traditional inorganic chemistry. For example, most transition metals have paramagnetic properties and are optically active, allowing for UV-Vis, EPR, MCD and other types of spectroscopic analysis. As a result of the presence of metals in proteins, many traditional chemists now consider themselves “biochemists,” conducting extensive research into the mechanisms of catalysis performed by metalloenzymes.

How are intracellular concentrations of metals controlled? Recent studies by O’Halloran and coworkers have had a major impact on the field of metal homeostasis, defined as the regulation of metal levels in the cellular environment. In contrast to previous theories that relied on a “pool” of free metal ions in the cell, O’Halloran has shown that the effective free zinc concentration is less than one atom per cell [1]. The implications of these findings are vast: Nature appears to have developed an intricate mechanism for uptake, storage and transport of essential trace elements. Integral to this mechanism is regulation of gene expression. In *E. coli*, the apparent metalloregulator for

zinc homeostasis is ZntR, which begs the question, how does ZntR distinguish zinc from other transition metals? Even after that question has been answered, one must also consider whether there are general rules for differentiation of transition metals by metalloregulators (and other proteins).

Research that we have conducted during the last several years has contributed to the understanding of the metal active site structure of *Synechococcus* PCC7942 SmtB, a zinc- and cobalt-responsive metalloregulator [2]. Similarly, we have characterized the cadmium-responsive regulator, CadC from *Staphylococcus aureus* p1258 [3]. These experiments, in conjunction with those planned for nickel-responsive NmtR and cobalt-responsive CztA, will allow our research group to answer critical questions about the mechanism of metal uptake and regulation. In particular, we hope to be able to decipher how proteins “sense” metals and select the metal of interest.

Specific Examples of Metalloproteins in Biology

Metalloproteins in Medicine. Metal cofactors play many and varied roles in medicinal biology. For example, a well known metalloprotein is hemoglobin, which contains an iron atom in its active site. Hemoglobin binds oxygen in the lungs and carries it to the tissues where it is released to myoglobin, another iron-containing protein. Myoglobin then carries oxygen to the extremities of the body, where it is consumed. Ironically, oxygen is an extremely reactive, and even toxic, substance. As evidence, consider a once powerful and sleek car, now converted to rust. Were oxygen to be unconstrained inside a cell, it would cause many unwanted reactions, not the least of which involves damage to DNA. Thus, the cell must tightly regulate the presence of oxygen within the cell. Since metals can be used to bind and release oxygen, they are ideal for oxygen transport.

Another example of a biomedically important metalloprotein is methionyl aminopeptidase. This protein represents a unique class of proteases that are capable of removing the N-terminal residues from nascent polypeptide chains. Removal of N-

terminal residues from nearly all newly synthesized peptides is essential for co-translational and post-translational modifications that are critical for fully functional enzymes, correct cellular localization and eventual degradation of proteins. Methionyl aminopeptidases are essential for cell growth and proliferation and therefore are potential molecular targets for anti-cancer drugs that inhibit angiogenesis, the formation of new blood vessels. Towards developing protein-specific drugs, we have studied the iron center of methionyl aminopeptidase in the active and fumagillin-inhibited forms. These studies have led to a better understanding of the mechanism for the methionyl aminopeptidase, which will ultimately be useful in developing drug candidates based on this protein target.

In a current initiative in our laboratory, we are studying the biochemical pathways in pathogenic bacteria as a source for new antibiotic targets. A recent report by the United States Center for Disease Control and Prevention has indicated that there are several strains of *Staphylococcus aureus* that are resistant to all antibiotics except vancomycin and there are more recent reports of strains that are also resistant to vancomycin. Thus, there is an immediate and urgent need for new antibiotics. It is preferable that new antibiotics do not contain a β -lactam functional unit to prevent the pathogens from quickly mutating to gain resistance to these drugs. Hence, we are studying N-succinyl-L,L-diaminopimelic acid desuccinylase (DapE) from *Haemophilus influenzae*. DapE is an essential enzyme in a biosynthetic pathway that is the *only* source of lysine, one of the twenty key amino acids, in bacteria. Since lysine is required for bacterial cell growth and proliferation, DapE is an attractive target for a novel class of antibiotic drugs. Towards the rational design of DapE inhibitors, we are studying the zinc active site, as well as various substrate-bound and transition-state analogues.

Metals in Bioremediation. Aromatic compounds have proven to be persistent environmental contaminants. These compounds are present in wood as lignin and in man-made forms such as pesticides, detergents, solvents, paints and oils. In particular, halogenated aromatic compounds are quite intractable biodegradation targets [4, 5].

Chlorinated aromatics, which are more stable than the non-chlorinated counterparts, were widely used as coolants for transformers, flame retardants, and hydraulic fluids. It is estimated that polychlorinated biphenyls, released in bulk until the 1970's, account for approximately 375,000 tons of environmental contaminants [6]. The widespread use of dichlorodiphenyl-trichloroethane (DDT) as an herbicide and polychlorinated benzodioxins as transformer fluids and plasticizers have also led to significant bioaccumulation of chlorinated aromatics, particularly in the fatty tissues of animals [7].

Despite the stability of aromatic compounds, microbiological pathways for their degradation have evolved. In the soil bacterium *Acinetobacter* sp. ADP1, the β -ketoadipate pathway has evolved to process degradation products of various aromatic compounds, for example benzoate, to substrates in the Krebs cycle [8, 9]. Not surprisingly, a key step in these pathways is the interaction of aromatic compounds with oxygen, mediated by iron-containing enzymes, to affect oxidative ring-cleavage of substituted dihydroxybenzenes [10, 11]. Catechol 1,2-dioxygenase (CTD), one example of an iron-containing, oxygen-activating enzyme, catalyzes the conversion of catechol to *cis,cis*-muconate [12, 13], while protocatechuate 3,4-dioxygenase catalyzes the conversion of protocatechuate to β -carboxy-*cis,cis*-muconate [14, 15].

What is the Purpose of Selenium in Biology? Selenium has received increased attention as a micronutrient and an essential component of an expanding number of important enzymes. The presence of selenium in biological systems is rare and when it occurs, the selenium appears to play a specific role in enzymatic catalysis. This poses several interesting questions: Why does nature choose to use selenium instead of sulfur in some cases? What are the properties of selenium that make it useful to an organism? What mechanisms are associated with the use of sulfur (and selenium)? Ongoing research in the Scott laboratory seeks to characterize naturally occurring selenium-dependent systems. In addition, since selenium occurs only rarely in nature, we have exploited it as a spectroscopic probe. In systems where a key sulfur atom plays a critical role in catalysis, selenium substitution can be used to characterize the structural environment of

that atom throughout a catalytic cycle. Of particular interest to us and our collaborators is the mechanism for radical generation in *S*-adenosyl-L-methionine (AdoMet)-dependent systems. This class of enzymes is characterized by a complex FeS active site, which is thought to interact with AdoMet to generate a cofactor-based radical. We have conducted Se XAS experiments on biotin synthase, pyruvate formate lyase-activating enzyme, and lysine 2,3-aminomutase [16]. These experiments are helping to define the interaction between AdoMet and the FeS cluster and the mechanism of radical generation in these enzymatic systems.

Overview of X-ray Absorption Spectroscopy (XAS)

The Role of XAS in Biology. Many reference sources contain the details necessary for the expert practitioner; this description is meant for the non-expert. XAS consists of measuring the x-ray photon energy-dependent absorption coefficient of a sample. (The need to scan the x-ray photon energy to obtain a spectrum explains the requirement for an intense tunable source of x-rays such as a synchrotron.) For spectroscopically dilute samples like the frozen aqueous solutions of metalloproteins that we examine, x-ray fluorescence excitation is normally used because of its greater sensitivity [17]. An x-ray absorption *edge* (a relatively sharp rise in the absorption coefficient) is observed at an x-ray photon energy characteristic of each element in the sample. For elements in the first transition series and beyond, these edges require photons with energies in the hard x-ray region (e.g., Fe @ 7.1 keV; Zn @ 9.7 keV; Cd @ 31 keV). The K edge results from the x-ray photon-induced dissociation of a 1s electron from an atom of a particular element (often, but not always, a metal). The valence electron distribution of the metal affects the shape and energy of the K edge, providing some sensitivity to the electronic structure and geometry of the metal site. Given this sensitivity, analysis of the x-ray absorption edge spectrum of a given metal site can provide information about the oxidation state, spin state, coordination geometry, and coordination number.

At x-ray photon energies beyond the edge, the absorbed photon energy above that needed for $1s$ ionization is converted into kinetic energy of the resulting photoelectron, which can be considered a *de Broglie* wave. This photoelectron wave scatters from electron density surrounding atoms in the vicinity of the metal that absorbed the photon, giving rise to modulation of the x-ray absorption coefficient, called EXAFS (extended x-ray absorption fine structure). The EXAFS therefore contains information about the "atomic neighborhood" within about 5 Å of the absorbing metal, and proper analysis of the EXAFS modulations provides information about *how many* of *what kind* of atoms are at *what distance* from the metal. Each nearby atom contributes a sinusoidal modulation to the EXAFS, the *amplitude* of which is related to coordination number and atomic number, the *phase* of which is related to atomic number, and the *frequency* of which is related to metal-atom distance. Thus, Fourier transformation of the EXAFS (essentially a sum of sine waves) results in FT peaks at distances (frequencies of the sine waves) corresponding to metal-atom distances in the site of interest. The photoelectron scattering is not sensitive to the geometric arrangement of atoms around the metal, providing essentially a radial distribution (concentric spherical "shells") of atoms around the metal. In this sense, the structural information available from EXAFS is more limited than the position of every atom in the protein obtained from an x-ray crystal structure. However, metal-atom (-ligand) distances can be determined to an accuracy of ± 0.02 Å – at least five times better than a moderate resolution crystal structure. In addition to the ease of application of XAS (spectra can be recorded in a few hours at most) and its applicability to samples without long-range order (frozen solutions rather than crystals), this distance accuracy makes XAS very complementary to x-ray crystallography as a structural technique for metalloproteins.

XAS is ideal for examining spectroscopically difficult metals. Many of the sophisticated metalloproteophysical spectroscopic techniques in current use prove to be excellent tools for examining a wide variety of metalloproteins that contain spectroscopically rich metals like Mn, Fe, Ni, Cu, Mo. Such metals that contain an

unfilled valence d shell, can take advantage of electronic and magnetic properties of these valence electron configurations, providing indirect evidence of the arrangement and types of ligands to the metal. Thus, Mössbauer spectroscopy is an excellent technique for Fe sites, magnetic circular dichroism (MCD) spectroscopy provides the most information for paramagnetic metal sites, electron paramagnetic resonance (EPR) spectroscopy is applicable only to paramagnetic metal sites, only recently becoming useful for integer-spin paramagnets. All of these techniques have "blind spots": specific metals or oxidation/spin states for which they are inapplicable. On the other hand, EXAFS can provide structural information on any metal in any oxidation/spin state.

A good example of this advantage is for metalloproteins with Zn^{2+} sites, which constitute a huge class of important metalloproteins and –enzymes [18]. Zn^{2+} is d^{10} and is considered to be "spectroscopically difficult", being diamagnetic and exhibiting no metal-based electronic transitions. Thus Mössbauer, MCD, EPR, and resonance Raman spectroscopies are all useless in studying Zn^{2+} metalloproteins. Nuclear magnetic resonance (NMR) spectroscopy can be used to provide structural information on the protein component of these molecules, but XAS is the only technique (short of x-ray crystallography) that can study the Zn^{2+} site directly, providing direct information about the coordination environment. XAS is poised to provide unique and essential structural information about the interaction of these elements with biological systems.

XAS limitations. Effective use of the XAS technique requires recognition of what it can and *cannot* do. First, XAS is still a relatively insensitive technique and metal concentrations of a few hundred micromolar still represent the lower limit (this is being improved with third-generation synchrotron sources). Second, there remain uncertainties in the structural parameters obtained that must be recognized. The scattering atoms C, N, O cannot be distinguished. Two shells of atoms that are at very similar distances from the metal cannot be resolved. EXAFS-derived coordination numbers are "soft" ($\pm 20\%$) unless confirmed by edge analysis. Third, XAS "sees" all occurrences of a given metal, regardless of binding site. The resulting metal-site structure is a weighted *average* of all

sites in the sample. This becomes especially important in cases of weak metal-binding affinity of protein sites. The biochemistry of sample preparation must be carefully controlled so that there is no significant unbound metal in the sample. This often requires substoichiometric metal addition, which is counterintuitive to biochemical thinking. In another example, if the element being investigated is part of a substrate or cofactor (e.g., Se), there can be no excess of this molecule in the sample. These limitations do not make the use of XAS ineffective; in contrast, the most effective use of XAS requires careful attention to its limitations.

Summary of Graduate Research

During the course of my graduate career, I have conducted experiments on a number of biological systems. Rather than attempt to describe each set of experiments separately and completely in this dissertation, I have chosen to give a summary of each major project in this chapter, followed by an in-depth description of a few highlighted projects in subsequent chapters.

Zinc-containing enzymes: SmtB. In collaboration with D. P. Giedroc (Texas A&M University), we have employed XAS to characterize the metal-binding sites of SmtB, a zinc-responsive transcriptional repressor and a member of the ArsR superfamily of prokaryotic metalloregulatory transcription factors. SmtB binds one equivalent of either Zn(II), Co(II), or Ni(II), in order of decreasing affinity. XAS results indicate that zinc and cobalt bind isomorphously, but that nickel binds in a different coordination environment [2]. The extent to which the binding of these cations modulates the affinity of SmtB for DNA or otherwise alters the initiation of transcription is yet unknown and currently being pursued. As these results become available, the structural description of the metal-binding sites in SmtB will provide a basis for interpreting the effects of each cation on transcription.

Zinc-containing enzymes: TFIIIB. In work previously supported by another grant in our laboratory, we generated XAS samples of human transcription factor (TF)IIB and

the [C10H] variant of *Pyrococcus furiosus* (Pf) TFB. The [C10H] variant of PfTFB was constructed to resemble the metal-binding motif of higher eucaryal TFIIB proteins by mutating the second cysteine ligand to a histidine. Using XAS, we have shown that the Zn coordination environments of these two samples are identical, revealing that there is a common zinc-binding motif in archaeal and eucaryal transcription factors and that this motif is likely a determining factor in the overall structure and therefore function, for this class of transcription factors [19].

Zinc-containing enzymes: Carbonic Anhydrases. Carbonic anhydrases catalyze the reversible hydration of carbon dioxide and are ubiquitous in all domains of life. In collaboration with J. G. Ferry (Pennsylvania State University), we have explored the zinc and cobalt coordination environments in archaeal γ - and β -class carbonic anhydrases. XAS has played a key role in determining the differences in first-shell coordination environments, in particular showing that the β -class of carbonic anhydrases contains two sulfur and two nitrogen ligands [20], whereas the γ -class is marked by three histidine ligands and three other oxygen- or nitrogen-containing ligands [21]. In conjunction with kinetic studies, our XAS experiments have demonstrated that these structurally distinct classes of carbonic anhydrases perform functionally equivalent roles in nature.

Heavy metal Cd resistance: CadC. CadC is an extrachromosomally encoded metalloregulatory repressor protein from the ArsR superfamily that negatively regulates expression of the *cad* operon in a metal-dependent fashion. The metalloregulatory hypothesis holds that direct binding of thiophilic cations including Cd(II), Pb(II), Bi(III), and Zn(II), by CadC allosterically regulates the DNA binding activity of CadC to the *cad* operator/promoter (O/P). In collaboration both with D. P. Giedroc (Texas A&M University) and B. P. Rosen (Wayne State University), we have been successful in identifying the Cd(II) ligands in CadC [3]. Binding of Cd(II) to this tetrathiolate center results in a decrease of the intrinsic affinity of CadC for the *cad* O/P site. Continued efforts are underway to determine the precise mechanism for Cd(II)-induced regulation of the initiation of transcription in the *cad* system.

Iron-containing enzymes: Zinc-containing ferredoxins. An unexpected result from the crystallographic characterization of ferredoxin from *Sulfolobus* sp. was the presence of a tetrahedrally coordinated Zn site [22]. A functionally equivalent ferredoxin was purified from *Thermoplasma acidophilum* [23] and spectroscopic investigation revealed the presence of a similar zinc site. In an attempt to understand the nature of the zinc site in these unusual ferredoxins, we collaborated with T. Iwasaki (Nippon Medical School, Japan) to characterize the Fe-S cluster and zinc-binding site in ferredoxins from both *Sulfolobus* sp. and *Thermoplasma acidophilum*. XAS experiments indicate that the zinc coordination environment identified by crystallographic data, three histidine ligands and the carboxylate from aspartate, is identical in the two ferredoxins [24]. We have also characterized the selective oxidative degradation of one of the Fe-S clusters in *Sulfolobus* sp. Fd, revealing that there is no change in the zinc site, despite the conversion of the nearby [4Fe-4S] cluster to a [3Fe-4S] cluster [25].

Iron-containing enzymes: NOS. Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to citrulline and nitric oxide through two stepwise oxygenation reactions involving N^o-hydroxy-L-arginine, an enzyme-bound intermediate. The N^o-hydroxy-L-arginine- and arginine-bound NOS ferriheme centers show distinct, high-spin electron paramagnetic resonance (EPR) signals. In collaboration with T. Iwasaki, XAS was used to examine the structures of these ferriheme sites in full length neuronal NOS (Figure 1.1; [26]). Our XAS results show that the two forms are strikingly similar. Furthermore, even though Cu(II) inhibition affects the spin-state equilibrium as measured by EPR, there is no XAS-observable change to the ferriheme coordination environment. These results indicate that the manner in which substrate is held in the active site, rather than the heme site structure and geometry, specify the mechanism for the two-step hydroxylation reactions in neuronal NOS.

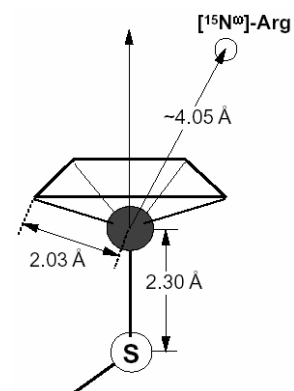


Figure 1.1. L-Arginine-bound form of neuronal nitric oxide synthase.

Iron-containing enzymes: TfdA. The first step in the degradation of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), by *Ralstonia eutropha* is catalyzed by the α -ketoglutarate (α -KG)-dependent dioxygenase, TfdA. Previously, EPR and ESEEM studies on inactive Cu(II)-substituted TfdA suggested a g -tensor rearrangement upon addition of 2,4-D [27]. In collaboration with R. P. Hausinger (Michigan State University), we have conducted XAS studies on various Cu(II) and Fe(II) forms of TfdA to determine the structural implications of this g -tensor rearrangement. Cu(II) has a d^9 valence electronic configuration, making it highly susceptible to Jahn-Teller distortion. This distortion results in longer axial bonds, making those ligands harder to detect by XAS and complicates the g -tensor description of the metal site. XAS does not have the paramagnetic requirements of the other two techniques, enabling us to study the active Fe(II) form of the enzyme. Fe(II) is d^6 which should display little Jahn-Teller distortion. XAS results indicate that the addition of 2,4-D to either Fe(II)- or Cu(II)-TfdA resulted in the loss of a histidine ligand [28]. Although the Cu(II) results could be explained by Jahn-Teller distortion, the changes at the Fe(II) site argue for loss of a histidine ligand, rather than simply a g -tensor rearrangement. Although the catalytic mechanism for TfdA remains unknown, our XAS results provide a structural backdrop against which future experiments will be interpreted.

Iron-containing enzymes: MetAP. Methionyl aminopeptidases (MetAPs) represent a unique class of proteases that are capable of removing the N-terminal methionine residue from nascent polypeptide chains. We have collaborated with R. C. Holz (Utah State University) to characterize the cobalt- and iron-binding sites in MetAP [29]. X-ray crystallographic studies of MetAPs from *E. coli*, *Homo sapiens*, and *Pyrococcus furiosus* have shown catalytic domains that contain a dinuclear cobalt core [30-33]. However, functional and kinetic experiments indicated the requirement for only one bound metal. Thus, XAS was used to establish the coordination sphere for both cobalt- and iron-bound forms of MetAP. Interestingly, the Fourier transform plots reveal no apparent metal-metal interaction, providing structural evidence for the hypothesis that MetAP is a

mononuclear enzyme. Given the XAS and biochemical evidence, the crystallographic results can be explained in terms of the excess metal that was present in the crystallization conditions.

Copper-containing enzymes: Cytochrome bo_3 . XAS has been used, in collaboration with R. B. Gennis (University of Illinois), to examine the structures of the Cu(II) and Cu(I) forms of the cytochrome bo_3 quinol oxidase from *E. coli* [34]. Cytochrome bo_3 is a member of the superfamily of heme-copper respiratory oxidases. Of particular interest is the fact that these enzymes function as redox-linked proton pumps, resulting in the net translocation of one H^+ per electron across the membrane. The molecular mechanism of how this pump operates and the manner by which it is linked to the oxygen chemistry at the active site of the enzyme are unknown. Several proposals have featured changes in the coordination of Cu_B during enzyme turnover that would result in sequential protonation or deprotonation events that are key to the functioning proton pump. Using XAS, we examined the structure of the Cu_B site in both the fully oxidized and fully reduced forms of the enzyme. The results show that in the oxidized enzyme, $Cu_B(II)$ is four-coordinate, consistent with three imidazoles and one hydroxyl (water). Upon reduction of the enzyme, the coordination of $Cu_B(I)$ is significantly altered, consistent with the loss of one of the histidine imidazole ligands in at least a substantial fraction of the population. These data add to the credibility that changes in the ligation of Cu_B might occur during catalytic turnover of the enzyme and therefore could be part of the mechanism of proton pumping.

Copper-containing enzymes: NosL. One of the accessory proteins, NosL, of the *nos* (nitrous oxide reductase) gene cluster has been structurally characterized, in collaboration with D. M. Dooley (Montana State University) [35]. The function of NosL is presently unknown, but the data indicate that NosL does not act as an electron transfer partner to nitrous oxide reductase. NosL is encoded on the same transcript as three other gene products (NosD, NosF, and NosY). These are required for assembly of the active site in nitrous oxide reductase, which is thought to be a copper cluster. Accordingly, it is

possible that NosL is a copper chaperone involved in metallocenter assembly. Our XAS results indicate that the copper ion in NosL is ligated by a cysteine, methionine, and histidine. Thus, NosL contains a novel type of biological copper site and further experimentation is necessary to establish the function of this protein in the nitrous oxide reductase system.

Nickel-containing enzymes: Urease. We have worked with R. P. Hausinger (Michigan State University) to structurally characterize enzymes responsible for the hydrolysis of urea into ammonia and carbamate. Urease is the primary catalyst in this reaction and is characterized by a dinuclear nickel site, first identified by XAS. In previous efforts in the Scott laboratory, XAS was used to describe the ligands to the dinuclear nickel site [36, 37]. This description was at odds with the crystal structure [38] and triggered the further refinement of the crystallographic information [39], resulting in the identification of additional water ligands that confirmed the XAS results. In a current research initiative, we expanded our investigation to include nickel and cobalt binding to wild type and (C319A) apo-urease [40]. In conjunction with crystallographic and kinetic experiments, we demonstrated that there are at least three distinct metal-bound species, only one of which is active. These results explain the observation that only 15% of the enzyme can be activated *in vitro* and underscores the importance of chaperone proteins that are involved in the proper formation of the dinuclear nickel site (UreD, -E, -F, -G).

Selenium in biology: Lysine 2,3-aminomutase. We have worked with S. J. Booker (Pennsylvania State University) and P. A. Frey (University of Wisconsin) to characterize lysine 2,3-aminomutase, which belongs to a class of enzymes that use FeS clusters and *S*-adenosyl-L-methionine (AdoMet) to initiate radical chemistry [16].

Using XAS, we have studied lysine 2,3-aminomutase at various stages of catalysis, in the

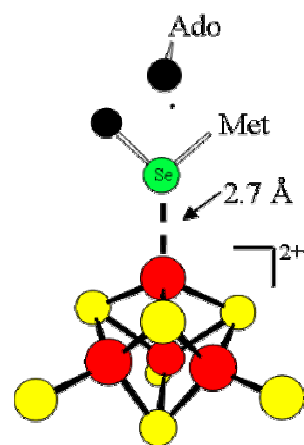


Figure 1.2. Proposed interaction between selenomethionine and the FeS cluster of lysine-2,3-aminomutase.

presence of selenomethionine or Se-adenosyl-L-selenomethionine (SeAdoMet), revealing that the cofactor is cleaved only in the presence of dithionite and the substrate analog *trans*-4,5-dehydrolysine. Strikingly, a new Fourier transform peak at 2.7 Å, interpreted as an Se–Fe interaction (Figure 1.2), appears concomitant with this cleavage. This is the first demonstration of a direct interaction of AdoMet, or its cleavage products, with the FeS cluster in this class of enzymes.

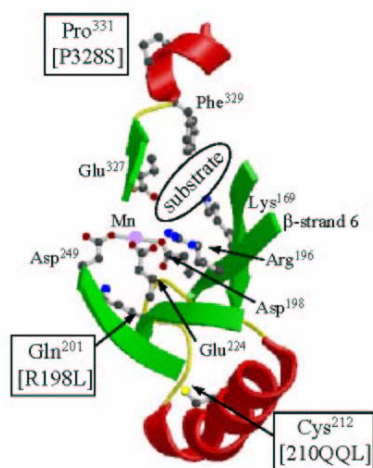


Figure 1.3. Expanded view of the active site of muconate cycloisomerase from *P. putida* (PDB code 1muc). Amino acids are numbered according to the *P. putida* convention. Altered residues in variant ADP1 muconate cycloisomerases are boxed.

Manganese-containing enzymes: Muconate Cycloisomerase.

Mutants of the bacterium *Acinetobacter* sp. ADP1 were selected to grow on benzoate without the BenM transcriptional activator. In the wild type, BenM responds to benzoate and *cis,cis*-muconate to activate expression of the *benABCDE* operon involved in benzoate catabolism. This operon encodes enzymes that convert benzoate to catechol, a compound subsequently degraded by *cat*-gene

encoded enzymes. Four spontaneous mutants were found to carry *catB* mutations that enabled BenM-independent growth on benzoate (Three of these mutations are highlighted in Figure 1.3). *CatB* encodes muconate cycloisomerase, an enzyme required for benzoate catabolism. Its substrate, *cis,cis*-muconate, is enzymatically produced from catechol by the *catA*-encoded catechol 1,2-dioxygenase. Muconate cycloisomerase was purified to homogeneity from the wild type and the *catB* mutants. Each purified enzyme was active, although there were differences in the catalytic properties of wild-type and variant muconate cycloisomerases. Strains with a chromosomal *benA::lacZ* transcriptional fusion

were constructed and used to investigate how *catB* mutations affected growth on benzoate. All the *catB* mutations increased *cis,cis*-muconate-activated *ben*-gene expression. A model was constructed in which the *catB* mutations reduce muconate cycloisomerase activity during growth on benzoate, thereby increasing intracellular *cis,cis*-muconate concentrations. This in turn may allow CatM, an activator similar to BenM in sequence and function, to activate *ben*-gene transcription. CatM normally responds to *cis,cis*-muconate to activate *cat*-gene expression. Consistent with this model, muconate cycloisomerase specific activities in cell-free extracts of benzoate-grown *catB* mutants were low relative to the wild type. Moreover, the catechol 1,2-dioxygenase activities of the mutants were elevated, which may result from CatM responding to the altered intracellular levels of *cis,cis*-muconate and increasing *cata* expression. Collectively, these results support the important role of metabolite concentrations in controlling benzoate degradation via a complex transcriptional regulatory circuit.

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CHAPTER 2

ALTERATION OF SUBSTRATE SPECIFICITY IN CATECHOL 1,2-DIOXYGENASE
FROM *ACINETOBACTER* SP. ADP1

Introduction

General background. Aromatic compounds have proven to be persistent environmental contaminants. These compounds are present in wood as lignin and in man-made forms such as pesticides, detergents, solvents, paints and oils. In particular, halogenated aromatic compounds are quite intractable biodegradation targets [2, 3]. Chlorinated aromatics, which are more stable than the non-chlorinated counterparts, were widely used as coolants for transformers, flame retardants, and hydraulic fluids. It is estimated that polychlorinated biphenyls, released in bulk until the 1970's, account for approximately 375,000 tons of environmental contaminants [4]. The widespread use of dichlorodiphenyl-trichloroethane (DDT) as an herbicide and polychlorinated benzodioxins as transformer fluids and plasticizers have also led to significant bioaccumulation of chlorinated aromatics, particularly in the fatty tissues of animals [5].

In part, these compounds are more prevalent as contaminants because the delocalization of π electrons imparts a marked increase in stability. The intrinsic stability of haloaromatics, coupled with the limited time they have been present in the biosphere, and the numerous variations of each compound, make them recalcitrant to efforts towards remediation. Even so, the reaction of aromatic compounds with oxygen is favored thermodynamically. However, since oxygen exists in a triplet ground state, characterized by two unpaired electrons, and aromatic compounds typically exist in a singlet ground state, with no unpaired electrons, uncatalyzed reactions are spin-forbidden. As oxygen is a highly reactive species and would be extremely toxic at the levels present in our atmosphere, this spin restraint protects cellular organisms by limiting the potential reactions available to oxygen. In particular, oxygen is capable of reacting with metals because of the unpaired electrons that may be associated with those metals. For example, oxygen reacts readily with iron to form rust.

Despite the stability of aromatic compounds, microbiological pathways for their degradation have evolved. In the soil bacterium *Acinetobacter* sp. ADP1, the β -keto adipate pathway has evolved to process degradation products of various aromatic

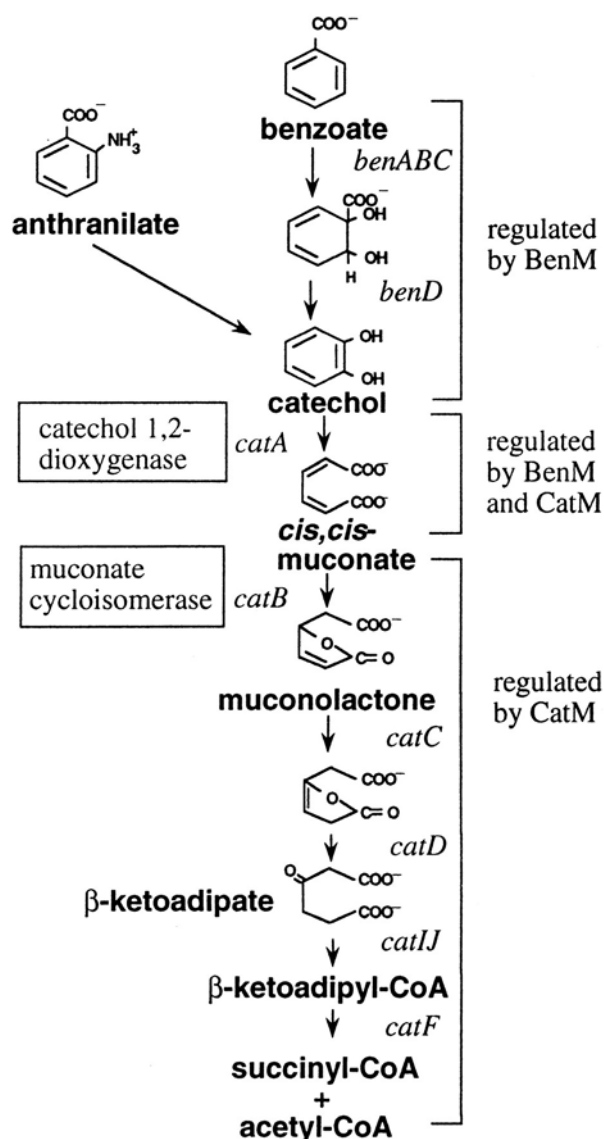


Figure 2.1. Catechol branch of the β -ketoadipate pathway of *Acinetobacter* sp. ADP1.

compounds (Figure 2.1), for example benzoate, to substrates in the Krebs cycle [6, 7]. Not surprisingly, a key step in these pathways is the interaction of aromatic compounds with oxygen, mediated by iron-containing enzymes, to effect oxidative ring-cleavage of substituted dihydroxybenzenes [8, 9]. Catechol 1,2-dioxygenase (CTD), one example of an iron-containing, oxygen-activating enzyme, catalyzes the conversion of catechol to *cis,cis*-muconate [10, 11], while protocatechuate 3,4-dioxygenase catalyzes the conversion of protocatechuate to β -carboxy-*cis,cis*-muconate [12, 13].

Mechanistic background.

A broad range of chemical reactions are catalyzed by oxygen-activating, nonheme iron-containing enzymes [14-17]. Within this group, a class of enzymes catalyzes aromatic ring cleavage of dihydroxylated benzene rings. Aromatic intradiol ring-cleaving dioxygenases exist in many evolutionary forms, *viz.*, there exist enzymes that cleave catechol, protocatechuate, gentisate, and many other substituted forms of these substrates (*e.g.* chlorinated catechols). These enzymes contain a nonheme iron, which is coordinated by two tyrosine and two histidine

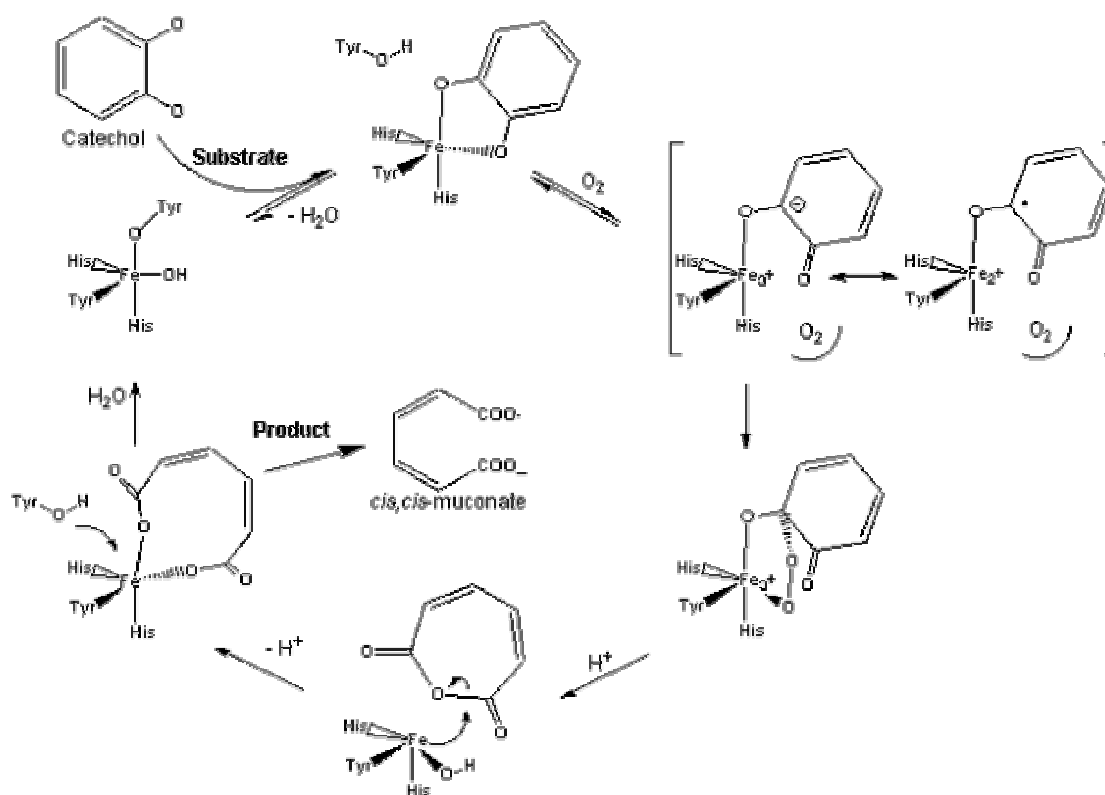


Figure 2.2. Proposed mechanism for catechol 1,2-dioxygenase. From reference [1].

ligands. The tyrosine ligands give rise to a ligand-to-metal charge transfer (LMCT) band at approximately 450 nm that causes the protein to have a reddish color. Several crystal structures have been determined for proteins in this class, *e.g.*, protocatechuate 3,4-dioxygenase [18, 19] and catechol 1,2-dioxygenases [1, 20]. A detailed reaction mechanism has been proffered on the basis of these structures and EPR [21-24], resonance Raman [25], MCD [26], and XAS [27] spectroscopic investigations (Figure 2.2). In numerous reports, Que and coworkers have verified this reaction mechanism using inorganic model complexes that are capable of catalyzing the same ring-cleaving reactions [28, 29]. Detailed spectroscopic and computational studies by Solomon and coworkers have described the importance of the strength of the tyrosine-iron bonds in the catalytic mechanism [30].

The reaction mechanism (Figure 2.2) has four major steps: 1) substrate interacts with the protein and binds via the two hydroxyl groups to the iron active site. This interaction causes the axial tyrosine ligand to leave the iron coordination sphere and results in an activated substrate complex. 2) The activated substrate attacks oxygen in a nucleophilic manner. 3) Through interaction with the activated substrate and the open axial position on the iron, oxygen cleaves catechol to yield *cis, cis*-muconate. 4) Substrate disassociates from the protein.

Rationale for mutations. There exist numerous methods for creating variants of an enzyme with altered properties. These methods can be divided into “random” and “rational” techniques. The random techniques involve methods for altering genes, such as error-prone PCR or bacterial strains devoid of the ability to repair replication errors. Rational techniques require the user to assimilate information regarding the structural and chemical properties of the enzyme and to design site-directed mutations that are expected to have some predetermined effect. Typically, rational techniques are only marginally, if at all, more successful than random efforts. This study involves the rational design of substrate specificity. An advantage of the chosen system is that the crystal structure of catechol 1,2-dioxygenase is available [1], as are sequence alignments of multiple catechol and chlorocatechol dioxygenases. The crystal structure provides information about the interaction of specific amino acid residues with the substrate. The sequence alignments suggest amino acids that are highly conserved in catechol dioxygenases and are also highly conserved, but different, in chlorocatechol dioxygenases. These sources of information were used to suggest several amino acid residues that could provide the basis for altering the active site of CTD to cleave chlorinated substrates. Some rationally designed mutations were successful and some were not. Specifically, [I105T] CTD, chosen based on the crystal structure because it appears to have hydrophobic interactions with substrate, was altered to increase the affinity of the active site for halogenated catechol. In contrast, mutations such as [F253C, A254C] CTD that were chosen on the basis of sequence alignment were less successful in altering substrate specificity.

Once [I105T] CTD was created, and found to have increased activity towards chlorocatechol, the nature of this change was probed through additional mutations at the same position. Serine and valine were chosen based the properties of the side chain (Figure 2.3), in order to determine whether the chemical or physical properties were most important in changing the substrate specificity. The results of this series of mutations are reported here.

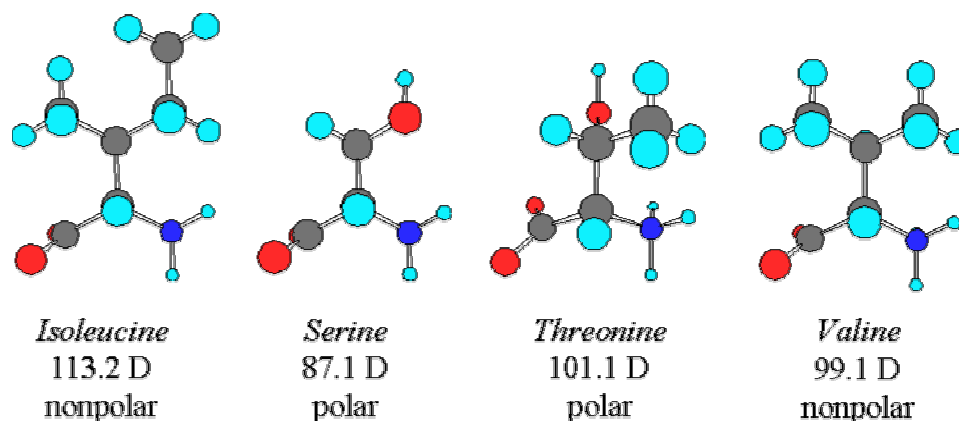


Figure 2.3. Ball and Stick drawing of key amino acids (Figure drawn in Chem3D, Cambridge Software).

Methods

Bacterial strains, growth conditions, and DNA manipulations. *Escherichia coli* BL21 (DE3) cells (Novagen) were used for expression and cloning. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene), according to the instructions. Primers were obtained from Integrated DNA Technology (Coralville, Iowa). Standard methods were used for plasmid DNA purifications, restriction enzyme digestions, electrophoresis, ligations, and *E. coli* transformation.

Induction of overexpression of CTD variants. 50 mL overnight cultures of BL21(DE3) Gold cells (Stratagene), containing the pET-21b overexpression vectors (Novagen) with gene inserts for each of the variants of CTD, were grown in sterile LB with 100 mg/L ampicillin. 2.8 L flasks containing 1 L LB and 100 mg/L ampicillin,

equilibrated at 37 °C, were inoculated with 10 mL of overnight culture and allowed to grow to an optical density (600 nm) of 0.85 – 1.10, at which point expression was induced by the addition of isopropyl 1-thio- β -D-galactopyranosidase (IPTG) powder to a final concentration of 100 mg/L. The culture was then incubated at 37 °C for 3-4 hours and harvested by centrifugation (Beckman JA-12 rotor, 5 min spin at 8,000 rpm). The pellet was stored at -80 °C until needed.

Purification of CTD variants. For each variant, the same procedure was followed: The cell pellet harvested from 4L of overexpressed cultures was resuspended on ice in approximately 30 mL of 50 mM Tris, pH = 7.5, buffer (Buffer A). The resuspension was sonicated for 2 minutes using a program consisting of 0.5 seconds of sonication followed by 0.5 sec pauses. The resulting solution was clarified by centrifugation (Beckman JA-21 rotor, 10 min spin at 10,000 rpm). The resulting supernatant was removed for further use and the pellet was resuspended in approximately the same volume of the same buffer and resonicated. Then, both the original supernatant and the resonicated solution were centrifuged (Beckman JA-21 rotor, 30 min spin at 15,000 rpm). The resulting supernatants were pooled and further clarified using a 0.22 μ m syringe filter. This filtrate represents the “crude extract.”

The crude extract was divided into two 25 mL aliquots which were diluted to 50 mL with Buffer A. Each solution was applied separately to either a 8mL Resource Q (BioRad) or 40 mL Q-Sepharose FF (Pharmacia) column and eluted with a gradient of 0-40% Buffer A + 1 M NaCl. The colored (red) fractions were pooled from both runs. This combined solution was concentrated, if necessary, using a YM-10 (10,000 MW cutoff filter) in an Amicon filtration system, to approximately 37.5 mL. To this solution, 12.5 mL of saturated ammonium sulfate was added to yield a final concentration of 25% (saturated) ammonium sulfate. This solution was clarified using a 0.22 μ m syringe filter and applied to a 40 mL Phenyl Sepharose FF (Pharmacia) column that had previously been equilibrated in Buffer A + 25% ammonium sulfate. The protein was eluted in a gradient of 0 – 100% Buffer A, and the colored fractions were pooled. These fractions

were concentrated in the Amicon system to a volume of approximately 2 mL. The concentrate was then applied to a 320 mL S75 Sephadex gel filtration column (Pharmacia) that had previously been equilibrated in Buffer A. After continuous flow of Buffer A, the protein was eluted in a symmetric peak, which corresponded to the colored fractions. The purity of the protein solution was verified by SDS-PAGE analysis.

Protein characterization (mass and metal content). Aliquots of each variant were submitted for liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis to the Chemical and Biological Sciences Mass Spectrometry Facility at the University of Georgia. For wild type CTD, the expected mass was 34,347 and the observed mass was 34,352. For [I105T] CTD, expected was 34,335 and observed was 34,339. For [I105S] CTD, expected was 34,321 and observed was 34,325. For [I105V] CTD, expected was 34,333 and observed was 34,336.

Determinations of protein concentrations were based on the calculated extinction coefficient for wt CTD at 280 nm ($28,110 \text{ M}^{-1} \text{ cm}^{-1}$). Absorbance measurements were performed on a Shimadzu UV2101PC scanning spectrophotometer. The accuracy of the calculated extinction coefficient was confirmed once by a Bradford protein determination assay. Subsequently, all concentration measurements were based on the absorbance of a sample at 280 nm. Iron concentrations were determined after protein digestion under reducing conditions, using bathophenanthroline, as described by Fish [31].

In addition, aliquots of each variant, along with commercial Fe standards, were submitted in triplicate for inductively coupled plasma – mass spectrometry analysis to the Chemical Analysis Laboratory of Research Services at the University of Georgia. Metal concentrations (and metal to monomer ratios) were determined based on Fe concentrations established from the standard curve obtained from the commercial standards and on the absorbance of the sample at 280 nm. All subsequent spectral and kinetic data are normalized to the amount of iron in the protein.

Enzymatic Assays of variants. Activity of CTD variants towards catechol and chlorocatechol was measured according to previously reported procedures [32]. Briefly,

the increase in absorbance at 260 nm was measured spectrophotometrically as catechol was converted to *cis,cis*-muconate (Figure 2.4). The reaction was carried out at room temperature in 1mL quartz cuvetts using various amounts of enzyme and substrate.

Spectroscopic characterization. UV-visible absorption spectra were collected on a Shimadzu UV3101PC spectrophotometer. Variable-temperature MCD spectra were collected on samples containing 55% glycerol using a Jasco J-715 (180 – 1000 nm) spectropolarimeter mated to an Oxford Instruments Spectromag 4000 (0-7 T) split-coil superconducting magnet. Experimental conditions for VTMCD data collection were as described elsewhere [33, 34].

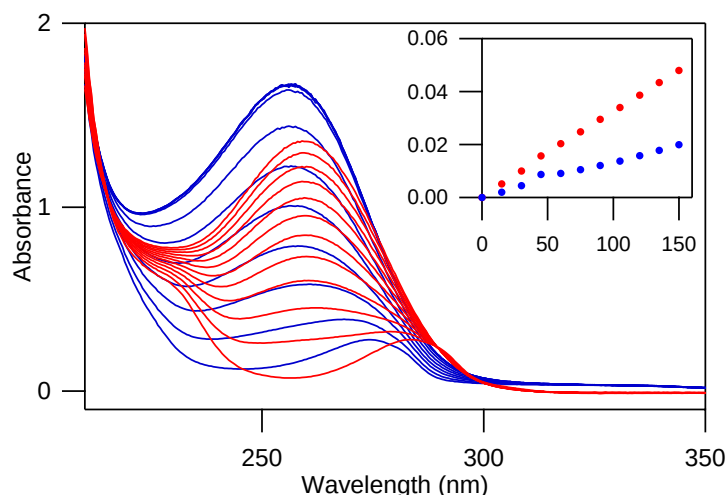


Figure 2.4. Conversion of catechol to *cis,cis*-muconate (Blue, 1 min intervals), and 4-Cl catechol to 3-Cl-*cis,cis*-muconate (Red, 3 minute intervals). Inset, increase in A₂₆₀ for wild type (blue) and variant (red) enzyme assayed with 4-Cl catechol.

Results

Enzymatic activity of variants. The enzymatic activity of wild type and variants of CTD towards catechol and substituted catechols was measured spectrophotometrically

Table 2.1. Enzymatic activity of wild type and variants of CTD.

	wt	[I105T]	[I105S]	[I105V]	[F254C, A254C]
catechol	218 ± 8	130 ± 20	110 ± 20	64 ± 3	71 ± 3
4-Me catechol	14 ± 3	50 ± 20	12 ± 5	10 ± 4	8.5 ± .5
4-Cl catechol	3.3 ± 0.3	29 ± 4	2 ± 1	1.3 ± .6	6.7 ± .3
3-Cl catechol	0.09 ± 0.02	0.18 ± .09	-	-	0.09 ± .04

Activities reported in mM/min per μmol enzyme, as an average of triplicate samples, accompanied by the deviation.

(Table 2.1). The activity of wild type enzyme with the native substrate, catechol, was the highest for any of the variants or substrates and each of the variants was most active towards catechol. However, [I105T] CTD was the most active towards both 3-methylcatechol and 4-chlorocatechol. [I105T] was the most active of the variant enzymes, both for catechol and substituted catechols. Interestingly, none of the proteins exhibited significant activity towards 3-chlorocatechol.

UV-visible spectra. UV-Visible spectra of wild type and variants of catechol 1,2-dioxygenase (CTD) are dominated by tyrosine-to-iron(III), ligand-to-metal charge transfer (LMCT) bands at about 450 nm (Figure 2.5). This band has been shown by resonance Raman excitation profiles to be composed of two distinct LMCT bands, one arising from each of two tyrosine ligands [25]. For wt CTD, the λ_{max} for this transition occurs at 452 nm. Likewise, for [I105S] and [I105V] CTD, λ_{max} occurs at 452 and 446 nm, respectively. However, the LMCT transition for [I105T] is shifted to 481nm.

UV-visible spectra of MCD samples. MCD samples were prepared for wt and [I105T] CTD by addition of glycerol to a final concentration of 50-70%

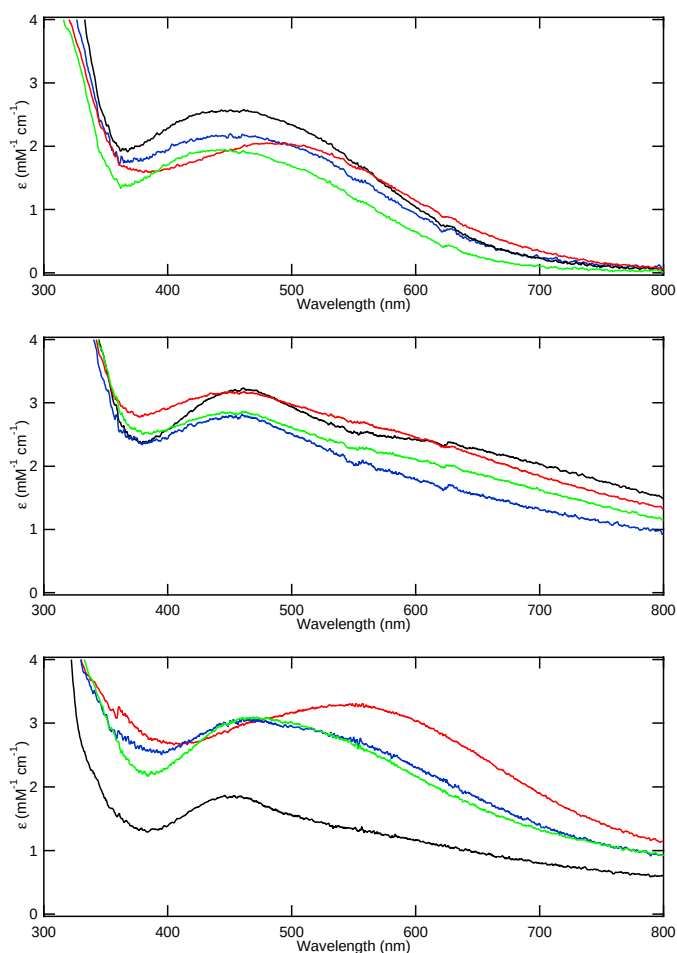


Figure 2.5. UV-Visible spectra of wild type (black), [I105T] (red), [I105S] (blue), and [I105V] (green) CTD as isolated (top) or upon addition of catechol (middle) or 4-chlorocatechol (bottom).

v/v. The addition of glycerol resulted in a shift in the absorption spectrum to higher energy (data not shown). Since anaerobic addition of substrate to wt CTD results in the hydroxyl ligands of the catechol binding to the iron and subsequent displacement of the axial tyrosine ligand, it is plausible that the addition of glycerol has a similar effect. The putative glycerol ligand would not be expected to contribute to the UV-Visible spectrum, allowing the assignment of the remaining absorption band to a LMCT band arising from one tyrosine ligand. The hydroxyl moieties on glycerol allow this compound to be considered a “substrate analogue.”

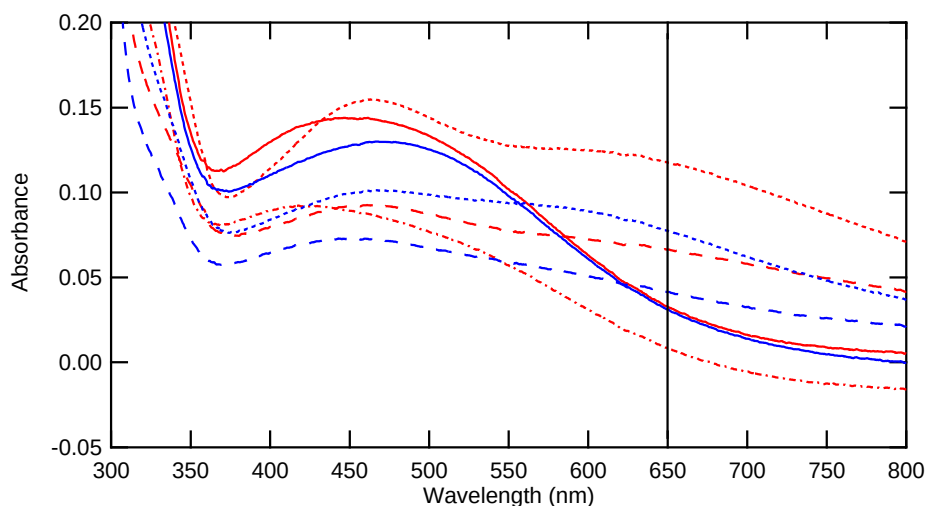


Figure 2.6. UV-Visible spectra of wild type (red) and [I105T] CTD (blue) as isolated (solid) or incubated with catechol (dotted) or catechol and glycerol (dashed).

Catechol-bound CTD samples were prepared by incubating the protein with catechol in an anaerobic environment, to prevent turnover. The UV-Visible spectrum of the complex of wt CTD and catechol revealed the appearance of a new LMCT band at approximately 620 nm (Figure 2.5,2.6). This band arises from a catechol-to-iron(III) CT transition. Upon addition of glycerol to the catechol-bound samples, no effect was observed on the UV-Visible spectrum, indicating that the coordination state of the iron had not been perturbed. Similarly, for [I105T] CTD, a new LMCT band appeared upon anaerobic addition of catechol. However, the position of this transition was shifted to higher energy relative to the wt CTD sample.

4-Chlorocatechol-bound CTD samples were similarly prepared by incubating the protein in an anaerobic environment. The UV-Visible spectrum of the 4-chlorocatechol-bound wt CTD complex reveals a catecholate-to-iron(III) CT transition with a λ_{max} of approximately 550 nm. The similar transition in [I105T] CTD is shifted to higher wavelength and is more intense (Figure 2.5, bottom panel).

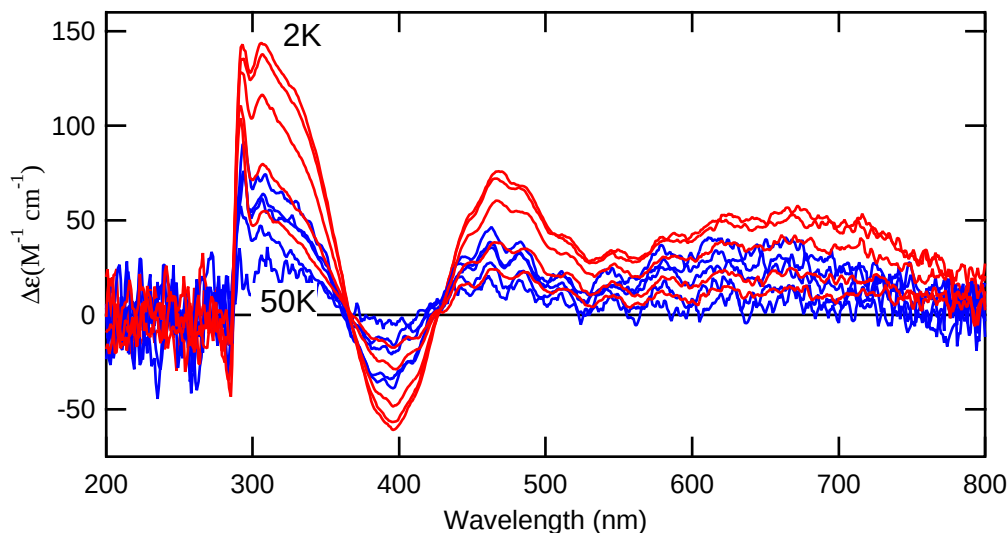


Figure 2.7. Temperature dependence of MCD spectra for wild type (red) and [I105T] (blue) catechol 1,2-dioxygenase in the presence of catechol.

MCD spectra. Variable-temperature MCD data for wt and [I105T] CTD reveal temperature-dependent absorption bands indicative of a paramagnetic chromophore (Figure 2.7). The MCD spectra are dominated by LMCT transitions at *ca.* 320 and 500 nm. The higher energy band likely arises from a histidine-to-iron(III) transition, while the lower energy (500 nm) band is a tyrosine-to-iron(III) transition. Upon anaerobic addition of catechol to these samples, the MCD spectra exhibit a new transition at *ca.* 660 nm for wt and *ca.* 600 nm for [I105T] CTD (Figure 2.8, bottom). This transition is assigned as a catechol-to-iron(III) CT transition. The 2K spectra for catechol bound forms of wt and [I105T] CTD (Figure 2.8) confirm the shift in energy of the catechol-to-iron(III) CT transition seen in the UV-Visible spectra (Figure 2.6).

Discussion

Molecular orbital considerations. *Ab initio* calculations for several substituted catechols indicate that the nature of the substitution (at the 4 position) dictates the energy of the highest occupied molecular orbital (HOMO), as would be predicted based on organic principles [35]. In the same study, the authors compared the $\ln(k_{\text{cat}})$ vs HOMO

and found a linear correlation. This indicates that a determining factor in the rate of catalysis for substituted catechols is the ability for iron to activate the substrate, which is dependent on the HOMO of that substrate.

The interaction of substrate with iron causes electron density to be shifted from the deprotonated, negatively charged substrate to the ferric iron site. Thus, the formal description of the active site is most likely a resonance state between ferric iron with two negatively charged hydroxyl ligands and ferrous iron with a radical, semiquinone substrate species. Quantum mechanical

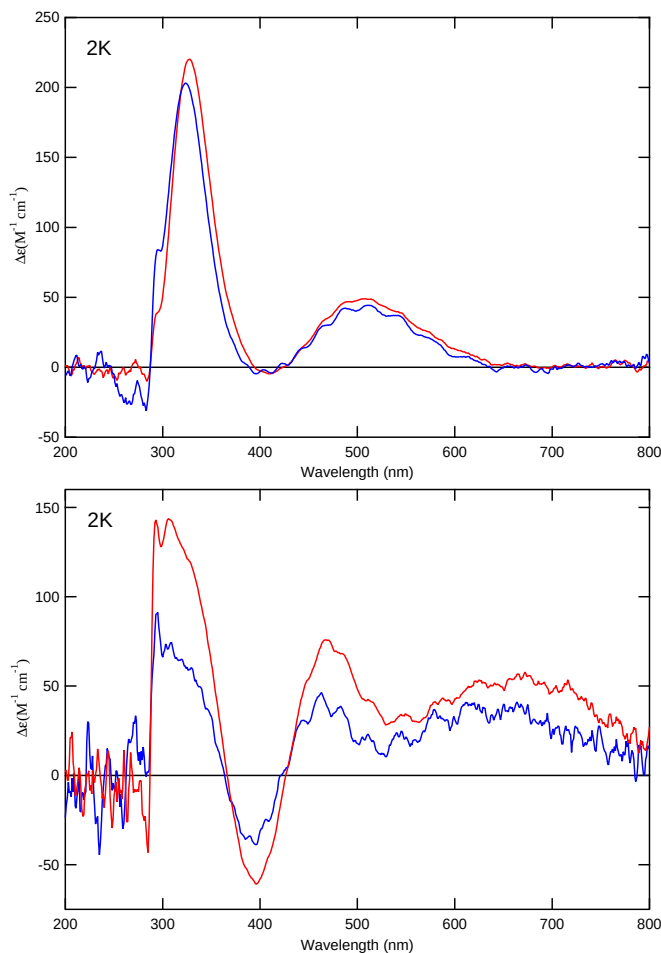


Figure 2.8. 2K MCD spectra of wild type (red) and [I105T] (blue) catechol 1,2-dioxygenase as isolated (top) and in the presence of catechol (bottom).

molecular orbital calculations, based on the crystallographic coordinates of the iron active site in protocatechuate 3,4-dioxygenase, indicate that $-0.8 e^-$ resides in the catecholate oxygen atoms [35].

In preparing various inorganic complexes as functional models for catechol dioxygenases, Que and co workers observed that there is a direct correlation between the position of the catechol-to-iron(III) charge transfer (CT) band and the redox potential of the Fe(II/III) couple [29]. This charge transfer band consists of a transition from the primarily ligand-based HOMO to the lowest unoccupied molecular orbital (LUMO), which has primarily iron character. Thus, the position of the CT band is a measure of the energy difference between the ligand-based HOMO and the iron-based LUMO. Changes in the position of this band can arise either by a change in energy of the LUMO or the HOMO, or a combination of the two. In the study by Que and coworkers, the catechol-like moiety is 3,5-di-*tert*-butylcatecholate (DBC), and is not altered between the various complexes. However, the other ligand to the iron *is* varied, resulting in a shift of the DBC-to-iron(III) CT band. Thus, it is reasonable to speculate that the changes in position of the CT band result from changes in the energy of the LUMO rather than changes in the energy of the HOMO, thereby explaining the correlation between the position of the CT band and the redox potential of the iron. In contrast, if the position of the charge transfer band was altered as a result of a substitution of the catechol ring, the most likely explanation is that the ligand-based HOMO has been altered.

Que and coworkers have further determined that there is a correlation between the reactivity of an inorganic complex towards DBC and the NMR shift (δ) of the 6-H and 4-H resonances for DBC [28]. These shifts have been explained as an increased semiquinone character of the Fe-DBC interaction, due to enhanced covalent interactions between the metal and catecholate moieties. In confirming these findings with another series of Fe-DBC complexes, Mialane and coworkers have further suggested that the paramagnetic shifts of the 4-H protons are due to an increased amount of Fe(II)(semiquinone) mixing in the ground state [36]. This mixing is attributed to the Fe(II)(semiquinone) excited state being closer in energy to the Fe(III)(catechol)ground state. Indeed, since an accurate measure of the difference in energy of the ground and

excited states is afforded by UV-Visible spectroscopy, there is a correlation between λ_{max} and $\delta(4\text{-H})$, and thus also a correlation between λ_{max} and reactivity.

To state the correlations above more quantitatively, the ground state wavefunction can be summarized as $\Phi_{\text{Fe(III)(catechol)}} + \alpha\Phi_{\text{Fe(II)(semiquinone)}}$ [36]. Furthermore, α can be described by perturbation theory as $|H_{\text{AB}}|/h\nu_{\text{max}}$, where H_{AB} is the transfer integral between ground and excited states and $h\nu_{\text{max}}$ is the energy difference between those levels [36]. Since λ_{max} is a measure of $h\nu_{\text{max}}$, it is correlated to the amount of Fe(II)(semiquinone) character in the ground state. Similarly, since ϵ_{max} is correlated to α , there is also a relationship between ϵ_{max} and the amount of ground and excited state mixing. Thus, we are afforded two tools, viz. ϵ_{max} and λ_{max} , which can be used to measure the amount of Fe(II)(semiquinone) character in the ground state. Since it is likely that the Fe(II)(semiquinone) moiety is the activated substrate complex, required for catalysis, these two tools are useful for understanding the reactivity of catechol 1,2-dioxygenase towards substituted catechols.

Spectroscopic characterization of variants. UV-Visible spectra of wt and [I105T] CTD reveal a shift towards lower energy of the tyrosine-to-iron(III) CT band in the latter species (Figure 2.5, top). This shift is indicative of a smaller energy separation of the tyrosine-based HOMO and the iron-based LUMO. The smaller energy difference can be explained either by a destabilization of the tyrosine-based HOMO or a stabilization of the iron-based LUMO. Our results do not favor one or the other of these possibilities. Perhaps the threonine, in conferring additional hydrophilicity to the active site, increases the amount of water in the active site thereby affecting hydrogen bonds which either stabilize the iron-based LUMO or destabilize the ligand-based HOMO.

Upon addition of glycerol to wt and [I105T] CTD, there is a shift to higher energy in the tyrosine-to-iron(III) CT band (data not shown). Since glycerol might reasonably be expected to coordinate the iron in a fashion similar to catechol, it is possible that the addition of glycerol results in the release of one tyrosine ligand. MCD of the glycerol-bound forms of CTD reveal that the transitions are in approximately the same position for

wt and [I105T] CTD (Figure 2.8, top), indicating that when one of the tyrosine ligands is dissociated, the remaining tyrosine-to-iron(III) CT transition is substantially unaffected. Taken together with the UV-Visible spectra of uncomplexed CTDs, these results suggest that any effect of the [I105T] mutation is “felt” only by the tyrosine that becomes dissociated during catalysis. Since this ligand is replaced by substrate during catalysis, the fact that it is affected by the [I105T] mutation offers an explanation for the altered substrate specificity of the variant.

UV-Visible spectra of catechol-bound forms of wt and [I105T] CTD reveal the appearance of a new catechol-to-iron(III) CT transition at approximately 575-650 nm (Figure 2.5, middle). MCD spectra confirm that this transition is shifted to higher energy for the [I105T] variant (Figure 2.8, bottom). Since the glycerol-bound form of the CTDs indicates that the tyrosine-to-iron(III) transition is in approximately the same position, we can assume that the iron-based LUMO is unaffected by the mutation (note also: these transitions are in approximately the same position in the MCD spectra). Therefore, the basis for the shift in energy of the catechol-to-iron(III) CT band must arise as a result of a stabilization of the catechol-based HOMO. Based on previous comparisons of $\ln(k_{\text{cat}})$ with the ligand-based HOMO (*vide supra*, [35]), it would be predicted that the stabilization of the HOMO would result in decreased catalytic ability of the affected CTD. Indeed, this decrease in activity towards catechol is observed for [I105T] CTD, compared with wt enzyme (k_{cat} of 130 and 218 mM/min, respectively; Table 2.1).

UV-Visible spectra of 4-chlorocatechol-bound forms of wt and [I105T] CTD are also marked by the appearance of a catecholate-to-iron(III) CT band. For wt CTD, this transition is at higher energy than the corresponding transition in the catechol-bound form of the enzyme (*cf.* Figure 2.5, middle and bottom). However, for [I105T] CTD, the 4-chlorocatechol-to-iron(III) CT transition is at lower energy than the corresponding catechol-to-iron(III) CT band. In keeping with our supposition that the iron-based orbitals are largely unaffected, the differences in energy of these transitions must arise from changes in energy of the 4-chlorocatechol-based HOMO. Based on the correlations

between HOMO and $\ln(k_{\text{cat}})$, we would predict that wt CTD would have decreased activity towards 4-chlorocatechol compared with catechol. This decrease in activity would be explained by a stabilization of the ligand-based HOMO which would decrease the propensity of the ligand to undergo a cleavage reaction. Similarly, it would be predicted that the [I105T] CTD variant would have increased activity towards 4-chlorocatechol because the ligand-based HOMO is destabilized by the mutation, making it more susceptible to ring cleavage. Indeed, these predictions are borne out by kinetic analysis of the enzymes (Table 2.1).

Therefore, it appears that a determining factor in the rate of catalysis is the ability of this enzyme to destabilize the substrate-bound form of the enzyme to facilitate conversion of the complex to the product-bound form. By rationally designing the active site of CTD to destabilize the 4-chlorocatechol-bound form of [I105T] CTD, we have increased the activity of the enzyme towards that substrate. Similarly, as the catechol-bound form of [I105T] CTD became more stabilized, the activity of the enzyme was decreased towards that substrate. Although there are many determining factors of substrate specificity in enzymatic catalysis, this appears to be one factor which can be rationally modulated to alter the catalytic properties of a given enzyme.

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CHAPTER 3

STRUCTURAL EVIDENCE THAT THE METHIONYL AMINOPEPTIDASE FROM
ESCHERICHIA COLI IS A MONONUCLEAR METALLOPROTEASE¹

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Abstract

The Co and Fe K-edge Extended X-ray Absorption Fine Structure (EXAFS) spectra, of the methionyl aminopeptidase from *Escherichia coli* (*EcMetAP*) have been recorded in the presence of one and two equivalents of either Co(II) or Fe(II) (*i.e.* [Co(II)](*EcMetAP*), [Co(II)Co(II)](*EcMetAP*), [Fe(II)](*EcMetAP*), and [Fe(II)Fe(II)](*EcMetAP*)). The Fourier transformed data of both [Co(II)](*EcMetAP*) and [Co(II)Co(II)](*EcMetAP*) are dominated by a peak at *ca.* 2.05 Å, which can be fit assuming 5 light atom (N,O) scatterers at 2.04 Å. Attempts to include a Co-Co interaction (in the 2.4 – 4.0 Angstrom range) in the curve-fitting parameters were unsuccessful. Inclusion of multiple-scattering contributions from the outer-shell atoms of a histidine-imidazole ring resulted in reasonable Debye-Waller factors for these contributions and a slight reduction in the goodness-of-fit value (χ^2). These data suggest that a dinuclear Co(II) center does not exist in *EcMetAP* and that the first Co atom is located in the histidine-ligated side of the active site. The EXAFS data obtained for [Fe(II)](*EcMetAP*), and [Fe(II)Fe(II)](*EcMetAP*) indicate that Fe(II) binds to *EcMetAP* in a similar site to Co(II). Since no X-ray crystallographic data are available for any Fe(II)-substituted *EcMetAP* enzyme, these data provide the first glimpse at the Fe(II) active site of MetAP enzymes. In addition, the EXAFS data for [Co(II)Co(II)](*EcMetAP*) incubated with the anti-angiogenesis drug fumagillin, is also presented.

Introduction

Methionyl aminopeptidases (MetAPs) represent a unique class of proteases that are capable of removing the N-terminal methionine residues from nascent polypeptide chains (1-4). In the cytosol of eukaryotes, proteins are initiated with an N-terminal methionine residue; however, proteins synthesized in prokaryotes, mitochondria, and chloroplasts are initiated with an N-terminal formyl-methionyl residue. The formyl group is initially removed by a peptide deformylase before MetAP's remove the N-terminal methionine (2). Removal of N-terminal methionine residues from nearly all newly synthesized peptides, depending on the nature of the penultimate amino acid, is essential for co-translational and post-translational modifications that are critical for fully functional enzymes (5), correct cellular localization, and the timely degradation of proteins (1-4). Deletion of the gene encoding MetAP is lethal to *Escherichia coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae*; therefore, MetAP's are essential for cell growth and proliferation (6-8). Recently, the type-2 MetAP from eukaryotes has been identified as the molecular target for the anti-angiogenesis drugs ovalicin and fumagillin (9-13). Thus, the inhibition of aminopeptidase activity in malignant tumors is critically important in preventing the growth and proliferation of these types of cells, and for this reason, have become the subject of intense efforts in inhibitor design.

The MetAP's from *E. coli*, *Homo sapiens*, and *Pyrococcus furiosus* have been crystallographically characterized (13-16). These MetAP's and all other MetAP's studied to date have been shown to have identical catalytic domains that contain a bis(μ -carboxylato)(μ -aquo/hydroxo)dicobalt core with an additional carboxylate residue at each metal site and a single histidine residue bound to one of the two metal ions (13-16). Recently, it was suggested that the *in vivo* metal ion for the MetAP from *E. coli* (*EcMetAP*) is Fe(II) based on a combination of whole cell metal analyses and activity measurements as well as *in vitro* activity measurements and substrate binding constants (17, 18). In addition, the observed catalytic activity as a function of divalent metal ion and the metal binding constants for both

Fe(II) and Co(II) *EcMetAP* led to the proposal that *EcMetAP* functions as a mononuclear enzyme *in vivo* (17). The high-affinity or catalytically relevant metal binding site was assigned as the histidine-containing site; however, no structural data exist to verify these kinetic and spectroscopic data. Extended X-ray absorption fine structure (EXAFS) spectroscopy is particularly well suited to clarify structural problems of this type (19, 20). EXAFS data are sensitive to heavy atom scatterers in the second coordination sphere providing direct evidence for dinuclear sites, if they exist. Reported herein are Co and Fe K-edge EXAFS data for the catalytically competent Co(II)- and Fe(II)-loaded *EcMetAP* and the catalytically inactive Co(III)- and Fe(III)-bound forms of *EcMetAP*. In addition, EXAFS data of the Co(II)-loaded form of *EcMetAP* bound by the anti-angiogenesis agent fumagillin are presented.

Materials and Methods

Protein Expression and Purification. Recombinant *EcMetAP* was expressed and purified as previously described from a stock culture kindly provided by Drs. Brian W. Matthews and W. Todd Lowther (12, 18). Purified *EcMetAP* exhibited a single band on SDS-PAGE and a single symmetrical peak in matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis indicating $M_r = 29630 \pm 10$. Protein concentrations were estimated from the absorbance at 280 nm using an extinction coefficient of $16,500 \text{ M}^{-1} \text{ cm}^{-1}$ (12, 18). Apo-MetAP samples were exchanged into 25 mM HEPES, pH 7.5, containing 150 mM KCl (Centricon-10, Millipore Corp). Apo-MetAP samples were incubated anaerobically with MCl_2 , where $\text{M} = \text{Co(II)}$ or Fe(II) , for 30 minutes as previously reported (18).

Enzymatic Assay of EcMetAP. *EcMetAP* was assayed for catalytic activity with Met-Gly-Met-Met as the substrate (8 mM) using an HPLC method described previously (18). This method is based on the spectrophotometric quantitation of the reaction product Gly-Met-Met at 215 nm following separation on a C8 HPLC column (Phenomenex, Luna;

5 μm , 4.6 x 25 cm). The kinetic parameter v (velocity) was determined at pH 7.5 by quantifying the tripeptide Gly-Met-Met at 215 nm in triplicate. Enzyme activities are expressed as units/mg, where one unit is defined as the amount of enzyme that releases 1 μmol of Gly-Met-Met at 30 $^{\circ}\text{C}$ in 1 min. Catalytic activities were determined with an error of $\pm 10\%$.

X-ray absorption spectroscopy. EXAFS samples of *EcMetAP* (1mM) were frozen in polycarbonate cuvettes, 24x3x1 mm with a 0.025 mm Mylar window covering one 24x3 mm face. XAS data were collected at Stanford Synchrotron Radiation Laboratory (SSRL) with the SPEAR storage ring operating in a dedicated mode at 3.0 GeV (Table 3.1). The edge regions for multiple scans obtained on the same sample were compared to ensure that the sample was not damaged by exposure to X-ray radiation. EXAFS analysis was performed using EXAFSPAK software (www-ssrl.slac.stanford.edu/exafspak.html),

Table 3.1. X-ray absorption spectroscopic data collection.

	Co EXAFS	Fe EXAFS
SR facility	SSRL	SSRL
beamline	7-3, 9-3	7-3, 9-3
current in storage ring	50-100 mA	50-100 mA
monochromator crystal	Si[220]	Si[220]
detection method	fluorescence	fluorescence
detector type	solid state array ^a	solid state array ^a
scan length, min	24	20
scans in average	10	10
temperature, K	10	10
energy standard	Co foil, 1 st inflection	Fe foil, 1 st inflection
energy calibration, eV	7709.5	7111.3
E_0 , eV	7715	7120
pre-edge background		
energy range, eV	7390-7670	6789-7075
Gaussian center, eV	6930	6403
Gaussian width, eV	750	750
spline background		
energy range, eV	7715-7952 (4)	7120-7354 (4)
(polynomial order)	7952-8189 (4)	7354-7589 (4)
	8189-8427 (4)	7589-7822 (4)

^aThe 13-element Ge solid-state X-ray fluorescence detector at SSRL is provided by the NIH Biotechnology Research Resource.

according to standard procedures (19). Multiple-scattering analysis was performed as described previously (21). Both single- and multiple-scattering paths ≤ 4.5 Å from either the Fe or Co atom were used to identify and quantify imidazole coordination due to histidine. Multiple scattering models, calculated using FEFF v7.02 (22), were based on either bis(*N*-methylimidazole)bis(diphenylborondimethylglyoximate)iron(II) dichloromethane solvate (23) or hexakis(imidazole)cobalt(II) carbonate pentahydrate (24). The model was edited to only the metal atom and one imidazole and the coordinates were imported into FEFF to calculate scattering amplitudes and phase shifts for each scattering path containing four or fewer legs. A constrained fitting process was then used with the following parameters: Coordination numbers were constrained to be integer or half-integer values (the latter representing a ligand bound to one of the two metal ions), the distances for outer-shell atoms of imidazole rings were constrained to be a constant difference with the inner-shell imidazole atom distance. Only a single ΔE_0 value was optimized. Scaling factors were determined from model compound analysis. Debye-Waller values for imidazole C₂, N₃, C₄, C₅ atoms were constrained to be multiples of one another, but were not tied to the first-shell [N₁, (N,O)] Debye-Waller values. This allows non-imidazole first-shell ligands to have Debye-Waller values independent of the imidazole ligand values. Possible coordination numbers of histidyl imidazole ligands were chosen from fits that yielded chemically and physically reasonable Debye-Waller factors for the outer-shell atoms, since goodness-of-fit values (*f*) were relatively insensitive to these coordination numbers.

Results and Discussion

Cobalt and iron K-edge X-ray absorption (XAS) spectra were acquired on 1 mM samples of *EcMetAP* with one or two equivalents of added Co(II) (*i.e.* [Co(II)₁(*EcMetAP*)] and [Co(II)₂(*EcMetAP*)], respectively), or one or two equivalents of added Fe(II) (*i.e.* [Fe(II)₁(*EcMetAP*)] and [Fe(II)₂(*EcMetAP*)],

respectively), as well as for the fully loaded Co(III) and Fe(III) forms of the protein and for [Co(II)Co(II)(*Ec*MetAP)] incubated with the inhibitor fumagillin. For fully loaded samples (*e.g.* [Co(II)Co(II)(*Ec*MetAP)]), the EXAFS data reveal an average of both metal ion environments. The 1s→3d pre-edge transitions for [Co(II)(*Ec*MetAP)] and [Co(II)Co(II)(*Ec*MetAP)] occur at 7709 eV with a peak intensity of 0.118 and 0.127 eV, respectively (Figure 3.1a). Since 1s→3d pre-edge transitions are Laporte forbidden in

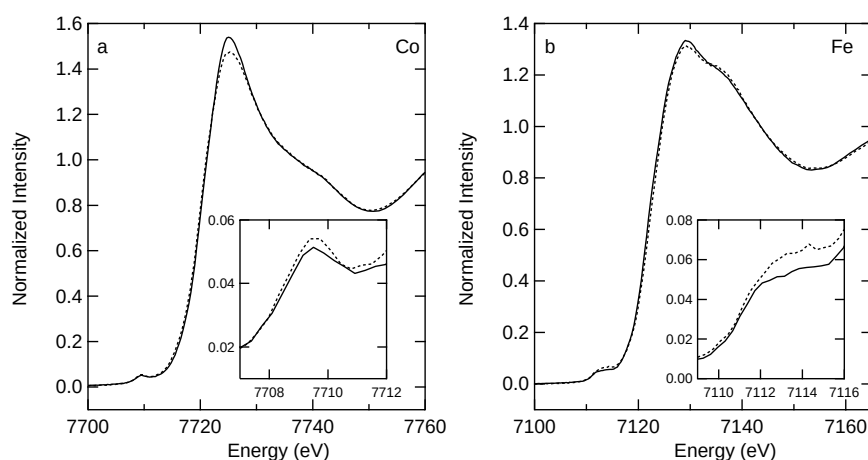


Figure 3.1. X-ray absorption K-edge spectra for *Ec*MetAP: a) [Co(II)Co(II)(*Ec*MetAP)] (solid) and [Co(II)(*Ec*MetAP)] (dotted). b) [Fe(II)Fe(II)(*Ec*MetAP)] (solid) and [Fe(II)(*Ec*MetAP)] (dotted). In the inset, the pre-edge 1s → 3d transition is expanded.

centrosymmetric environments (*e.g.*, octahedral, but not tetrahedral), the intensity of the 1s→3d pre-edge transitions is inversely proportional to coordination number (assuming tetrahedral four-coordination). The intensities of the observed transitions for [Co(II)(*Ec*MetAP)] and [Co(II)Co(II)(*Ec*MetAP)] are consistent with, on average, five- or six-coordinate Co(II) sites (25, 26).

Fourier transforms (FTs) of the EXAFS data for both [Co(II)(*Ec*MetAP)] and [Co(II)Co(II)(*Ec*MetAP)] are dominated by a peak at *ca.* 2.05 Å (Figure 3.2). Excellent single-shell fits of EXAFS spectra for both [Co(II)(*Ec*MetAP)] and

[Co(II)Co(II)(*EcMetAP*)] were obtained with 6 ± 1 N/O scatterers at 2.04 Å, (Fits 2-4,8-10; Table 3.2). Attempts to include a Co-Co interaction (in the 2.4 – 4.0 Å range) in the curve-fitting parameters were unsuccessful. Inclusion of multiple-scattering contributions from the outer-shell atoms of a histidine-imidazole ring result in reasonable Debye-Waller factors for these contributions and a slight reduction in the goodness-of-fit value (χ^2) (Fits 5,11; Table 3.2). This is consistent with the suggestion of a single histidine ligand from the crystallographic analyses (13-16). The Debye-Waller factor values are higher for [Co(II)Co(II)(*EcMetAP*)] than for [Co(II)_(*EcMetAP*)], suggesting that the first Co atom is located in the histidine-ligated site.

The observed EXAFS spectra of Co(II)-loaded *EcMetAP* suggest that the Co(II) ions reside in a distorted penta- or hexacoordinate geometry, containing a histidine ligand. This is consistent with the X-ray crystal structure of Co(II)-loaded *EcMetAP* which indicates that the histidine-ligated Co(II) ion resides in a distorted trigonal bipyramidal coordination environment while the second Co(II) ion is either trigonal bipyramidal or distorted octahedral (16, 27). The average bond distance obtained by EXAFS for both *EcMetAP* samples are in excellent agreement with the crystallographically determined bond lengths for [Co(II)Co(II)(*EcMetAP*)] of 2.04 Å (16). Additionally, it was recently reported, based on ^1H NMR data that upon the addition of Co(II) to *EcMetAP*, the first Co(II) bound to the lone histidine residue in the active site (17). The previously reported electronic absorption spectrum of *EcMetAP* upon the addition of one equivalent of Co(II) under anaerobic conditions, exhibited three resolvable d-d transitions at 580, 630, and 690 nm ($\epsilon = 60, 50, \text{ and } 20 \text{ M}^{-1}\text{cm}^{-1}$, respectively) (17). These data are also consistent with the first Co(II) ion residing in a pentacoordinate environment. Similarly, the observed EPR spectrum of [Co(II)_(*EcMetAP*)] was shown to be a broad, featureless signal suggesting an unconstrained ligand-field (17). Thus, there is a great deal of flexibility in the ligand environment (28, 29). Moreover, the low E/D value of 0.09 (in general, $1/3 \geq E/D \geq 0$) reported for [Co(II)_(*EcMetAP*)] also indicates a fairly high degree of axial symmetry.

Table 3.2. Curve-fitting results for Co *Ec*MetAP EXAFS^a

	Fit	Shell	N _S	R _{as} (Å)	σ _{as} ² (Å ²)	ΔE ₀ (eV)	f ^b
[Co(II)](<i>Ec</i> MetAP) MC20C, 2-12 Å ⁻¹ Δ <i>k</i> ³ χ = 10.75	1	Co-O	4	2.05	0.0060	-1.17	0.094
	2	Co-O	5	2.04	0.0078	-1.28	0.084
	3	Co-O	6	2.05	0.0096	-1.43	0.082
	4	Co-O	7	2.04	0.0114	-1.57	0.084
	5	Co-(O,N)	6	2.05	0.0078	-1.29	0.077
		Co-C	1	2.85	0.0041		
		Co-C	1	[2.96]	[0.0043]		
		Co-C	1	[3.93]	[0.0057]		
		Co-N	1	[3.99]	[0.0057]		
	6	Co-O	5	2.05	0.0078	-1.25	0.083
		Co-Co	1	3.19	0.0140		
[Co(II)Co(II)](<i>Ec</i> MetAP) MC22C, 2-12 Å ⁻¹ Δ <i>k</i> ³ χ = 11.42	7	Co-O	4	2.06	0.0052	-0.74	0.090
	8	Co-O	5	2.05	0.0068	-0.84	0.078
	9	Co-O	6	2.05	0.0084	-1.03	0.072
	10	Co-O	7	2.05	0.0103	-1.21	0.073
	11	Co-(O,N)	6	2.05	0.0085	-0.99	0.069
		Co-C	1	2.83	0.0063		
		Co-C	1	[2.95]	[0.0066]		
		Co-C	1	[3.90]	[0.0087]		
		Co-N	1	[3.97]	[0.0088]		
	12	Co-O	5	2.06	0.0068	-0.83	0.077
		Co-Co	1	3.14	0.0250		
[Co(III)Co(III)](<i>Ec</i> MetAP) MC33C, 2-12 Å ⁻¹ Δ <i>k</i> ³ χ = 11.23	13	Co-O	4	2.05	0.0076	0.25	0.087
	14	Co-O	5	2.05	0.0097	0.02	0.078
	15	Co-O	6	2.04	0.0120	-0.35	0.076
	16	Co-O	7	2.04	0.0139	-0.67	0.077
	17	Co-(O,N)	5	2.05	0.0097	0.05	0.076
		Co-C	1	2.85	0.0133		
		Co-C	1	[2.96]	[0.0138]		
		Co-C	1	[3.92]	[0.0183]		
		Co-N	1	[3.99]	[0.0187]		
	18	Co-O	5	2.05	0.0097	-0.07	0.078
		Co-Co	1	3.07	0.0299		

^a Shell is the chemical unit defined for single- and multiple-scattering calculations.N_S is the number of scatterers per shell. R_{as} is the metal-scatterer distance. σ_{as}² is a mean square deviation in R_{as}. ΔE₀ is the shift in E₀ for the theoretical scattering functions. Numbers in square brackets were constrained to be multiples of the value above.^b f^b is a normalized error (chi-squared): $f^b = \{\sum_i [k^3(\chi^{\text{obs}} - \chi^{\text{calc}})]^2 / N\}^{1/2} / [(k^3\chi^{\text{obs}})_{\text{max}} - (k^3\chi^{\text{obs}})_{\text{min}}]$.

The combination of the electronic absorption, EPR, and EXAFS data suggests that the single catalytically competent Co(II) ion in *EcMetAP* resides in a site that is consistent with the histidine-containing site reported in the X-ray crystal structure of [Co(II)Co(II)(*EcMetAP*)] (16). Furthermore, the EXAFS data do not detect a Co-Co interaction, providing no support for a dinuclear Co(II) site in *EcMetAP*, as seen in the published X-ray crystal structures for all MetAP's (15, 16).

Table 3.3. Curve-fitting results for Fe *EcMetAP* EXAFS^a

	Fit	Shell	N _S	R _{as} (Å)	σ_{as}^2 (Å ²)	ΔE_0 (eV)	f
[Fe(II)](<i>EcMetAP</i>)	1	Fe-O	4	2.04	0.0080	−0.98	0.10
MF20C, 2-12 Å ^{−1}	2	Fe-O	5	2.03	0.0101	−1.43	0.10
$\Delta k^3\chi = 8.42$	3	Fe-O	6	2.03	0.0122	−1.81	0.10
	4	Fe-O	7	2.03	0.0144	−2.14	0.10
[Fe(II)Fe(II)](<i>EcMetAP</i>)	6	Fe-O	4	2.03	0.0074	−2.21	0.09
MF22C, 2-12 Å ^{−1}	7	Fe-O	5	2.03	0.0095	−2.27	0.08
$\Delta k^3\chi = 8.35$	8	Fe-O	6	2.03	0.0116	−2.47	0.08
	9	Fe-O	7	2.03	0.0137	−2.65	0.09
[Fe(III)Fe(III)](<i>EcMetAP</i>)	10	Fe-O	4	2.02	0.0071	0.08	0.08
MF33C, 2-12 Å ^{−1}	11	Fe-O	5	2.02	0.0091	−0.44	0.07
$\Delta k^3\chi = 11.37$	12	Fe-O	6	2.01	0.0110	−0.92	0.07
	13	Fe-O	7	2.01	0.0129	−1.33	0.07

^a See footnotes to Table 3.2.

The 1s→3d pre-edge transitions are observed at 7113 eV with intensities of 0.130 and 0.151 eV for [Fe(II)](*EcMetAP*) and [Fe(II)Fe(II)](*EcMetAP*), respectively (Figure 3.1B). The intensities of the 1s→3d transitions are consistent with Fe(II) sites that are, on average, five-coordinate (30). These data suggest that Fe(II) binds to *EcMetAP* in a similar site to Co(II). The Fourier transforms for [Fe(II)](*EcMetAP*) and [Fe(II)Fe(II)](*EcMetAP*) (Figure 3.3, bottom), similar to the Co FTs, are dominated by peaks at *ca.* 2.03 Å. Excellent single-shell fits of this peak in both [Fe(II)](*EcMetAP*) and [Fe(II)Fe(II)](*EcMetAP*) were obtained with 5 or 6 N/O scatterers per Fe atom at 2.04 or 2.03 Å (Fits 2,3,7,8; Table 3.3). Attempts to include either a Fe-Fe or a Fe-imidazole interaction in the curve-fitting parameters resulted in fits that did not converge.

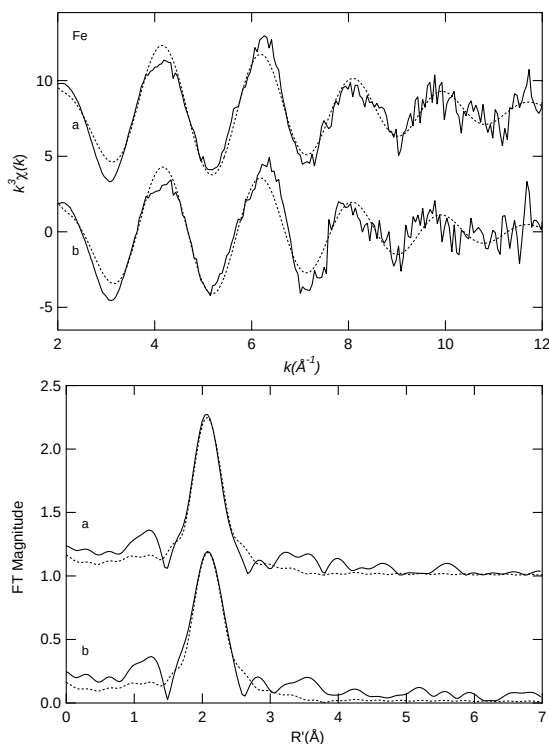


Figure 3.3. k^3 -weighted Fe EXAFS (top) and Fourier transforms (bottom, over $k=2$ – $12 \text{ \AA}^{-1}$) for: a) $[\text{Fe(II)Fe(II)}](\text{EcMetAP})$ (solid) and the calculated spectra for Fe- O_5 (dotted; Fit 7, Table 3.3) and b) $[\text{Fe(II)}](\text{EcMetAP})$ (solid) and the calculated spectra for Fe- O_5 (dotted; Fit 2, Table 3.3).

made up of oxygen or nitrogen donor ligands.

The lack of M-M FT peaks in the second-shell of both Fe(II) and Co(II)-loaded *EcMetAP* are consistent with the recently reported metal binding constants. The first metal binding event for Co(II)- and Fe(II)-substituted *EcMetAP* exhibited K_d values of 300 and $200 \pm 200 \text{ nM}$, respectively (17). In addition, it was shown that the binding of excess metal ions (< 50 equivalents) resulted in the loss of $\sim 50 \%$ of the catalytic activity. The second metal-binding event for Co(II)-*EcMetAP* was shown to have a K_d value of $2.5 \pm 0.5 \text{ mM}$ (17). Therefore, under the conditions in which these EXAFS samples used in this study were prepared (1 mM *EcMetAP* plus one or two equivalents of divalent

The observed Fe(II) bond distances are in agreement with X-ray crystallographic data for the Co(II)-loaded *EcMetAP* (16) and are also similar to those derived from fits of Co(II)-loaded *EcMetAP*. Since Fe(II)-loaded *EcMetAP* is colorless, air sensitive, and EPR silent, no structural information has been reported for the catalytically competent $[\text{Fe(II)}](\text{EcMetAP})$ enzyme. Therefore, the EXAFS data of $[\text{Fe(II)}](\text{EcMetAP})$ and $[\text{Fe(II)Fe(II)}](\text{EcMetAP})$ provide the first structural glimpse of the Fe(II) active site of *EcMetAP* and reveal that the first Fe(II) ion likely resides in a penta- or hexacoordinate geometry

metal ion) one would not expect the second metal binding site to be occupied, consistent with the EXAFS data.

The lack of a second-shell metal ion scatterer is also consistent with the reported EPR signal for [Co(II)Co(II)(*Ec*MetAP)]. The EPR spectra of both [Co(II)](*Ec*MetAP)] and [Co(II)Co(II)(*Ec*MetAP)] are broad, featureless, and indistinguishable in form suggesting an unconstrained ligand-field. The observed EPR signal for [Co(II)](*Ec*MetAP)] integrated to one Co(II) ion per MetAP enzyme and this signal doubled in intensity upon the addition of a second equivalent of Co(II). Moreover, the observed EPR signals followed Curie Law over the temperature range 4 to 60 K at non-saturating microwave powers (17).

These data suggest that the Co(II) ions in [Co(II)Co(II)(*Ec*MetAP)] exhibit no detectable spin-spin interaction, consistent with lack of a Co-Co active site. In support of these data, no integer spin signal could be detected in the parallel mode for *Ec*MetAP at pH 7.5 (17). These data clearly indicate no M-M interaction exists in either the mono- or dimetal Co(II) and Fe(II) EXAFS samples. Therefore, a dinuclear center is not formed upon addition of one or two equivalents of either Co(II) or Fe(II) to *Ec*MetAP at enzyme concentrations of 1 mM, which is clearly higher than *in vivo* MetAP concentrations.

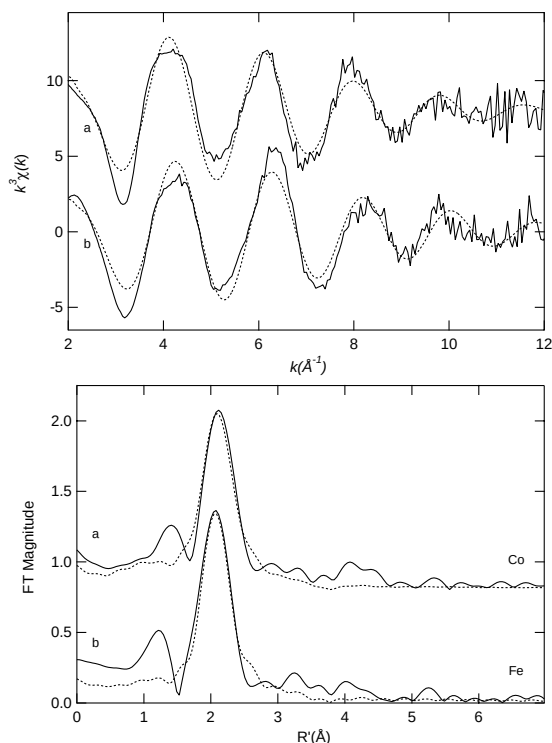


Figure 3.4. k^3 -weighted EXAFS (top) and Fourier transforms (bottom, over $k=2$ -12 $\text{\AA}^{-1}$) for a) [Co(III)Co(III)(*Ec*MetAP)] (solid) and the calculated spectra for Co-O₅ (dotted; Fit 14, Table 3.2) and b) [Fe(III)Fe(III)(*Ec*MetAP)] and the calculated spectra for Fe-O₅ (dotted; Fit 11, Table 3.3).

Similarly, the two Fe(III) ions in Fe(III)-loaded *EcMetAP* do not exhibit any significant spin-spin interaction based on EPR spectroscopic studies, similar to the two Co(II) ions in Co(II)-substituted MetAP, further indicating that a dinuclear active site does not exist. These data are also consistent with EXAFS spectra of [Co(III)Co(III)(*EcMetAP*)] and [Fe(III)Fe(III)(*EcMetAP*)] which show no M-M interaction (Figure 3.4; Tables 3.2,3.3).

The range of temperatures over which the EPR signals from Co(II)-loaded *EcMetAP* were detectable can be compared with the temperature range of the detectable EPR signal from the Co(II)-loaded aminopeptidase from *Aeromonas proteolytica* ([Co(II)Co(II)(AAP)]) (28, 29). The two cobalt ions in [Co(II)Co(II)(AAP)] were shown to be spin-coupled, providing a spin-spin relaxation pathway that results in the spectrum of [Co(II)Co(II)(AAP)] obeying $1/T$ dependence over only a narrow temperature range (9 to 15 K). This electronic communication is likely mechanistically important for [Co(II)Co(II)(AAP)] in that a pathway for the modulation of the Lewis acidity of one metal ion by the other is present. Moreover, spin-spin interactions also reveal structural motifs such as μ -OH(H) ligands. Since the observed EPR signal of [Co(II)Co(II)(*EcMetAP*)] was detectable at temperatures up to 60 K and the signal intensity was found to be inversely proportional to the absolute temperature, following Curie law dependence at non-saturating microwave powers, one can then speculate that the proposed bridging water molecule observed in the X-ray crystal structure of *EcMetAP* is incapable of mediating detectable spin-spin coupling, presumably because the second metal ion does not exist in the active site in EPR-analyzed samples.

There is precedent for metallohydrolases that have crystallographically characterized dinuclear active sites to exhibit catalytic activity with only one metal ion bound. For instance, AAP, which has been crystallographically characterized as well as the aminopeptidase from porcine kidney have long been known to be catalytically active with only one divalent metal ion present (31-34). For *EcMetAP*, the addition of up to 200 equivalents of either Co(II) or Fe(II) resulted in a decrease in the catalytic activity, similar

to the metal binding properties of the type-I MetAP from *S. cerevisiae* but, different to those of AAP and porcine kidney (31-35). These data suggest that the binding of a second metal ion to MetAP's is actually inhibitory, which would imply that the second metal ion does not have a catalytic role. Inhibition of catalytic activity by excess divalent metal ions has also been observed for other mononuclear metalloenzymes such as carboxypeptidase *Taq* when overexpressed in *E. coli* (36), bovine carboxypeptidase A (37, 38), and thermolysin (39). Inhibition of carboxypeptidase A was attributed to excess metal ion binding to an amino acid residue in the vicinity of the metallo-active site that was involved in catalysis (37). In addition, the authors proposed that a bridging water/hydroxide, inserted between the two metal ions, which enhanced the formation of a dinuclear site. This proposal was corroborated by X-ray crystallography where the structures of carboxypeptidase A, as well as thermolysin in the presence of excess metal ion revealed two coordinated metal ions forming a (μ -hydroxo)dizinc(II) core with a Zn-Zn distance of 3.48 and 3.2 Å, respectively (39-41). Therefore, the observation that the addition of excess metal ions to *EcMetAP* inhibited enzymatic activity suggests that the inhibition is likely due to the occupation of a non-catalytically relevant metal-binding site, similar to carboxypeptidase A.

An important class of MetAP inhibitors is based on natural products of fungal origin, namely, fumagillin and ovalicin. Ovalicin and a synthetic analog of fumagillin (AGM-1470) have been demonstrated to preferentially inhibit endothelial cell growth in tumor vasculature *in vivo* (42). Based on fumagillin-specific affinity reagents and mass spectroscopic studies on MetAP-fumagillin complexes, MetAP's were identified as the specific target of fumagillins (10, 11).

The mode of inhibition was shown to be *via* the formation of a covalent bond between a conserved histidine residue in MetAP's and an epoxide carbon moiety on fumagillin (10-12, 43). Confirmation that fumagillin reacts with the type-I MetAP from *E. coli* comes from mass spectrometric and N-terminal sequence analysis which indicated that fumagillin covalently binds to an active site histidine residue (His79) that is not a ligand at the dinuclear active site cluster (12). In order to determine the interaction between the active site Co(II) ion of *EcMetAP* and the anti-angiogenesis drug fumagillin, the EXAFS spectrum of

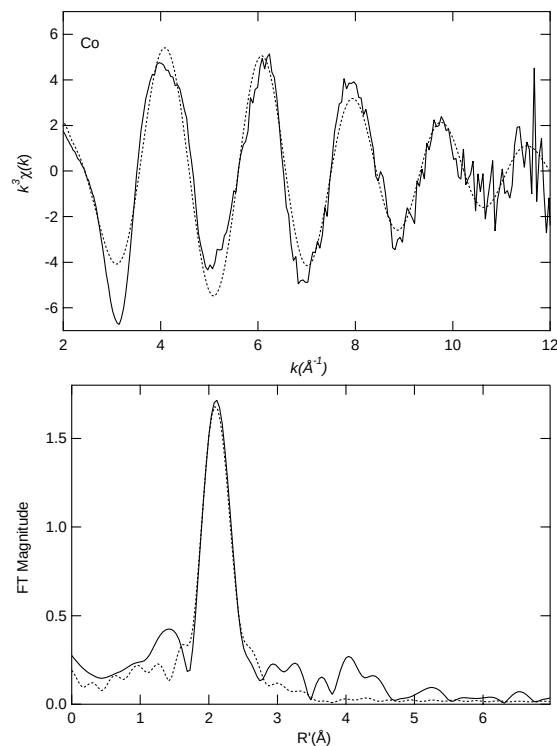


Figure 3.5. k^3 -weighted Co EXAFS (top) and Fourier transforms (bottom, over $k=2-12 \text{ \AA}^{-1}$) for $[\text{Co(II)Co(II)(EcMetAP)}]$ plus fumagillin (solid) and the calculated spectra for Co-O₆ (dotted; Fit 3, Table 3.4).

$[\text{Co(II)Co(II)(EcMetAP)}]$ was recorded after reaction with fumagillin. That fumagillin was covalently bound to a divalent metal ion loaded *EcMetAP* was verified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) spectrometric analysis

which revealed a mass shift of 451 Da. in excellent agreement with the mass of fumagillin (458 Da.).

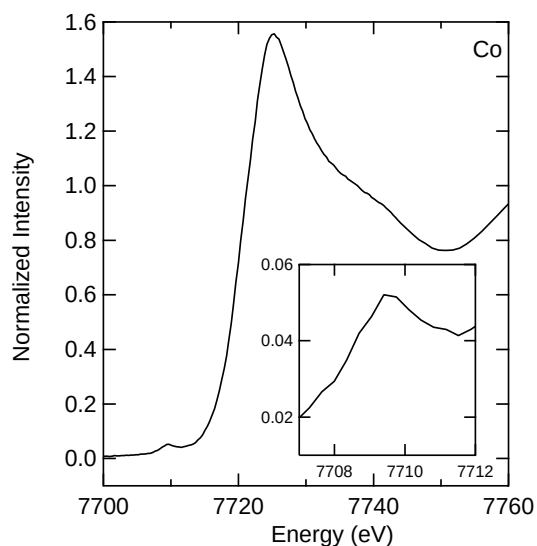


Figure 3.6. X-ray absorption K-edge spectra for $[\text{Co(II)Co(II)}(\text{EcMetAP})]$ plus fumagillin. In the inset, the pre-edge $1s \rightarrow 3d$ transition is expanded.

Interestingly, but perhaps not surprisingly, no significant change in the XAS data for $[\text{Co(II)Co(II)}(\text{EcMetAP})]$ in the presence of fumagillin was observed (Figure 3.5). The $1s \rightarrow 3d$ pre-edge transition observed for $[\text{Co(II)Co(II)}(\text{EcMetAP})]$ -fumagillin occur at 7709 eV with a peak intensity of 0.127 eV suggesting five- or six-coordinate Co(II) sites (Figure 3.6) (25, 26). Excellent single-shell fits of EXAFS spectrum of $[\text{Co(II)Co(II)}(\text{EcMetAP})]$ -fumagillin were obtained

with 5 or 6 N/O scatterers at 2.05 Å, based on Debye-Waller factors. The residuals of the fits (r^2 for Fits 2,3; Table 3.4) were similar to those observed for $[\text{Co(II)Co(II)}(\text{EcMetAP})]$ without added fumagillin. Fits that include either a Co-Co interaction or the multiple-scattering contributions from outer-shell atoms of a histidine ligand resulted in unreasonably high Debye-Waller factors (Fits 5,6; Table 3.4). These data suggest that a dinuclear Co(II) site does not exist in $[\text{CoCo}(\text{EcMetAP})]$ -fumagillin, contrary to the published X-ray crystal structures for the MetAP from *Homo sapiens* (13). These data strongly suggest that upon fumagillin binding, there is little, if any change in the coordination sphere of the average Co(II) site.

Comparison of the EXAFS spectroscopic results for $[\text{Co(II)Co(II)}(\text{EcMetAP})]$ -fumagillin with the recent 1.8 Å X-ray crystal structure of the type-II MetAP from *H. sapiens* complexed with fumagillin (13), reveals striking similarities and differences. In

the X-ray structure, the epoxide-bearing side chain of fumagillin occupies the putative substrate-binding pocket of *HsMetAP*. The long unsaturated side-chain is analogous to the COOH-terminal peptide chain in the X-ray structure of a substrate analog inhibited

Table 3.4. Curve-fitting results for [CoCo(*EcMetAP*)]+Fumagillin EXAFS^a

Sample	Fit	Shell	N _S	R _{as} (Å)	σ _{as} ² (Å ²)	ΔE ₀ (eV)	f ^b
[Co(II)Co(II)(<i>EcMetAP</i>)]+fum. MC2FA, 2-12 Å ⁻¹ Δ k ³ χ = 11.87	1	Co-O	4	2.06	0.0048	-0.53	0.095
	2	Co-O	5	2.05	0.0064	-0.59	0.084
	3	Co-O	6	2.05	0.0080	-0.84	0.079
	4	Co-O	7	2.05	0.0096	-1.02	0.080
	5	Co-(O,N)	6	2.06	0.0080	-0.76	0.077
		Co-C	1	2.88	0.0143		
		Co-C	1	[3.00]	[0.0148]		
		Co-C	1	[3.97]	[0.0196]		
		Co-N	1	[4.04]	[0.0200]		
	6	Co-O	6	2.06	0.0080	-0.83	0.078
		Co-Co	1	3.04	0.0196		

^aSee footnotes to Table 3.2.

form of *EcMetAP* (15). The crystallographic results also verify that a covalent bond is formed between the reactive ring epoxide of fumagillin and His231 in the active site of the type-II MetAP. The oxygen atom liberated from the breaking of the epoxide bond, is 3.28 Å away from Co1, the Co(II) ion bound by His331, Glu364, and the two bridging carboxylate residues Asp262 and Glu459. This alkoxide oxygen atom was suggested to be directly coordinated to Co. The EXAFS results presented herein clearly indicate that

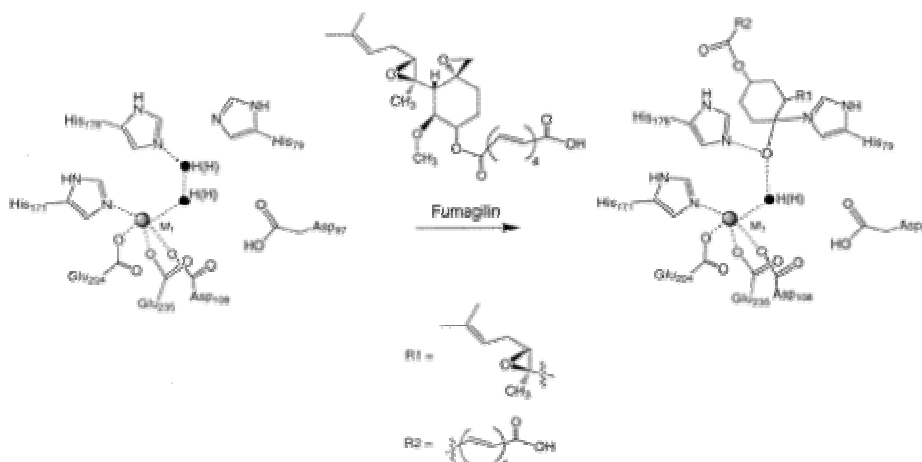


Figure 3.7. Proposed structure of the mono-Co(II) or mono-Fe(II) forms of *EcMetAP* in the presence of fumagillin.

the alkoxide oxygen atom of fumagillin is *not* an additional ligand to the Co(II) ion bound in the active site of *EcMetAP*, contrary to the suggestion by Liu *et al.* (13). Closer inspection of the X-ray crystal structure of *HsMetAP* complexed by fumagillin indicates that the approximate location of the alkoxide oxygen of fumagillin is where a water molecule resided at $> 3 \text{ \AA}$ from the Co(II) ion in the uncomplexed structure. Therefore, we propose that the oxygen atom liberated upon the addition of fumagillin to *EcMetAP*, displaces the water molecule that bridges between His178 and the water molecule bridging the two Co(II) ions in the X-ray structure of native *EcMetAP* (Figure 3.7). Thus, fumagillin does *not* provide a ligand to the metal ion in the *EcMetAP* active site. Since fumagillin has two reactive epoxide moieties, it is quite cytotoxic probably due to alkylation of other biomolecules within the cell. Therefore, understanding the molecular mechanism of the MetAP-catalyzed cleavage of N-terminal methionine residues as well as the binding mode of known anti-angiogenesis drugs will facilitate the rational design of new, more potent MetAP inhibitors with improved *in vivo* stability, specificity, and lower cytotoxicity.

Acknowledgments

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CHAPTER 4

DIRECT FE-S CLUSTER INVOLVEMENT IN GENERATION OF A RADICAL IN
LYSINE 2,3-AMINOMUTASE¹

¹ Cosper, N.J., S. J. Booker, F. Ruzicka, P. A. Frey, R. A. Scott. 2000. Accelerated publication in Biochemistry. 39:15668-15673.

Abstract

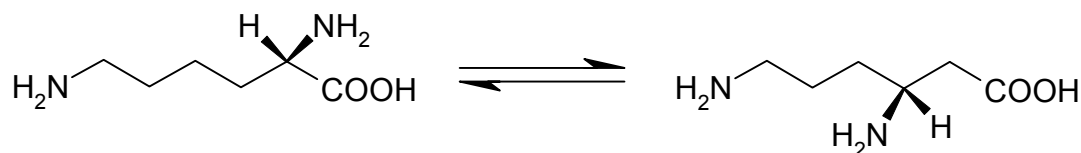
Lysine 2,3-aminomutase (KAM) belongs to a class of enzymes that use FeS clusters and S-adenosyl-L-methionine to initiate radical-dependent chemistry. Selenium K-edge x-ray absorption spectroscopic analysis of KAM poised at various stages of catalysis, in the presence of selenomethionine or Se-adenosyl-L-selenomethionine, reveals that the cofactor is cleaved only in the presence of dithionite and the substrate analog *trans*-4,5-dehydrolysine. A new Fourier transform peak at 2.7 Å, assigned as a Se-Fe interaction, appears concomitant with this cleavage. This is the first demonstration of a direct interaction of S-adenosyl-L-methionine, or its cleavage products, with the FeS cluster in this class of enzymes.

Introduction

In recent years, mechanistic details of a new class of S-adenosyl-L-methionine (AdoMet)-dependent enzymes have begun to emerge (1, 2). These enzymes use Fe_4S_4 clusters in combination with AdoMet to generate enzyme-bound, carbon-centered radicals, which are obligatory intermediates in the corresponding reactions. The importance of this radical-generating system is underscored by the diversity of reactions and the difficult chemistry in which it participates. Many of these reactions are anaerobic counterparts to those that are typically catalyzed by copper- or iron-dependent monooxygenases and dioxygenases. Although others are presumed to exist, four enzymes within this class have been characterized in moderate detail. They include biotin synthase (3), pyruvate formate-lyase activating enzyme (PFL-activase) (4, 5), anaerobic ribonucleotide reductase activating enzyme (ARR-activase) (6), and lysine 2,3-aminomutase (KAM) (7, 8). Very recent studies suggest that these and other biosynthetic and metabolic enzymes form a superfamily of radical-generating AdoMet-dependent enzymes (personal communication, H. J. Sofia). Other potential members of this superfamily are also involved in vitamin (thiamin) and cofactor (heme, bacteriochlorophyll, molybdopterin, nitrogenase) biosynthesis.

Biotin synthase, the apparent product of the *bioB* gene, catalyzes the final step, insertion of sulfur into dethiobiotin, in the biosynthesis of this essential vitamin. Two unactivated hydrogens from the precursor are removed in the process and two moles of AdoMet are expended per mole of biotin synthesized (9, 10). PFL-activase and ARR-activase catalyze the formation of a stable radical that is situated on the backbone of a glycine residue of the respective cognate proteins, pyruvate formate-lyase (PFL) and anaerobic ribonucleoside triphosphate reductase (ARR) (11-13). PFL catalyzes the reversible condensation of acetyl-CoA and formate, producing pyruvate and CoA (14), while ARR catalyzes the production of deoxyribonucleoside triphosphates from the corresponding ribonucleoside triphosphate precursors (15). Both of these enzymes are central to the anaerobic metabolism of *E. coli*, and are present in other obligate or

facultative anaerobes. In each case, the glycyl radical acts as an initiator of chemistry that is postulated to proceed by radical-dependent mechanisms (14, 15). One mole of AdoMet is expended per mole of glycyl radical that is generated; however, the glycyl radical is regenerated after each turnover, and therefore serves as a cofactor (6, 16, 17). KAM, isolated from *Clostridium subterminale* SB4, catalyzes the interconversion of L- α -lysine and L- β -lysine (Scheme 4.1). This is the initial step in the catabolism of the amino acid to acetyl-CoA and ammonia, which are usable carbon and nitrogen sources for the bacterium (18, 19). Thus, the enzymes in this class catalyze a range of chemical conversions that are essential to biosynthetic and metabolic pathways in numerous organisms.



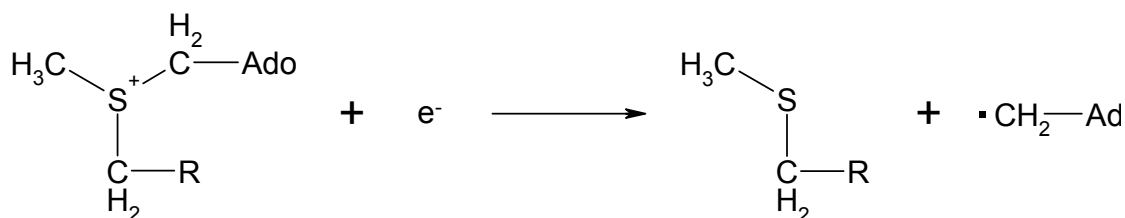
Scheme 4.1. Conversion of L- α -lysine to L- β -lysine, catalyzed by lysine 2,3-aminomutase.

The KAM holoenzyme is composed of 6 identical subunits of M_r 47 kDa, and contains 1 pyridoxal 5'-phosphate (PLP) and 1 Zn per subunit, in addition to the iron and sulfide that constitute the Fe_4S_4 centers (8, 19-21). The reaction it catalyzes is functionally equivalent to those that have been historically considered to lie exclusively within the domain of coenzyme B_{12} -containing enzymes; however, the enzyme neither contains this cofactor nor is activated by it (22). Instead, stoichiometric amounts of AdoMet are sufficient to render it maximally active in the presence of a suitable reductant (dithionite or deazaflavin and light) (7, 23).

Numerous mechanistic studies have led to a model in which the 5'-deoxyadenosyl moiety of AdoMet acts as an intermediate carrier of hydrogen during the reaction (22, 24, 25). This is realized via the reductive cleavage of the cofactor to methionine and 5'-deoxyadenosine 5'-yl, which initiates catalysis in the forward direction by abstracting the

3-*proR* hydrogen of α -lysine (24). After rearranging to the product radical via a PLP-stabilized azocyclopropylcarbinyl radical, the product radical re-abstracts a hydrogen atom from 5'-deoxyadenosine to complete the reaction, and reafford 5'-deoxyadenosine 5'-yl (3, 26).

The cleavage of AdoMet to 5'-deoxyadenosine 5'-yl is an unprecedented biochemical reaction. The stoichiometry of the reaction requires input of an electron, which is provided by the reduced iron-sulfur cluster ($[\text{Fe}_4\text{S}_4]^{+1}$) (6, 7). Several mechanisms to account for the cleavage of AdoMet can be envisioned (1, 3). In the



Scheme 4.2. Generation of methionine and 5'-deoxyadenosyl radical by cleavage of a sulfur-carbon bond in AdoMet. Replacement of the sulfur by selenium supports KAM catalysis and provides a “spectroscopic handle.”

simplest case, the iron-sulfur cluster transfers an electron into the sulfonium of AdoMet, causing it to be fragmented into methionine and 5'-deoxyadenosine (Scheme 4.2). Alternatively, AdoMet might serve to adenosylate a bridging sulfide of the cluster. Homolytic cleavage of the sulfur-carbon bond would then yield a 5'-deoxyadenosyl radical, and an oxidized FeS cluster. Other proposals invoke participation of an iron atom of the cluster. For instance, a 5'-deoxyadenosyl radical could derive from homolytic cleavage of an Fe-carbon bond.

To obtain insight into the mechanism of AdoMet cleavage in KAM, we used selenium K-edge x-ray absorption spectroscopy (XAS) in combination with the selenium derivative of AdoMet, Se-adenosyl-L-selenomethionine (AdoSeMet), to follow the course of the cleavage reaction. AdoSeMet is a known substrate for many AdoMet-dependent methylases and AdoMet decarboxylases, and in some cases supports faster turnover than the normal substrate (27-30). The rate of turnover of KAM with the AdoSeMet is more

than half of that with AdoMet, verifying that this is an appropriate analog with which to study the cleavage reaction (23).

Materials and Methods

XAS data were collected at Stanford Synchrotron Radiation Laboratory (SSRL), beamline 7-3, with the SPEAR storage ring operating in a dedicated mode at 3.0 GeV and 60-100 mA. Fluorescence data were collected using a Ge solid state array detector and a Si(220) double-crystal monochromator that was 50% detuned. Calibration was achieved using a Se foil (first inflection, 12658 eV). EXAFS analysis was performed with the EXAFSPAK software (www-ssrl.slac.stanford.edu/exafspak.html), according to standard procedures (31). Fourier transform plots were generated with sulfur-based phase correction. Both Se and Zn XAS data were collected on the same samples and in most cases, duplicate preparations of similar samples were analyzed.

Typical EXAFS samples contained 200 mM sodium EPPS buffer, pH 8.0, 536 μ M KAM (holoenzyme, 3 FeS clusters per hexamer), 1.6 mM AdoSeMet or SeMet, 3.4 mM *trans*-4,5-dehydrolysine or L-lysine. When included, the concentration of 5'-deoxyadenosine was 3.6 mM, and the concentration of sodium dithionite was 2.6 mM. The enzyme was reductively incubated in the absence of iron, desalted by gel filtration, concentrated, and added to a mixture of the other components of the reaction. After 10 min at ambient temperature, the reaction was mixed with an equal volume of anaerobic 50% glycerol, loaded into an XAS cuvet, and frozen in liquid N₂. The concentration of all components of the reaction mixture was therefore diluted by a factor of 2. All steps involving preparation of XAS samples, including freezing, were carried out inside of a Coy anaerobic chamber.

The use of *trans*-4,5-dehydrolysine, *in lieu* of the normal substrate, was essential to generate a sufficient quantity of the intermediate state for successful XAS analysis. Upon abstraction of the 3-*proR* hydrogen by 5'-deoxyadenosine 5'-yl, a stable allylic radical is formed, which is not of sufficient energy to partition backwards by

reabstraction of a hydrogen atom from 5'-deoxyadenosine. The result is that near stoichiometric amounts of the products of AdoMet cleavage are generated (32). In

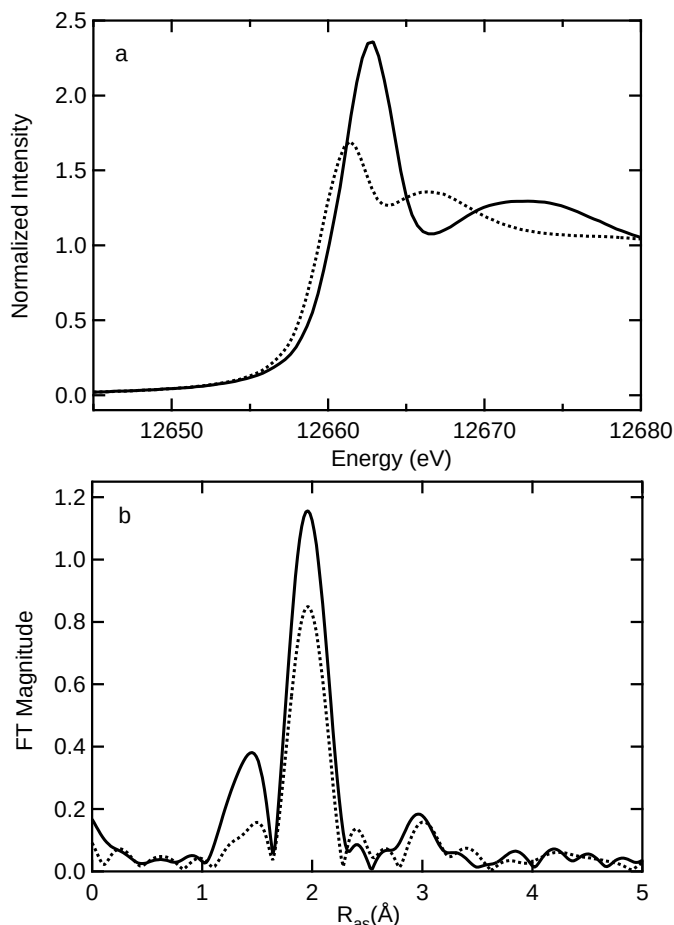


Figure 4.1. Se K-edge x-ray absorption spectra (a) and Fourier transforms (b; over $k = 2\text{--}12.5 \text{ \AA}^{-1}$) of Se-adenosyl-L-selenomethionine (AdoSeMet; solid) and L-selenomethionine (SeMet; dotted).

investigation of SeMet and AdoSeMet reveals the expected change in edge position and a significant change in edge shape (Figure 4.1), making Se edges a diagnostic fingerprint for distinguishing between samples that resemble these two compounds. The shift in absorption edge position between SeMet (12659.6 eV inflection) and AdoSeMet (12661.2 eV) is indicative of a change in the oxidation state of the sample (33).

contrast, with the normal substrate less than 10% of this intermediate accumulates if the reaction is frozen in the steady state. Substantially lesser amounts accumulate as the reaction approaches equilibrium.

Results

XAS spectra were acquired for selenomethionine (SeMet) and AdoSeMet, and then compared with spectra of these molecules bound to KAM under different conditions, and poised at various stages in the catalytic cycle. Se K-edge XAS

Concomitant with this edge change is a reduction in Fourier transform (FT) peak intensity, which is indicative of the lower coordination number for SeMet compared with AdoSeMet. First-shell EXAFS for SeMet are fit best assuming *two* carbon atoms at 1.93 Å (Fits 2,6; Table 4.1), while first-shell EXAFS for AdoSeMet are fit best assuming *three* carbons at 1.94 Å (Fits 9,12; Table 4.1). In the Fourier transforms of both SeMet and AdoSeMet there is a small peak at ca. 3 Å (Figure 4.1b). The EXAFS contribution to this

Table 4.1. Curve fitting results for EXAFS of Se models^a

Sample filename (<i>k</i> range) $\Delta k^3\chi$	Fit	Shell	N_s	R_{as} (Å)	σ_{as}^2 (Å ²)	ΔE_0 (eV)	f^b
Se-methionine	1	Se-C	1	1.94	−0.0017	−0.03	0.100
EMETA (2-12.5 Å ^{−1})	2	Se-C	2	1.93	0.0015	−3.44	0.088
$\Delta k^3\chi = 5.34$	3	Se-C	3	1.92	0.0041	−7.29	0.116
	5	Se-C	2	1.93	0.0015	−4.64	0.086
		Se-C	1	2.89	0.0052		
EMETB (2-12.5 Å ^{−1})	6	Se-C	2	1.93	0.0012	−4.00	0.091
$\Delta k^3\chi = 5.45$	7	Se-C	2	1.93	0.0012	−4.00	0.087
		Se-C	1	2.84	0.0043		
Se-adenosyl-L-seleno-	8	Se-C	2	1.94	0.0003	0.28	0.095
methionine (SeSAM)	9	Se-C	3	1.93	0.0025	−1.97	0.097
ESAMA (2-12.5 Å ^{−1})	10	Se-C	4	1.92	0.0044	−4.95	0.122
$\Delta k^3\chi = 6.45$	11	Se-C	3	1.92	0.0024	−3.69	0.093
		Se-C	1	2.85	0.0021		
ESAMB (2-12.5 Å ^{−1})	12	Se-C	3	1.94	0.0020	−0.73	0.097
$\Delta k^3\chi = 6.32$	13	Se-C	3	1.94	0.0020	−0.85	0.091
		Se-C	1	2.86	0.0011		

^a Group is the chemical unit defined for the multiple scattering calculation. N_s is the number of scatterers (or groups) per metal. R_{as} is the metal-scatterer distance. σ_{as}^2 is a mean square deviation in R_{as} . ΔE_0 is the shift in E_0 , which is the energy at which the EXAFS begin, for the theoretical scattering functions. $\Delta k^3\chi$ is the amplitude of the EXAFS oscillations, which is used to normalize the goodness-of-fit values.

^b f' is a normalized error (chi-squared):

$$f' = \{\sum_i [k^3(\chi^{obs} - \chi^{calc})]^2 / N\}^{1/2} / [(k^3\chi^{obs})_{max} - (k^3\chi^{obs})_{min}].$$

peak can be fit, with reasonable Debye-Waller factor values, assuming a carbon scatterer at 2.8-2.9 Å (Fits 5,7,11,13; Table 4.1).

Incubating KAM with stoichiometric amounts of AdoSeMet with (Figure 4.2, solid) or without dithionite, yields Se edge and FT spectra that are similar to that of

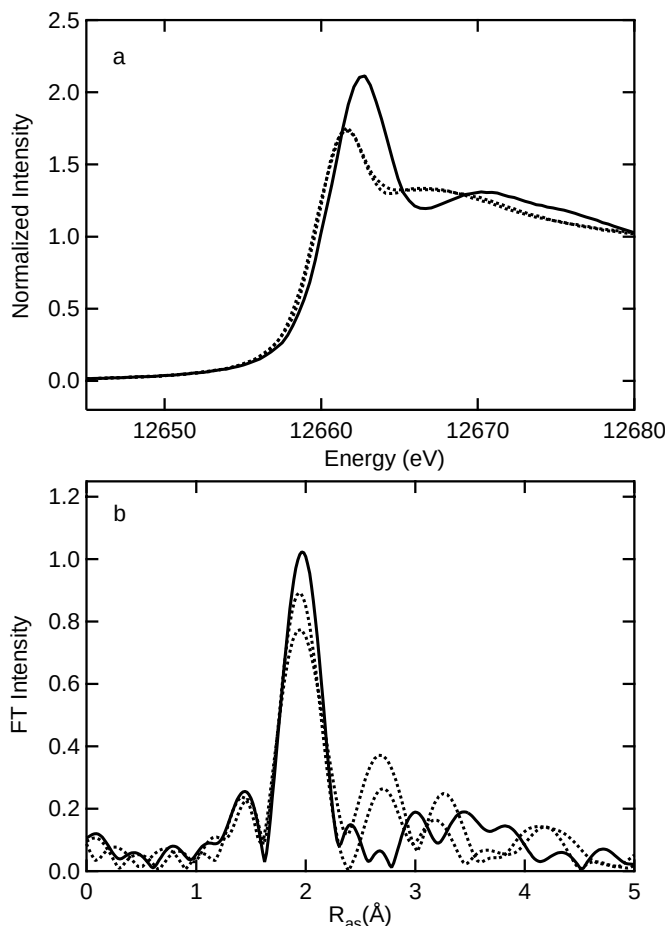


Figure 4.2. Se K-edge x-ray absorption spectra (a) and Fourier transforms (b; over $k = 2-12.5 \text{ Å}^{-1}$) of KAM incubated with AdoSeMet and dithionite (solid), or AdoSeMet, dithionite and *trans*-3,4-dehydrolysine (dotted; duplicate samples).

AdoSeMet alone (Figure 4.1, solid). The EXAFS for this sample are best fit assuming *three* carbon scatterers at 1.93 Å (Fits 2,6; Table 4.2). As with SeMet and AdoSeMet, this spectrum exhibits a peak at ca. 3 Å, which can be fit assuming a single carbon scatterer at 2.88 Å (Fits 5,7; Table 4.2).

In contrast, incubating KAM with AdoSeMet, dithionite, *and* the substrate analog, *trans*-4,5-dehydrolysine, yields a Se edge spectrum that is reminiscent of SeMet (Figure 4.2, dotted). This indicates that AdoSeMet has been cleaved to form SeMet and 5'-deoxyadenosine. Importantly,

the Se environment in this sample differs from free SeMet by the presence of a new, reproducible peak at ca. 2.7 Å in the FT (Figure 4.2b, dotted). The EXAFS for KAM incubated with AdoSeMet, dithionite, and *trans*-4,5-dehydrolysine are best fit assuming *two* carbon scatterers at 1.93 Å (Fits 9,14; Table 4.2). The new ca. 2.7 Å FT peak can be

Table 4.2. Curve fitting results for Se EXAFS of KAM incubated with Se compounds^a

Sample filename (<i>k</i> range) $\Delta k^3\chi$	Fit	Shell	N _S	R _{as} (Å)	σ_{as}^2 (Å ²)	ΔE_0 (eV)	f
KAM + AdoSeMet	1	Se-C	2	1.94	0.0000	0.56	0.104
EL0EA (2-12.5 Å ⁻¹)	2	Se-C	3	1.93	0.0021	-1.33	0.101
$\Delta k^3\chi = 8.03$	3	Se-C	4	1.92	0.0039	-3.96	0.115
	4	Se-C	3	1.93	0.0021	-3.15	0.098
		Se-Fe	1	2.88	0.0168		
	5	Se-C	3	1.93	0.0021	-2.01	0.100
		Se-C	1	2.89	0.0047		
EL0EB (2-12.5 Å ⁻¹)	6	Se-C	3	1.93	0.0012	-2.22	0.092
$\Delta k^3\chi = 10.00$	7	Se-C	3	1.94	0.0013	-1.65	0.089
		Se-C	1	2.92	0.0029		
KAM + AdoSeMet + dith. + dehydrolysine	8	Se-C	1	1.93	-0.0022	-1.65	0.101
	9	Se-C	2	1.93	0.0010	-4.48	0.092
ELAEA (2-12.5 Å ⁻¹)	10	Se-C	3	1.92	0.0033	-7.53	0.102
$\Delta k^3\chi = 8.21$	11	Se-C	2	1.93	0.0010	-5.18	0.086
		Se-Fe	1	2.65	0.0121		
	12	Se-C	2	1.92	0.0010	-5.63	0.093
		Se-C	1	2.97	0.0002		
	13	Se-C	2	1.92	0.0006	-4.89	0.082
		Se-Fe	1	2.67	0.0113		
		Se-C	1	2.94	-0.0007		
ELAEB (2-12.5 Å ⁻¹)	14	Se-C	2	1.93	0.0019	-5.40	0.119
$\Delta k^3\chi = 8.16$	15	Se-C	3	1.92	0.0044	-7.36	0.126
	16	Se-C	2	1.93	0.0020	-4.18	0.099
		Se-Fe	1	2.67	0.0088		
		Se-C	1	2.95	-0.0017		
KAM+SeMet+	17	Se-C	1	1.96	-0.0009	2.20	0.135
5'deoxyadenosine	18	Se-C	2	1.95	0.0025	0.49	0.127
ELSEA (2-12.5 Å ⁻¹)	19	Se-C	3	1.93	0.0054	-5.31	0.142
$\Delta k^3\chi = 6.33$	20	Se-C	2	1.94	0.0025	-2.59	0.126
		Se-Fe	1	2.86	0.0166		
	21	Se-C	2	1.94	0.0025	-1.69	0.124

		Se-C	1	2.88	0.0009		
KAM+ SeMet +	22	Se-C	1	1.92	-0.0020	-5.07	0.132
5'deoxyadenosine +	23	Se-C	2	1.92	0.0011	-6.48	0.127
dehydrolysine	24	Se-C	3	1.92	0.0035	-8.58	0.133
ELQEA (2-12.5 Å ⁻¹)	25	Se-C	2	1.93	0.0010	-4.58	0.101
$\Delta k^3\chi = 8.75$		Se-Fe	1	2.64	0.0059		
	26	Se-C	2	1.91	0.0014	-9.23	0.121
		Se-C	1	2.95	-0.0035		
	27	Se-C	2	1.93	0.0011	-4.62	0.098
		Se-Fe	1	2.64	0.0065		
		Se-C	1	2.94	-0.0005		

^aSee footnotes to Table 4.1.

successfully modeled as a first-row transition metal. Since Zn K-edge XAS shows that the divalent cation site in KAM does not change at any stage of catalysis (data not shown), this peak is interpreted as a selenium-iron interaction with an interatomic distance of 2.67 Å (Fits 11,13,16; Table 4.2). Although inclusion of the 2.9 Å Se-C scatterer does not significantly improve the goodness of fit value, f^2 , this shell is needed as a baseline parameter for subsequent fits. XAS "sees" an average coordination environment for all molecules of a given element in the sample. Thus, Fe XAS would see an average of the four Fe atoms in the Fe₄S₄ cluster and would not be sensitive to the addition of a Se atom at only one Fe.

This intermediate cannot be generated simply by adding SeMet and 5'-deoxyadenosine to the [Fe₄S₄]²⁺ state of KAM (Figure 4.3, solid); however, it is formed if *trans*-4,5-dehydrolysine (Figure 4.3, dotted) or lysine is included (data not shown). The EXAFS for KAM incubated with SeMet and 5'-deoxyadenosine are best fit assuming two carbon atoms at 1.95 Å (Fit 18; Table 4.2). The EXAFS for KAM incubated with SeMet, 5'-deoxyadenosine, and *trans*-4,5-dehydrolysine are best fit assuming two carbon scatterers at 1.93 Å and a first row transition metal at 2.64 Å, which simulates the EXAFS contribution for the new 2.7 Å peak (Fits 25,27; Table 4.2). As with KAM

samples incubated with AdoSeMet, dithionite, and *trans*-3,4-dehydrolysine, this metal is interpreted as an iron atom. This behavior is completely consistent with a true intermediate state rather than adventitious binding, especially since no more than stoichiometric amounts of selenomethionine were used.

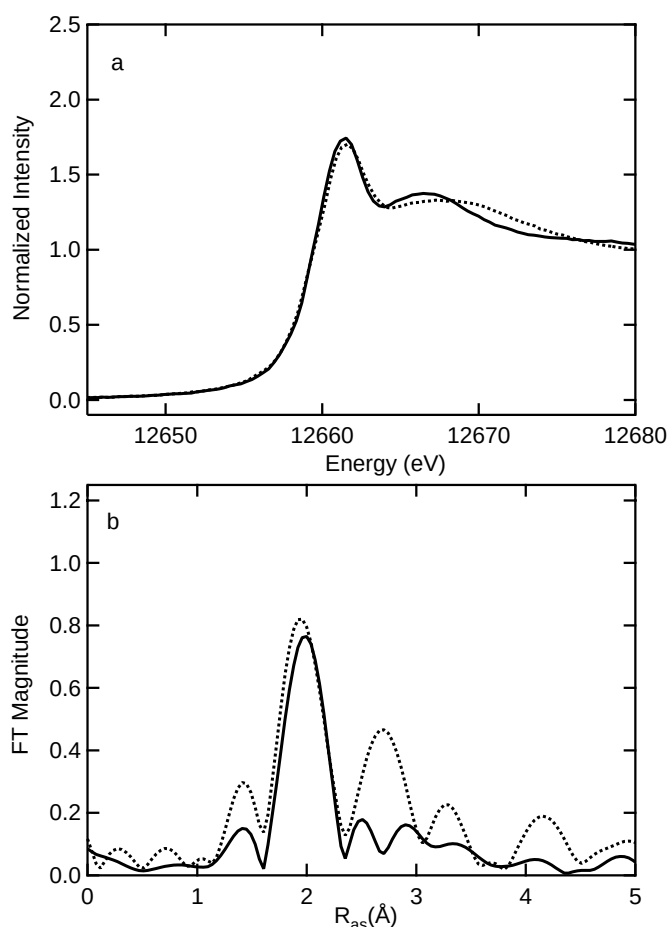


Figure 4.3. Se K-edge x-ray absorption spectra (a) and Fourier transforms (b; over $k = 2\text{--}12.5 \text{ \AA}^{-1}$) of KAM incubated with SeMet and 5'-deoxyadenosine (solid) or SeMet, 5'-deoxyadenosine, and *trans*-3,4-dehydrolysine (dotted).

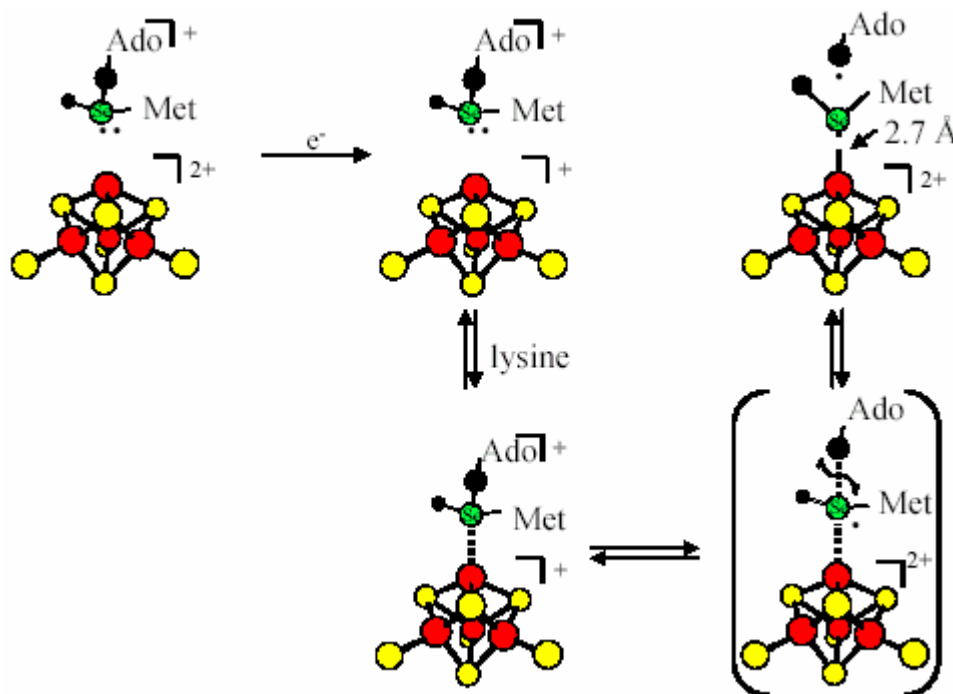
Discussion

The need for lysine or *trans*-4,5-dehydrolysine to effect cleavage of AdoMet and to observe the subsequent interaction is consistent with recent results with S-3',4'-anhydroadenosyl-L-methionine (3'4'-anAdoMet). This AdoMet analog supports turnover, albeit at a highly reduced rate. More importantly, it allows observation of the surrogate 5'-deoxyadenosyl radical via its allylic stabilization.

However, no cleavage of the cofactor is observed unless substrate or substrate analog is present (34). This suggests a

mechanism for cleavage in which substrate binding induces a conformational change that brings the non-bonding electron pair of the sulfonium in proximity to one of the irons of the FeS cluster. This might raise the redox potential of AdoMet to that which would

allow inner-sphere electron transfer from the FeS cluster, with concerted cleavage of the carbon-sulfur bond.



Scheme 3. Proposed mechanism for generation of 5' deoxyadenosyl radical in lysine 2,3-aminomutase. This scheme is shown with an empty coordination site for the top Fe in the cube. Although this coordination site is most likely filled during some stages of catalysis, our XAS data do not provide any evidence for the identity or occupancy of this putative ligand.

The appearance of a FT peak at 2.7 Å, which is explained as a selenium-iron interaction, concomitant with the change in edge position and reduction in intensity of the main FT peak, indicates that AdoSeMet is cleaved to SeMet, which associates with the FeS cluster. This suggests that the mechanism for generation of the 5'-deoxyadenosyl radical in KAM involves iron-based chemistry (Scheme 4.3) and renders an intermediate involving an iron-carbon bond unlikely. The interaction of selenomethionine to the iron-sulfur cluster suggests a unique Fe site in the Fe₄S₄ cluster. This is consistent with previous electron paramagnetic resonance (EPR) spectroscopic studies on KAM, in which nearly stoichiometric amounts of [Fe₃S₄]⁺¹ clusters were generated when the enzyme was treated with oxygen or ferricyanide (8). This behavior is reminiscent of

aconitase, which is known to cycle between Fe_3S_4 and Fe_4S_4 clusters, with loss of the iron that has a water or hydroxide ligand in place of a protein-derived cysteine ligand.

KAM is distinct within this class of AdoMet-dependent enzymes in that the cleavage of the cofactor is freely reversible. We postulate that the interaction of methionine with the FeS cluster might aid not only in cleaving the cofactor, but also in maintaining the methionine in place for the back reaction, and influencing the energetics of this process.

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CHAPTER 5

STRUCTURAL CONSERVATION OF THE ISOLATED ZINC SITE IN ARCHAEAL ZINC-CONTAINING FERREDOXINS AS REVEALED BY X-RAY ABSORPTION SPECTROSCOPIC ANALYSIS AND ITS EVOLUTIONARY IMPLICATIONS¹

¹ Cosper, N. J., C. M. V. Stålhandske, H. Iwasaki, T. Oshima, R. A. Scott, and T. Iwasaki. 1999. *Journal of Biological Chemistry*. 274:23160-23168. Reprinted here with permission of publisher.

Abstract

The *zfx* gene encoding a zinc-containing ferredoxin from *Thermoplasma acidophilum* strain HO-62 was cloned and sequenced. It is located upstream of two genes encoding an archaeal homolog of nascent polypeptide-associated complex α subunit and a tRNA nucleotidyltransferase. This gene organization is not conserved in several euryarchaeotal genomes. The multiple sequence alignments of the *zfx* gene product suggest significant sequence similarity of the ferredoxin core fold to that of a low-potential 8Fe-containing dicluster ferredoxin without a zinc center. The tightly bound zinc site of zinc-containing ferredoxins from two phylogenetically distantly related Archaea, *T. acidophilum* HO-62 and *Sulfolobus* sp. strain 7, was further investigated by X-ray absorption spectroscopy. The Zn K-edge X-ray absorption spectra of both archaeal ferredoxins are strikingly similar. The EXAFS are best fit assuming a coordination environment of $\text{Zn}(\text{imid})_{3,4}(\text{COO}^-)$. The Zn-N and Zn-O bond distances (2.01 and 1.90 Å, respectively) obtained are in agreement with the crystallographically derived distances found in the 6Fe form of *Sulfolobus* sp. ferredoxin (1.96 and 1.90 Å, respectively) [T. Fujii, Y. Hata, T. Wakagi, N. Tanaka, and T. Oshima, *Nat. Struct. Biol.* 3:834-837, 1996]. Thus the X-ray absorption spectroscopic results show that the same zinc site is found in *T. acidophilum* ferredoxin as in *Sulfolobus* sp. ferredoxin, suggesting the structural conservation of isolated zinc binding sites among archaeal zinc-containing ferredoxins. The sequence and spectroscopic data provide the common structural features of the archaeal zinc-containing ferredoxin family.

Introduction

The archaeal domain contains organisms having the most extraordinary optimal growth conditions, with members flourishing at the extremes of pH, temperature and salinity. As oxygen is often scarce in these conditions, the majority of Archaea are anaerobic organisms (1-3). For the more unusual aerobic Archaea, one of the characteristic features in the central metabolic pathways is the involvement in electron transport of small iron-sulfur (FeS) proteins called ferredoxins. Ferredoxins take the place of NAD(P)⁺, a typical electron carrier in Bacteria and Eucarya (4-7). The physiological significance of bacterial-type ferredoxins in several aerobic and thermoacidophilic Archaea was first recognized by Kerscher *et al*, when it was demonstrated that ferredoxins are an effective electron acceptor of a coenzyme A-acylating 2-oxoacid:ferredoxin oxidoreductase (8), which is a key enzyme of the tricarboxylic acid cycle and of coenzyme A-dependent pyruvate oxidation in aerobic Archaea (5-7,9).

The primary structures of archaeal ferredoxins differ from those of regular bacterial-type monocluster and dicluster ferredoxins in that they contain a central loop region and an N-terminal extension, composed of three β -strands and one α -helix (10-14). An unexpected result from recent X-ray structural analysis of the ferredoxin from the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7 (optimal growth conditions, pH 2.5-3.0 and 80° C (5,15)) was that four amino acid residues in the extra regions (His¹⁶, His¹⁹, His³⁴ and Asp⁷⁶) serve as ligands to a tetragonally coordinated, novel zinc center (16). This isolated center is buried within the molecule and connects the two FeS cores and the N-terminal extension region.

The thermoacidophilic euryarchaeote, *Thermoplasma acidophilum*, represents one of the longest evolutionary lineages, within the euryarchaeota, of the archaeal domain, and uniquely lacks the S-layer (2,17-19). Unlike methanogenic euryarchaeotes, it is a facultative aerobic thermoacidophile that grows optimally at pH 1-2 and 56-59° C. Several new isolates of *T. acidophilum* have been obtained from hot sulfur springs at the

Ohwakudani solfataric field in Hakone, Japan, and marked morphological variations among different isolates were recognized (20). Although the energy metabolism of this euryarchaeote has not been studied in detail, preliminary studies have suggested that *T. acidophilum* contains at least two major redox systems, one being the cytosolic ferredoxin-dependent redox system for saccharolytic and peptide fermentation (8,14) and the other being the membrane-bound aerobic respiratory chain containing multiple *b*- and *d*-type cytochromes (21) (T. Iwasaki, unpublished results). The pioneering work by Kerscher and coworkers (8) has shown that *T. acidophilum* strain DSM 1728 contains a bacterial-type ferredoxin functioning as an electron acceptor of the cognate 7-oxoacid:ferredoxin oxidoreductase. The amino acid sequence of this ferredoxin was previously determined by Edman degradation of proteolytically generated peptides (10).

Recently, we purified the functionally equivalent ferredoxin from *T. acidophilum* strain HO-62 (20). Through chemical analysis, electron paramagnetic resonance (EPR) and low-temperature resonance Raman spectroscopy, it was demonstrated that the ferredoxin contains one $[3\text{Fe-4S}]^{1+,0}$ cluster, one $[4\text{Fe-4S}]^{2+,1+}$ cluster, and one tightly bound zinc center (14), thus indicating the existence of "zinc-containing ferredoxins" among phylogenetically diverse members of several thermoacidophilic Archaea (14). Although the presence of a tightly bound zinc center is one of the most unique properties of the archaeal zinc-containing ferredoxins, the structural details of the zinc site have been characterized only for ferredoxin from *Sulfolobus* sp. strain 7, which was analyzed by X-ray diffraction (16,20).

X-ray absorption spectroscopy (XAS) is ideally suited for the investigation of the metric structural environment of specific metal sites in biomolecules (22). The edge region provides information concerning the electronic environment of the absorbing atom, while the extended X-ray absorption fine structure (EXAFS) region provides structural information concerning the number, type, and average distance of atoms in close proximity to the metal site. Hence, we report the XAS analysis of zinc-containing ferredoxins from these two phylogenetically distantly related Archaea, *Thermoplasma*

acidophilum strain HO-62 and *Sulfolobus* sp. strain 7 (6,14,15), to characterize the structural properties of the zinc and iron coordination environments. We also report cloning and sequencing of the *zfx* gene encoding zinc-containing ferredoxin of *T. acidophilum* strain HO-62 (*zfx* for Zinc-containing FerredoXin) and its flanking regions, to clarify its gene organization and the distribution of zinc-containing ferredoxin homologs in thermophilic organisms. The gene sequence and spectroscopic data provide the basis for comparison of the structural features among the archaeal zinc-containing ferredoxin family.

Materials and Methods

DEAE-Sephacel and Sephadex G-50 were purchased from Pharmacia LKB Biotechnology Inc. Water was purified by the Milli-Q purification system (Millipore). Other chemicals used in this study were purchased commercially and were of analytical grade.

Thermoplasma acidophilum strain HO-62 cells, originally isolated from hot sulfur springs at Ohwakudani solfataric field in Hakone, Japan, were routinely cultivated at pH 1.8 and at 56 °C in 10- and 30-liter acid-resistant fermenters as described by Yasuda *et al.* (20), and zinc-containing ferredoxin was purified as described previously (14). *Sulfolobus* sp. strain 7 cells, originally isolated from Beppu hot springs, Japan, were cultivated aerobically and chemoheterotrophically at pH 2.5-3 and 75-80 °C (23), and the 7Fe form of the cognate ferredoxin was purified as described previously (6,15).

Escherichia coli strain DH5 α , used for cloning, was grown in LB or TB medium, with 50 μ g/ml ampicillin when required. Plasmids pGEMT and pGEM3Zf(+) (Promega) were used for cloning and sequencing. DNA was manipulated by standard procedures (24).

The N-terminal 15 amino acid residues of *T. acidophilum* HO-62 ferredoxin (VKLEELDFKPKPIDE) (14) have been confirmed in the previous work to be identical to the amino acid sequence of a different strain (DSM 1728) of *T. acidophilum*

determined by Edman degradation of proteolytically generated peptides (accession number P00218) (10). A DNA fragment encoding the *zfx* gene was obtained by PCR from template genomic DNA of *T. acidophilum* strain HO-62, using the following two oligonucleotide primers: TFP1 (corresponding to the N-terminal KPKPIDEH sequence (10,14)), 5'-AA(AG) CC(AGCT) AA(AG) CC(AGCT) AT(ACT) GA(CT) GA(AG) CA(TC) TT-3', and TFP2 (corresponding to the DCIFCMAC sequence at the cluster-binding site (10)), 5'-TC(AG)CA(ACGT) GCC AT(AG) CA(AG) AA(GAT) AT(AG) CA(AG) TC-3'. The resultant PCR product with expected length (~370 bp) was amplified, subcloned into pGEMT vector, and sequenced with the vector-specific T7 and SP6 primers. PCR was then performed using a set of the TFP1/TFP2 and SP6/T7 primers, on a template genomic library generated by the ligation of *Bam*HI-digested *T. acidophilum* genomic DNA and pGEM3Zf(+). The resultant PCR products were size-fractionated on an agarose gel, extracted, subcloned into pGEMT vectors, and sequenced with primers designed from nucleotide sequence of the initial genomic PCR product. Finally, a genomic fragment was amplified using PCR primers corresponding to the 5'- and 3'-untranslated regions resulting in an intact *zfx* gene.

The sequence determination was performed by Sanger dideoxy sequencing with an automated DNA sequencer, ABI model 373A (Applied Biosystems Inc.). The DNA sequence was processed using the DNASIS ver. 3.6 software (Hitachi Software Engineering Co., Ltd). Database searches were performed with BEAUTY and BLAST network services (25). Multiple sequence alignments were performed using a CLUSTAL X graphical interface (26) followed by small manual adjustment.

Purified zinc-containing ferredoxins in 20 mM potassium phosphate buffer, pH 6.8, were concentrated by pressure filtration with an Amicon YM-3 membrane. Further concentration was achieved by placing the samples under a stream of dry nitrogen gas. The resultant samples (~2-3 mM), containing 30% (v/v) glycerol, were frozen in a 24x3x2 mm polycarbonate cuvet with a Mylar-tape front window for XAS studies.

XAS data were collected at Stanford Synchrotron Radiation Laboratory (SSRL) with the SPEAR storage ring operating in a dedicated mode at 3.0 GeV (Table 1). EXAFS analysis was performed with the EXAFSPAK software (courtesy of G. N. George; www-ssrl.slac.stanford.edu/exafspak.html) according to standard procedures (22). Curve-fitting analysis was performed as described previously (27). Multiple scattering models, calculated using Feff v7.02 (28), were based on bis(acetato)-bis(imidazole)-zinc(II) (29) or tetra(imidazole) zinc(II) perchlorate (30).

Table 5.1. X-ray absorption spectroscopic data collection for Fe and Zn analysis.

	Fe EXAFS	Zn EXAFS
SR facility	SSRL	SSRL
beamline	7-3	7-3
current in storage ring	80-100 mA	50-60 mA
monochromator crystal	Si[220]	Si[220]
detection method	fluorescence	fluorescence
detector type	solid state array ^a	solid state array
scan length, min	28	25
scans in average	16	10
temperature, K	10	10
energy standard	Fe foil, 1 st inflection	Zn foil, 1 st inflection
energy calibration, eV	7111.3	9660.7
E ₀ , eV	7120	9670
pre-edge background		
energy range, eV	6789-7075	8657-9625
Gaussian center, eV	6403	8638
Width, eV	750	750
spline background		
energy range, eV	7120-7354 (4)	9333-9902 (4)
(polynomial order)	7354-7589 (4)	9902-10134 (4)
	7589-7822 (4)	10134-10366

(4)

^aThe 13-element Ge solid-state X-ray fluorescence detector at SSRL is provided by the NIH Biotechnology Research Resource.

Absorption spectra were recorded with a Hitachi U-3210 spectrophotometer equipped with a thermoelectric cell holder. Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry of purified apoferredoxin (made in distilled water) was performed by a Finnigan MAT VISION 2000 instrument at an accelerating potential of 5.0 kV, using a 2,5-dihydroxybenzoic acid matrix. EPR measurements were performed using a JEOL JEX-RE1X spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system and a Scientific Instruments series

5500 temperature indicator/controller. The spectral data were processed using KaleidaGraph v3.05 (Abelbeck Software).

Results and Discussion

Sequence analysis of the *zfx* gene and flanking regions. Previous studies have shown that a ferredoxin purified from the moderately thermoacidophilic euryarchaeote *T. acidophilum* strain HO-62 is a zinc-containing ferredoxin with one [3Fe-4S] cluster, one [4Fe-4S] cluster, and one zinc center (14). It is constitutively expressed as the predominant ferredoxin in cells grown chemoheterotrophically, and could be obtained regardless of the different growth phases; even where small changes in compositions of the membrane-bound cytochromes could be detected (data not shown).

The *zfx* gene utilizes a translational start codon, GTG (positions 121-123, Fig. 1), and the corresponding valine residue is absent in zinc-containing ferredoxin isolated from the *T. acidophilum* HO-62 cells (Fig. 1), indicating post-translational modification. The single open reading frame encodes a protein with a deduced molecular mass of 15,955 Da (excluding the initial residue), which is in agreement with the average mass $[M + H]^{1+}$ of 15,961 Da (estimated error, ± 10 Da) for purified apoferredoxin by MALDI-TOF mass spectrometry. The *zfx* gene sequence predicts an amino acid sequence containing the three consensus histidine residues, His³⁰, His³³, and His⁵⁷, and a remote Asp¹¹⁶ (doubly-underlined in Fig. 1A). The equivalent residues in *Sulfolobus* sp. ferredoxin (Fig. 2) serve as ligands to the isolated zinc center (14). The deduced amino acid sequence is essentially identical to the reported sequence of *T. acidophilum* DSM 1728 ferredoxin determined by Edman degradation of proteolytically generated peptides (accession number P00218) (10). The two discrepancies, Glu¹⁰¹ and Ala¹⁰⁵, located in the central loop region (underlined residues in Fig. 1), most likely reflects the difference in strains used (strain HO-62 versus DSM 1728).

Similarity searches against available databases (GenEMBL, PIR, and SWISS-PROT) indicate a high sequence homology of the *zfx*-gene product with other zinc-

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1                                     GGATCCACAGTAGAAGAATA      20
21                                     BoxA      RBS
CGTAAATGTTCCATCTTAATAGGGAATTTCCAAAAAATTTCTGAAGGAGTTGCGTATAGTTTAAATAAAAAATATAAAATATCTGTGCGGTGAGTGTATT 120
zfx ->
121 GTGGTTAAGCTAGAGGAGCTTGATTTCAGGCCAAAGCCCATAGATGAGCATTTTCTTGAGAACGACAAGGACTATCCCGTGACCGGTGAGCACAATGGCC 220
1 (V) V K L E E L D F K P K P I D E H F L E N D K D Y P V T G Q H N G      33
221 ATGATGTAAGGGCCGAGGGAATGCAGAGGCTGGAGCCCGATGAAAAACCTATCCACAAAGCTGGGCATACATGGTACCCATGTTGGGTTGACTGGGA 320
34 H D V R A E G M Q R L D A D G K P Y P T K L G I H G T H V A V D W D      67
321 CTGCTGCATTGCAGATGGTGCATGCATGGATGTCTGCCCTGTCAATCTCTATGAGTGGAACTTAATCCCGAAAAATCAGGCACGGGAAACGATCACAAG 420
68 C C I A D G A C M D V C P V N L Y E W N L N P G K S G T G N D H K      100
421 ATAGAAAAGGCGAGCGGAGTGGAAACAAGTACAGGACAGACAAATGTGATCCTGTCCGTGAATCGGACTGCATATTCTGCATGGCTTGTGAGAGCGTTT 520
101 I E K G S A E W N K Y R T D K C H P V R E S D C I F C M A C E S V      133
521 GCCCTGTGAGAGCAATAAAGATAACTCCGtaaatATATCAAATCCATTTTATATATATATCTGTTCATTCGCAGTCATGGTTTTTGTGATGCATTTTCGCACA 620
134 C P V R A I K I T P end term      143
orfl ->
621 ATAAAAACTCAACTTTATTTACGATTTAATGATCAGTGCATAAATGATACCCGGCAGGATGAACCTCAAGGGAGATGCCCGGCTGATGCCGAGATGGGC 720
M I S A K M I P G R M N S R E M R R L M A Q M G
721 ATAAAGTCCACAGAGATGGACGATGTACGAAGGTAATTAITCAAGGGAAGACAAAGGATTACGTTATCGACAACGCCAGGTACGATGATCGAAGCAC 820
I K S T E M D D V T K V I F K G K T K D Y V I D N A Q V T M I E A
821 AGGCGGTGAAGACCTTTTCAGGTTGTGCGTACGATGAGGGAAGTCCCAAGGAGCCAGAAGAGAAGGAAGAAATCCCTCCAGAGGATGACATAAAGCT 920
Q G V K T F Q V V G T M R E V P K E P E E K K E E S F P E D D I K L
921 TGTATGATGATCAGGCAAGCGTTTCCAGGGAAAAGCGATAGAGGCACTGAAAGCGCTCTGGCGGAGAGCCTGCTCAGGCCATAATAAACCTTTATCGAGAA 1020
V M D Q A S V S R E K A I E A L K A S G G E P A Q A I I N L M Q K
ca ->
1021 tgaTAGATTATCAGGAAGTACTAAGCAGATACAGGCCAACGCAAGGAAGAAATAGGCTGAGGATCATATCAGATGATATTATCCGAAAGATAAACAG 1120
end *
M I D Y Q E V L S R Y R P T Q E E E N R L R I I S D D I I R K I N S
1121 CATCTGCAGATCAAGAAGGCTCAGGCCGAAGCCGTTATAGTAGGATCATATGCAAAGGCGACGAATCTCAGGGACGGGGATCTGGATATATTATCATAGCA 1220
I C R S R R L R A E A V I V G S Y A K G T N L R D G D L D I F I A
1221 TTCACCGCGATTTATCCCGAGGAAATATCAATACGGAGGGGCTCCACATAGGTCACGCCGTGATACCCCAATGGAAGGAGAAATACGCAGAGCATCCCT 1320
F D R D Y P E E I I N T E G L H I G H A V I P N G R E K Y A E H P
1321 ATGTCAGCGGCGAGATTGGCGGTGTGAAGATCGATGTGTCTCTGTACAAAGATGAGCTTCGGCGACAGAAAGATAAGCGCAGTTGACAGGACGCTGCT 1420
Y V S G E I G G V K I D V V P C Y K M S F G D R K I S A V D R T L L
1421 TCATACCGAATACGTGAACGGGCATCTCGACGAAAAAGCGGATGAAGTCAGGCTGCTGAAGATATTACGAAATCTATAGGGGTATACGGAGCTGAG 1520
H T E Y V N G H L D E K G R D E V R L L K I F T K S I G V Y G A E
1521 GCCAGGACATTTGGTTTTTCTGGATACCTGTGCGAGCTTCTGGTATCAGGTTCCGAAGTTTCGAAAAACGTCATAAGGTACTTTTCAAAGGCCAAGGGGA 1620
A R T F G F S G Y L C E L L V I R F R S F E N V I R Y F S K A K G
1621 GAGTTCTCATAGACCCGTGATGAACGCTTCCGTGATCCGATGCTCCTAATAGATCCCGTGGATCC 1684
R V L I D P D E R F R D P M L L I D P V D ...

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Figure 5.1 Nucleotide sequence and derived amino acid sequence of the 1684-bp BamHI-digested DNA fragment containing the *zfx* and *orfl* genes and a part of the *cca* gene pf *T. acidophilum* strain HO-62. Underlined nucleic acids represent the putative Box A, ribosome binding site (RBS), and terminating structures (term). The stop codon is over- and underlined. The predicted amino acid sequence is shown below the nucleotide sequence in the one letter code. Amino acid residues are numbered beginning with the valine, the putative first amino acid residue of the translation product that is removed post-translationally. Underlined residues were previously determined by N-terminal sequencing (ref 14). The probable ligand residues to an isolated zinc center of ZFX (dotted and underlined residues), and those to the two FeS clusters (dotted) are illustrated. Two other cysteine residues conserved in the *zfx* gene product are also shown (bold residues). The 3' half of the *cca* gene, which is not included in the 1684-bp BamHI-digested DNA fragment, was not sequenced in this study.



Figure 5.2. Multiple amino acid sequence alignments of selected bacterial-type ferredoxins of *Archaea* and bacteria. Conserved amino acid residues (shaded) and potential ligand residues to the isolated zinc center in archeal zinc-containing ferredoxins (open circles) are shown. Zinc-containing ferredoxins are shaded and PsAC homologs are boxed. The amino acid sequences used are: T.acid.HO-62 (*T. acidophilum* HO-62 zinc-containing ferredoxin), this work; T.acid.DSM1728 (*T. acidophilum* DSM1728 zinc-containing ferredoxin), P00218; Sul.sp.7 (*Sulfolobus* sp strain 7 zinc-containing ferredoxin), O32423; S.acidocaldarius (*S. acidocaldarius* zinc-containing ferredoxin), P00219; D.ambivalens (*Acidianus (Desulfurolobus) ambivalens* N-terminal partial amino acid sequence of probable zinc-containing ferredoxin), P49949; Des.africanus_III (*Desulfovibrio africanus* 7Fe ferredoxin (ferredoxin III)), P08812; Des.vulgaris_I (*Desulfovibrio vulgaris* 7Fe ferredoxin (ferredoxin I)), Q46600; C.pasteurianum (*Clostridium pasteurianum* 8Fe ferredoxin), M11214; Mc.jannaschi_MJ1302 (hyperthermophilic *Methanococcus jannaschii* PsAC isolog), Q58698 (MJ1302); S.elongatus_psaC (thermophilic cyanobacterium *Synechococcus elongatus* Naegeli photosystem I FeS protein PsAC), P18083; C.paradoxa_psaC (cyanelle *Cyanophora paradoxa* photosystem I FeS protein PsAC) U30821; P.furiosus (hyperthermophilic *Pyrococcus furiosus* 4Fe ferredoxin), X79502; T.maritima (hyperthermophilic *Thermotoga maritima* 4Fe ferredoxin), P46797; and D.gigas_II (mesophilic *Desulfovibrio gigas* 3Fe ferredoxin (ferredoxin II)), P00209.

containing ferredoxins of several fast-clock crenarchaeotes (*Sulfolobales*, Fig. 2), which are distantly related to the euryarchaeote *T. acidophilum* on the basis of the universal 16S rRNA sequence tree (2,3,19). On the other hand, no *zfx* gene homolog with the consensus N-terminal extension sequence could be identified in the genomes of hyperthermophilic euryarchaeotes such as *Methanococcus jannaschii* (31), *Methanobacterium thermoautotrophicum* (32), *Pyrococcus horikoshii* (*shinkaj*) (33), *Archaeoglobus fulgidus* (34), and a hyperthermophilic bacterium *Aquifex aeolicus* (35) by either amino acid or nucleotide sequence similarity searches (data not shown). Clearly, distribution of zinc-containing ferredoxins in hyperthermophilic and extremely thermophilic organisms is limited even in the archaeal domain.

A promoter-like element (box A) (36) was found immediately upstream of the *zfx* gene at positions 81-86 (Fig. 1), and a putative ribosome binding sequence (RBS) (5'-GGTGAG-3') complementary to the 3' end of the 16S rRNA (19) at positions 109-114 (underlined in Fig. 1). Because the *zfx* gene product is abundantly produced in *T. acidophilum* (8,14), the proximal promoter region of the *zfx* gene might be useful to express a foreign gene efficiently in this euryarchaeote. A T-rich terminator-like element (37) was found shortly after the stop codon at positions 565-573 (underlined in Fig. 1). Apparently, the *zfx* gene of *T. acidophilum* strain HO-62 does not have an operonic structure.

Two other open reading frames were found shortly after the *zfx* gene (Fig. 1). The first structural gene, *orf1*, encodes a 13.9 kDa protein with a relatively high methionine content in the N-terminal region. The Orf1 protein is strictly conserved in several thermophilic Archaea (as unknown ORF in Refs. (31-35)), and has a domain weakly homologous to that of yeast GAL4 enhancer protein, EGD2, and mammalian nascent polypeptide-associated complex α subunit (α -NAC) (38-42) (Fig. 3A). Mammalian α -NAC is a constituent of the heterodimeric nascent polypeptide-associated complex, whose heterodimerization partner has been identified as the transcription factor BTF3b (38), and has been suggested to serve as a transcriptional coactivator (41). Nascent polypeptide-associated complex is involved in ensuring signal-sequence-specific protein sorting and translocation, and is proposed to contribute to the fidelity of the recognition by modulating interactions that occur between the ribosome-nascent chain complex, the signal recognition particle and the endoplasmic reticulum membrane (38-40,42-44). The similarity of the Orf1 protein of *T. acidophilum* to eucaryal α -NAC homologs suggests that the archaeal protein might also serve as a putative transcriptional coactivator.

The second gene, *cca*, was found immediately downstream of *orf1*, and was partially sequenced in this study (Fig. 1). It predicts the N-terminal half of a *T. acidophilum* homolog of class I tRNA nucleotidyltransferase (Fig. 3B), which repairs the 3'-terminal CCA sequence of all tRNAs (45,46). Interestingly, the archaeal tRNA

nucleotidyltransferases are similar to eucaryal poly(A) polymerases and DNA polymerase β , but distantly related to either the bacterial or eucaryal CCA-adding enzymes (45-47). The unique feature of the *cca* gene of *T. acidophilum* is its one-base pair overlap with the *orf1* gene, implying an operonic structure; this gene organization is not observed for other hyperthermophilic euryarchaeotes with known genome sequences (31-34) (data not shown). The two structural genes downstream of the *zfx* gene are likely involved in translation or tRNA modification system, and apparently unrelated to the *zfx* gene, which is involved in cytoplasmic electron transport.

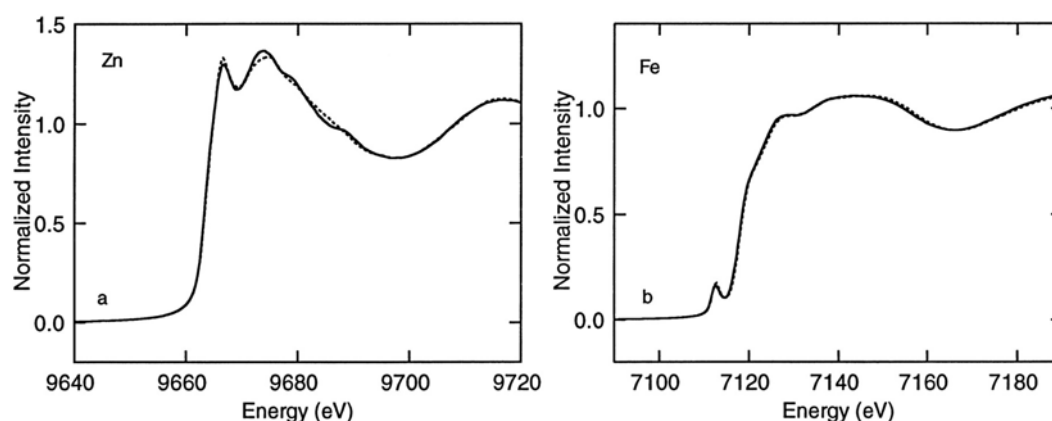


Figure 5.4. Fe (a) and Zn (b) X-ray absorption spectra of ferredoxin from *T. acidophilum* (solid line) and *Sulfolobus* sp. (dotted line).

Zn K-edge XAS analysis. The Zn K-edge X-ray absorption spectra of the 7Fe form of zinc-containing ferredoxins purified from the two phylogenetically distantly related Archaea, *T. acidophilum* strain HO-62 and *Sulfolobus* sp. strain 7, are very similar (Fig. 4a). The absorption edge position (9663.3 for *T. acidophilum*; 9663.2 for *Sulfolobus* sp) for both samples fall at the expected energy for Zn(II) with all light elements (nitrogen or oxygen) in the coordination sphere (48,49). Edge position energies were calculated by determining the maxima of the first derivative of the absorption edge. The intensity of the edge is most reminiscent of four-coordinate compounds and the peak area of the second XANES peak is not as intense as expected for tetra-imidazole coordination, nor is it as weak as seen in a ZnO_4 compound (48).

Curve-fitting analyses of Zn EXAFS of each of the two archaeal zinc-containing ferredoxins suggest the presence of three or four imidazoles. However, such

$\text{Zn}(\text{imid})_{3,4}(\text{N},\text{O})_1$ fits simulate FT peaks of about the same height at 3 and 4 Å, while the observed data have a much larger FT peak at 4 Å (Fig. 5). This suggests that some other scatterer interferes destructively with the ~ 3 -Å imidazole contribution, resulting in an absence of FT intensity. This interference can be modeled with a carboxylate group, in which the average Zn-N and Zn-O bond distances are 2.01 Å and 1.90 Å, respectively. The data were modeled with a Zn-O-C angle of either $\sim 180^\circ$ (data not shown) or $\sim 126^\circ$ (Fits 3,4 & 7,8, Table 2), the two most common conformations found for Zn-carboxylate coordination in the Cambridge Structural Database. The latter provides better fits of the data. Thus, the Zn K-edge EXAFS spectra of both archaeal zinc-containing ferredoxins

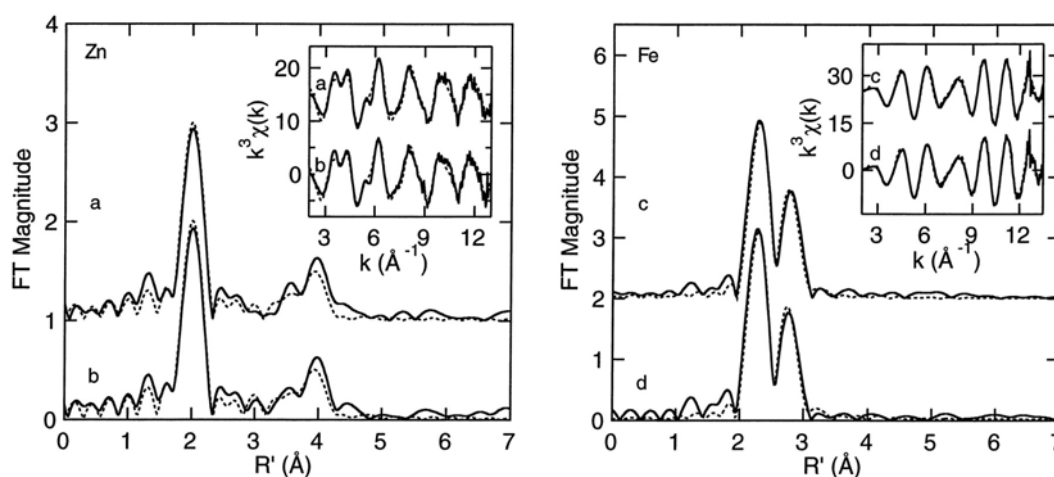


Figure 5.5. k^3 -weighted Zn EXAFS (left, inset) and Fourier transforms (left, over $k = 2$ -13 Å⁻¹) of ferredoxin from (a) *T. acidophilum* (solid line) and the predicted results for $\text{Zn}(\text{imid})_4(\text{COO}^-)$ (dashed line; Fit 8, Table 2), and (b) *Sulfolobus* (solid line) and the predicted results for $\text{Zn}(\text{imid})_4(\text{COO}^-)$ (dashed line; Fit 4, Table 2). k^3 -weighted Fe EXAFS (right, inset) and Fourier transforms (right, over $k = 2$ -13.5 Å⁻¹) of ferredoxin from (c) *T. acidophilum* (solid line) and the predicted results for FeS_4Fe_2 (dashed line; Fit 11, Table 2), and (d) *Sulfolobus* (solid line) and the predicted results for FeS_4Fe_2 (dashed line; Fit 9, Table 2).

can be best fit assuming a $\text{Zn}(\text{imid})_{3,4}(\text{COO}^-)_1$ coordination environment (Fig. 5). The number of imidazoles from this analysis is not absolute and probably depends on the exact geometry enforced on the carboxylate ligand. The Zn XAS results clearly show that the zinc site found in the *zfx* gene product of *T. acidophilum* strain HO-62 is very similar to that of *Sulfolobus* sp. ferredoxin. The XAS-determined bond distances and bond angles are also in agreement with the crystallographically determined Zn-N and Zn-O bond distances (1.96 Å and 1.90 Å, respectively) and Zn-O-C angle ($\sim 126^\circ$) (16).

Table 5.2. EXAFS curve fitting results^a

Sample filename (<i>k</i> range)	Fit	Shell	R _{as} (Å)	σ _{as} ² (Å ²)	f	Sample filename (<i>k</i> range)	Fit	Shell	R _{as} (Å)	σ _{as} ² (Å ²)	f
? <i>k</i> ³ χ						? <i>k</i> ³ χ					
<i>Sulfolobus</i> Zn-ferredoxin ZSFDA (2-13 Å ⁻¹) Δ <i>k</i> ³ χ = 13.05	1	4 Zn-(N/O)	2.00	0.0016	0.085	<i>Th. acido</i> Zn-ferredoxin ZTFBA (2-13 Å ⁻¹) Δ <i>k</i> ³ χ = 13.07	5	4 Zn-(N/O)	1.99	0.0015	0.089
		3 Zn-C	2.99	0.0054				3 Zn-C	2.96	0.0074	
		3 Zn-C	[3.05]	[0.0054]				3 Zn-C	[3.02]	[0.0074]	
		3 Zn-C	[4.14]	[0.0068]				3 Zn-C	[4.10]	[0.0093]	
		3 Zn-N	[4.18]	[0.0068]				3 Zn-N	[4.14]	[0.0093]	
	2	5 Zn-(N/O)	1.99	0.0027	0.083		6	5 Zn-(N/O)	1.99	0.0027	0.086
		3 Zn-C	2.98	0.0053				3 Zn-C	2.97	0.0073	
		3 Zn-C	[3.05]	[0.0053]				3 Zn-C	[3.04]	[0.0073]	
		3 Zn-C	[4.13]	[0.0066]				3 Zn-C	[4.12]	[0.0091]	
		3 Zn-N	[4.18]	[0.0066]				3 Zn-N	[4.17]	[0.0091]	
	3	1 Zn-O	1.91	0.0007	0.069		7	1 Zn-O	1.91	0.0002	0.070
		1 Zn-C	[2.82]	[0.0010]				1 Zn-C	[2.82]	[0.0030]	
		1 Zn-O	[3.08]	[0.0011]				1 Zn-O	[3.08]	[0.0032]	
		3 Zn-(N/O)	2.01	-0.0002				3 Zn-(N/O)	2.01	-0.0001	
		3 Zn-C	2.94	-0.0002				3 Zn-C	2.93	0.0001	
		3 Zn-C	[3.07]	[-0.0003]				3 Zn-C	[3.07]	[0.0002]	
		3 Zn-C	[4.10]	[-0.0004]				3 Zn-C	[4.10]	[0.0003]	
		3 Zn-N	[4.18]	[-0.0004]				3 Zn-N	[4.18]	[0.0003]	
	4	1 Zn-O	1.89	0.0017	0.067		8	1 Zn-O	1.90	0.0017	0.067
		1 Zn-C	[2.79]	[0.0025]				1 Zn-C	[2.81]	[0.0025]	
		1 Zn-O	[3.05]	[0.0027]				1 Zn-O	[3.08]	[0.0028]	
		4 Zn-(N/O)	2.01	0.0009				4 Zn-(N/O)	2.01	0.0013	
		4 Zn-C	2.93	0.0011				4 Zn-C	2.93	0.0013	
		4 Zn-C	[3.06]	[0.0017]				4 Zn-C	[3.07]	[0.0019]	
		4 Zn-C	[4.09]	[0.0022]				4 Zn-C	[4.09]	[0.0025]	
		4 Zn-N	[4.17]	[0.0022]				4 Zn-N	[4.17]	[0.0025]	
<i>Sulfolobus</i> Zn-ferredoxin FSFDA (2-13.5 Å ⁻¹) Δ <i>k</i> ³ χ = 22.37	9	4 Fe-S	2.26	0.0022	0.036	<i>Th. acido</i> Zn-ferredoxin FTFBA (2-13.5 Å ⁻¹) Δ <i>k</i> ³ χ = 20.86	11	4 Fe-S	2.25	0.0018	0.045
		2 Fe-Fe	2.72	0.0016				2 Fe-Fe	2.71	0.0015	
	10	4 Fe-S	2.26	0.0021	0.040		12	4 Fe-S	2.25	0.0017	0.048
		2.5 Fe-Fe	2.72	0.0027				2.5 Fe-Fe	2.71	0.0027	

^a R_{as} is the metal-scatterer distance. σ_{as}² is a mean square deviation in R_{as}. The shift in E₀ for the theoretical scattering functions was optimized, but did not vary more than 1.5 eV. Numbers in square brackets were constrained to be either a multiple of the above value (σ_{as}²) or to maintain a constant difference from the above value (R_{as}). f is a normalized error (chi-squared):

$$f = \frac{\left\{ \sum_i \left[k^3 (\chi_i^{obs} - \chi_i^{calc}) \right]^2 / N \right\}^{1/2}}{\left[(k^3 \chi^{obs})_{max} - (k^3 \chi^{obs})_{min} \right]}$$

Fe K-edge XAS analysis. The Fe K-edge X-ray absorption spectra for zinc-containing ferredoxin from *T. acidophilum* strain HO-62 are almost identical to that from *Sulfolobus* sp. strain 7 (Fig. 4b). The integrated peak area (0.206 eV for *T. acidophilum* and 0.289 eV for *Sulfolobus*), for the $1s \rightarrow 3d$ transition at ~ 7113 eV, falls in the range expected for tetrahedral compounds (50-52).

Curve-fitting analysis of both archaeal ferredoxins reveals the presence of a 2.25-2.26 Å Fe-S and a 2.71-2.72 Å Fe-Fe interaction. The best fit (by goodness-of-fit values) is obtained from calculated EXAFS for FeS_4Fe_2 (Fits 1 and 3, Table 3; Fig 6). However, the data can also be fit assuming $\text{FeS}_4\text{Fe}_{2.5}$ (Fits 2 and 4, Table 3), as expected for one 3Fe and one 4Fe cluster.

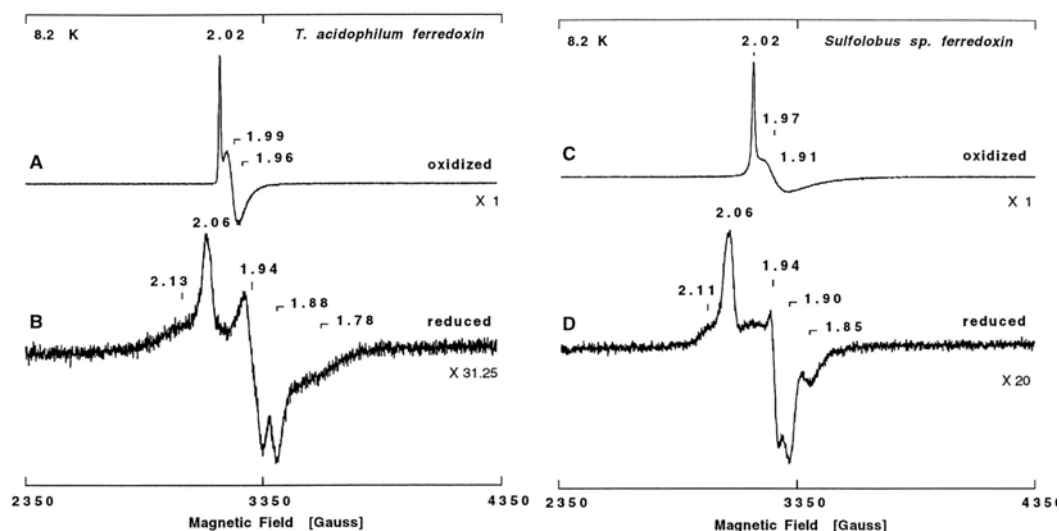


Figure 5.6. EPR spectra of ferredoxin from *T. acidophilum* (a and b) and *Sulfolobus* sp (c and d) in the air-oxidized (a and c) and dithionite-reduced (b and d) states at pH = 9.3.

EPR spectroscopy. The air-oxidized form of both ferredoxins (*Sulfolobus* and *T. acidophilum*) elicited the sharp $g = 2.02$ EPR signals with slightly different lineshapes (0.9-1.0 spin/mol), which are attributable to a $[\text{3Fe-4S}]^{1+}$ cluster as reported previously (6,14) (Figs. 7A and 7C). Upon reduction of these ferredoxins by excess dithionite under anaerobic conditions, the sharp $g = 2.02$ EPR signals disappeared, and a broad low-field resonance at $g = 12$ appeared; this signal is characteristic of the reduced $S = 2$ $[\text{3Fe-4S}]^0$

cluster (data not shown). In addition, rhombic EPR signals at $g = 2.06$, 1.94 , and 1.88 (Fig. 7B) and $g = 2.06$, 1.94 , and 1.90 (Fig. 7D), both attributed to a reduced $S = 1/2$ $[4\text{Fe-4S}]^{1+}$ cluster, were detected up to 30 K for *T. acidophilum* and *Sulfolobus* sp. ferredoxins, respectively, together with additional wings on the high- and low-field sides of the main EPR signals due to magnetic interactions with the reduced $S = 2$ $[3\text{Fe-4S}]^0$ cluster (Figs. 7B and 7D).

Taken together, the XAS and EPR results indicate that the two archaeal zinc-containing ferredoxins contain one $[3\text{Fe-4S}]^{1+,0}$ cluster and one $[4\text{Fe-4S}]^{2+,1+}$ cluster, and that the average Fe environments are nearly identical in the two proteins (Figs. 6,7 and Table 3). The *zfx* gene product of *T. acidophilum* contains three cysteine residues arranged in a $\text{Cys}^{67}\text{-Cys}^{68}\text{-Ile-Ala-Asp}^{71}\text{-Gly-Ala-Cys}^{74}$, and remote $\text{Cys}^{133}\text{-Pro}$ motif, which could serve as ligands to a $[3\text{Fe-4S}]$ cluster, and four cysteine residues in another motif, $\text{Cys}^{123}\text{-Ile-Phe-Cys}^{126}\text{-Met-Ala-Cys}^{129}$, and remote $\text{Cys}^{78}\text{-Pro}$, which are likely ligands to a $[4\text{Fe-4S}]$ cluster (dotted cysteines in Fig. 1). The same spacing of consensus cysteine residues was found in other zinc-containing ferredoxin sequences (6,10,11,13,53), and was proposed to be attributed to the similarity of the pattern of hyperfine-shifted resonances of $^1\text{H-NMR}$ spectra of the 7Fe form of zinc-containing ferredoxins (T. Iwasaki, manuscript in preparation) to those of the 3Fe-, 4Fe-, and 8Fe-containing ferredoxins (53,54). In the *Azotobacter*-type 7Fe-containing ferredoxins with a long C-terminal region, the cysteine ligand residues are arranged more asymmetrically due to the insertion of a short amino acid sequence stretch at the cluster binding motif (54-58). The *zfx* sequence also shows the presence of two additional cysteine residues, Cys^{66} and Cys^{115} (bold residues in Fig. 1), which are not present in *Sulfolobus* sp. ferredoxin sequence (Fig. 2), and hence most likely do not serve as ligands to the clusters.

Conclusion

The sequence and spectroscopic data reported herein provide detailed structural information of the metal binding sites in *T. acidophilum*. The tightly bound zinc atom of archaeal zinc-containing ferredoxins constitutes an isolated and structurally conserved zinc center. The zinc is tetrahedrally coordinated with (most likely) three histidine imidazoles and one carboxylate, with average Zn-N and Zn-O bond distances of 2.01 and 1.90 Å, respectively. The sequence comparisons suggest that the three conserved histidine residues in the N-terminal extension region and one conserved aspartate in the ferredoxin core fold (Fig. 2) serve as ligands to the Zn. The similarity search for zinc-containing ferredoxin homologs with these consensus sequence motifs against nucleotide and amino acid sequence data bases indicated their limited distribution among hyperthermophilic organisms, even within the archaeal domain (Fig. 2). This implies that early zinc-containing ferredoxins might have appeared shortly after divergence of the early Archaea, which is also in line with previous phylogenetic analysis (14).

The overall protein fold of archaeal zinc-containing ferredoxins is largely asymmetric due to the presence of a long N-terminal extension and the insertion of central loop region, as compared with those of regular bacterial-type ferredoxins (Fig. 2). However, close inspection of the ferredoxin core fold suggests the strict conservation of a pseudo-two-fold symmetry with respect to the local two FeS cluster binding sites. Thus, in spite of the presence of one $[3\text{Fe-4S}]^{1+,0}$ cluster and one $[4\text{Fe-4S}]^{2+,1+}$ cluster in purified proteins (6,14,15) (Fig. 2), the distribution of the conserved cysteine ligand residues in

archaeal zinc-containing ferredoxins is similar to those of regular 8Fe-containing dicluster ferredoxins, except for the presence of an aspartate residue (Asp⁷¹ in *T. acidophilum* ferredoxin) in place of cysteine (Fig. 2). In fact, the ferredoxin core-fold of archaeal zinc-containing ferredoxins exhibited 55-65% homology to various PsuC proteins (also called F_A/F_B proteins) from some phototrophic organisms and a PsuC homolog of a hyperthermophilic euryarchaeote *Mc. jannashii* (MJ 1302 (31)) (Fig. 2).

PsaC is a 8Fe ferredoxin homolog found as a part of photosystem I and carries two $[4\text{Fe-4S}]^{2+,1+}$ clusters, namely centers F_A and F_B , which serve as an electron donor to another FeS center, F_x (59-62). The redox potentials of the centers F_A and F_B of PsaC are both well below -500 mV (52), as in the cases reported for a lower-potential $[4\text{Fe-4S}]^{2+,1+}$ cluster (cluster II) of archaeal zinc-containing ferredoxins (6,12,63).

Interestingly, PsaC and its archaeal analog contain a central loop region as found in archaeal zinc-containing ferredoxins, but lack the N-terminal histidine-rich stretch that contains the zinc site (Fig. 2). Because a *zfx* gene homolog with the consensus histidine-rich motif in the N-terminal extension region has not been found in any of the genome sequences available for aerobic and anaerobic hyperthermophiles (31-35), it seems plausible to postulate that early zinc-containing ferredoxins might have evolved as an 8Fe-containing low-potential two-electron carrier similar to the PsaC homolog, to which the N-terminal extension and central loop regions were attached in the later stage of molecular evolution, presumably shortly after divergence of the archaeal domain. This putative evolutionary scheme seems to be in line with the physiological function of zinc-containing ferredoxins of thermoacidophilic Archaea, serving as an electron acceptor of 2-oxoacid:ferredoxin oxidoreductases as do hyperthermophile monocluster ferredoxins without the zinc center (64-66).

The *zfx* gene homologs apparently exhibit limited distribution in the archaeal domain, and have been found exclusively from the aerobic and thermoacidophilic Archaea so far (14). In thermophilic euryarchaeotes, the *zfx* gene product has been found only in the *Thermoplasmales*, an unexpected result based on the universal 16S rRNA based sequence tree (2,3). Analogous observation has been reported for the functionally equivalent ferredoxins of extremely halophilic and aerobic euryarchaeotes (4,67), which contain a single plant-type $[2\text{Fe-2S}]$ cluster and exhibit the amino acid and base sequence similarity to those of the extremely halophilic cyanobacteria (68).

In the aerobic and thermoacidophilic Archaea, the intracellular pH is maintained at pH 5.5-6.5, by the membrane bound aerobic respiratory system operating at high

temperature (23,69-71), implying that the cytoplasmic FeS proteins should be protected against long-term exposure to the microaerobic and fairly acidic conditions during cell growth. The structurally conserved isolated zinc site of archaeal zinc-containing ferredoxins allows tight binding of the extra extension regions to one side of the ferredoxin core fold, thereby possibly providing a means to protect against gradual degradation of the bound FeS clusters under physiological conditions.

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CHAPTER 6
CONCLUSION

During the course of my graduate career, I have conducted experiments on a number of biological systems. Rather than attempt to describe each set of experiments separately and completely in this dissertation, I have chosen to give a complete description of only a few highlighted projects. The preceding chapters describe those results. In this conclusion chapter, a brief overview is given of all of the projects.

Zinc-containing enzymes: SmtB. In collaboration with D. P. Giedroc (Texas A&M University), we have employed XAS to characterize the metal-binding sites of SmtB, a zinc-responsive transcriptional repressor and a member of the ArsR superfamily of prokaryotic metalloregulatory transcription factors. SmtB binds one equivalent of either Zn(II), Co(II), or Ni(II), in order of decreasing affinity. XAS results indicate that zinc and cobalt bind isomorphously, but that nickel binds in a different coordination environment [1]. The extent to which the binding of these cations modulates the affinity of SmtB for DNA or otherwise alters the initiation of transcription is yet unknown and currently being pursued. As these results become available, the structural description of the metal-binding sites in SmtB will provide a basis for interpreting the effects of each cation on transcription.

Zinc-containing enzymes: TFIIB. In work previously supported by another grant in our laboratory, we generated XAS samples of human transcription factor (TF)IIB and the [C10H] variant of *Pyrococcus furiosus* (*Pf*) TFB. The [C10H] variant of *Pf*TFB was constructed to resemble the metal-binding motif of higher eucaryal TFIIB proteins by mutating the second cysteine ligand to a histidine. Using XAS, we have shown that the Zn coordination environments of these two samples are identical, revealing that there is a common zinc-binding motif in archaeal and eucaryal transcription factors and that this motif is likely a determining factor in the overall structure and therefore function, for this class of transcription factors [2].

Zinc-containing enzymes: Carbonic Anhydrases. Carbonic anhydrases catalyze the reversible hydration of carbon dioxide and are ubiquitous in all domains of life. In collaboration with J. G. Ferry (Pennsylvania State University), we have explored the zinc

and cobalt coordination environments in archaeal γ - and β -class carbonic anhydrases. XAS has played a key role in determining the differences in first-shell coordination environments, in particular showing that the β -class of carbonic anhydrases contains two sulfur and two nitrogen ligands [3], whereas the γ -class is marked by three histidine ligands and three other oxygen- or nitrogen-containing ligands [4]. In conjunction with kinetic studies, our XAS experiments have demonstrated that these structurally distinct classes of carbonic anhydrases perform functionally equivalent roles in nature.

Heavy metal Cd resistance: CadC. CadC is an extrachromosomally encoded metalloregulatory repressor protein from the ArsR superfamily that negatively regulates expression of the *cad* operon in a metal-dependent fashion. The metalloregulatory hypothesis holds that direct binding of thiophilic cations including Cd(II), Pb(II), Bi(III), and Zn(II), by CadC allosterically regulates the DNA binding activity of CadC to the *cad* operator/promoter (O/P). In collaboration both with D. P. Giedroc (Texas A&M University) and B. P. Rosen (Wayne State University), we have been successful in identifying the Cd(II) ligands in CadC [5]. Binding of Cd(II) to this tetrathiolate center results in a decrease of the intrinsic affinity of CadC for the *cad* O/P site. Continued efforts are underway to determine the precise mechanism for Cd(II)-induced regulation of the initiation of transcription in the *cad* system.

Iron-containing enzymes: Zinc-containing ferredoxins. An unexpected result from the crystallographic characterization of ferredoxin from *Sulfolobus* sp. was the presence of a tetrahedrally coordinated Zn site [6]. A functionally equivalent ferredoxin was purified from *Thermoplasma acidophilum* [7] and spectroscopic investigation revealed the presence of a similar zinc site. In an attempt to understand the nature of the zinc site in these unusual ferredoxins, we collaborated with T. Iwasaki (Nippon Medical School, Japan) to characterize the Fe-S cluster and zinc-binding site in ferredoxins from both *Sulfolobus* sp. and *Thermoplasma acidophilum*. XAS experiments indicate that the zinc coordination environment identified by crystallographic data, three histidine ligands and the carboxylate from aspartate, is identical in the two ferredoxins [8]. We have also

characterized the selective oxidative degradation of one of the Fe-S clusters in *Sulfolobus* sp. Fd, revealing that there is no change in the zinc site, despite the conversion of the nearby [4Fe-4S] cluster to a [3Fe-4S] cluster [9].

Iron-containing enzymes: NOS. Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to citrulline and nitric oxide through two stepwise oxygenation reactions involving N^o-hydroxy-L-arginine, an enzyme-bound intermediate. The N^o-hydroxy-L-arginine- and arginine-bound NOS ferriheme centers show distinct, high-spin electron paramagnetic resonance (EPR) signals. In collaboration with T. Iwasaki, XAS was used to examine the structures of these ferriheme sites in full length neuronal NOS (Figure 1.1; [10]). Our XAS results show that the two forms are strikingly similar. Furthermore, even though Cu(II) inhibition affects the spin-state equilibrium as measured by EPR, there is no XAS-observable change to the ferriheme coordination environment. These results indicate that the manner in which substrate is held in the active site, rather than the heme site structure and geometry, specify the mechanism for the two-step hydroxylation reactions in neuronal NOS.

Iron-containing enzymes: TfdA. The first step in the degradation of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), by *Ralstonia eutropha* is catalyzed by the α -ketoglutarate (α -KG)-dependent dioxygenase, TfdA. Previously, EPR and ESEEM studies on inactive Cu(II)-substituted TfdA suggested a *g*-tensor rearrangement upon addition of 2,4-D [11]. In collaboration with R. P. Hausinger (Michigan State University), we have conducted XAS studies

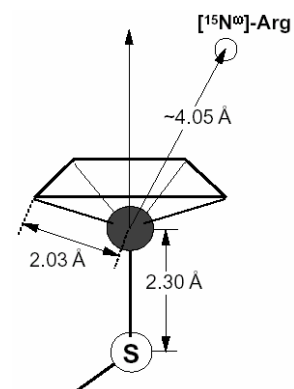


Figure 1.1. L-Arginine-bound form of neuronal nitric oxide synthase.

on various Cu(II) and Fe(II) forms of TfdA to determine the structural implications of this *g*-tensor rearrangement. Cu(II) has a d^9 valence electronic configuration, making it highly susceptible to Jahn-Teller distortion. This distortion results in longer axial bonds, making those ligands harder to detect by XAS and complicates the *g*-tensor description of the metal site. XAS does not have the paramagnetic requirements of the other two

techniques, enabling us to study the active Fe(II) form of the enzyme. Fe(II) is d^6 which should display little Jahn-Teller distortion. XAS results indicate that the addition of 2,4-D to either Fe(II)- or Cu(II)-TfdA resulted in the loss of a histidine ligand [12]. Although the Cu(II) results could be explained by Jahn-Teller distortion, the changes at the Fe(II) site argue for loss of a histidine ligand, rather than simply a g -tensor rearrangement. Although the catalytic mechanism for TfdA remains unknown, our XAS results provide a structural backdrop against which future experiments will be interpreted.

Iron-containing enzymes: MetAP. Methionyl aminopeptidases (MetAPs) represent a unique class of proteases that are capable of removing the N-terminal methionine residue from nascent polypeptide chains. We have collaborated with R. C. Holz (Utah State University) to characterize the cobalt- and iron-binding sites in MetAP [13]. X-ray crystallographic studies of MetAPs from *E. coli*, *Homo sapiens*, and *Pyrococcus furiosus* have shown catalytic domains that contain a dinuclear cobalt core [14-17]. However, functional and kinetic experiments indicated the requirement for only one bound metal. Thus, XAS was used to establish the coordination sphere for both cobalt- and iron-bound forms of MetAP. Interestingly, the Fourier transform plots reveal no apparent metal-metal interaction, providing structural evidence for the hypothesis that MetAP is a mononuclear enzyme. Given the XAS and biochemical evidence, the crystallographic results can be explained in terms of the excess metal that was present in the crystallization conditions.

Copper-containing enzymes: Cytochrome bo_3 . XAS has been used, in collaboration with R. B. Gennis (University of Illinois), to examine the structures of the Cu(II) and Cu(I) forms of the cytochrome bo_3 quinol oxidase from *E. coli* [18]. Cytochrome bo_3 is a member of the superfamily of heme-copper respiratory oxidases. Of particular interest is the fact that these enzymes function as redox-linked proton pumps, resulting in the net translocation of one H^+ per electron across the membrane. The molecular mechanism of how this pump operates and the manner by which it is linked to the oxygen chemistry at the active site of the enzyme are unknown. Several proposals

have featured changes in the coordination of Cu_B during enzyme turnover that would result in sequential protonation or deprotonation events that are key to the functioning proton pump. Using XAS, we examined the structure of the Cu_B site in both the fully oxidized and fully reduced forms of the enzyme. The results show that in the oxidized enzyme, Cu_B(II) is four-coordinate, consistent with three imidazoles and one hydroxyl (water). Upon reduction of the enzyme, the coordination of Cu_B(I) is significantly altered, consistent with the loss of one of the histidine imidazole ligands in at least a substantial fraction of the population. These data add to the credibility that changes in the ligation of Cu_B might occur during catalytic turnover of the enzyme and therefore could be part of the mechanism of proton pumping.

Copper-containing enzymes: NosL. One of the accessory proteins, NosL, of the *nos* (nitrous oxide reductase) gene cluster has been structurally characterized, in collaboration with D. M. Dooley (Montana State University) [19]. The function of NosL is presently unknown, but the data indicate that NosL does not act as an electron transfer partner to nitrous oxide reductase. NosL is encoded on the same transcript as three other gene products (NosD, NosF, and NosY). These are required for assembly of the active site in nitrous oxide reductase, which is thought to be a copper cluster. Accordingly, it is possible that NosL is a copper chaperone involved in metallocenter assembly. Our XAS results indicate that the copper ion in NosL is ligated by a cysteine, methionine, and histidine. Thus, NosL contains a novel type of biological copper site and further experimentation is necessary to establish the function of this protein in the nitrous oxide reductase system.

Nickel-containing enzymes: Urease. We have worked with R. P. Hausinger (Michigan State University) to structurally characterize enzymes responsible for the

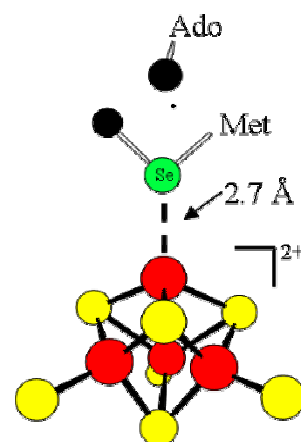


Figure 1.2. Proposed interaction between selenomethionine and the FeS cluster of lysine-2,3-aminomutase.

hydrolysis of urea into ammonia and carbamate. Urease is the primary catalyst in this reaction and is characterized by a dinuclear nickel site, first identified by XAS. In previous efforts in the Scott laboratory, XAS was used to describe the ligands to the dinuclear nickel site [20, 21]. This description was at odds with the crystal structure [22] and triggered the further refinement of the crystallographic information [23], resulting in the identification of additional water ligands that confirmed the XAS results. In a current research initiative, we expanded our investigation to include nickel and cobalt binding to wild type and (C319A) apo-urease [24]. In conjunction with crystallographic and kinetic experiments, we demonstrated that there are at least three distinct metal-bound species, only one of which is active. These results explain the observation that only 15% of the enzyme can be activated *in vitro* and underscores the importance of chaperone proteins that are involved in the proper formation of the dinuclear nickel site (UreD, -E, -F, -G).

Selenium in biology: Lysine 2,3-aminomutase. We have worked with S. J. Booker (Pennsylvania State University) and P. A. Frey (University of Wisconsin) to characterize lysine 2,3-aminomutase, which belongs to a class of enzymes that use FeS clusters and S-adenosyl-L-methionine (AdoMet) to initiate radical chemistry [25]. Using XAS, we have studied lysine 2,3-aminomutase at various stages of catalysis, in the presence of selenomethionine or Se-adenosyl-L-selenomethionine (SeAdoMet), revealing that the cofactor is cleaved only in the presence of dithionite and the substrate analog *trans*-4,5-dehydrolysine. Strikingly, a new Fourier transform peak at 2.7 Å, interpreted as an Se–Fe interaction (Figure 1.2), appears concomitant with this cleavage. This is the first demonstration of a direct interaction of AdoMet, or its cleavage products, with the FeS cluster in this class of enzymes.

Manganese-containing enzymes: Muconate Cycloisomerase. Mutants of the bacterium *Acinetobacter* sp. ADP1 were selected to grow on benzoate without the BenM transcriptional activator. In the wild type, BenM responds to benzoate and *cis,cis*-muconate to activate expression of the *benABCDE* operon involved in benzoate catabolism. This operon encodes enzymes that convert benzoate to catechol, a compound

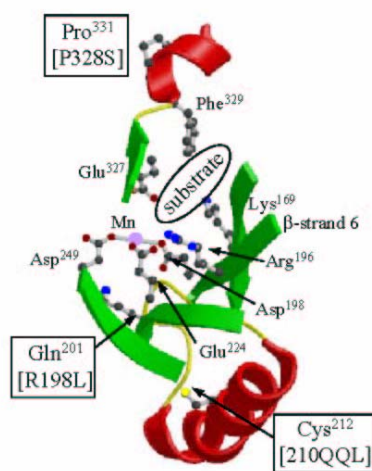


Figure 1.3. Expanded view of the active site of muconate cycloisomerase from *P. putida* (PDB code 1muc). Amino acids are numbered according to the *P. putida* convention. Altered residues in variant ADP1 muconate cycloisomerases are boxed.

subsequently degraded by *cat*-gene encoded enzymes. Four spontaneous mutants were found to carry *catB* mutations that enabled BenM-independent growth on benzoate (Three of these mutations are highlighted in Figure 1.3). *CatB* encodes muconate cycloisomerase, an enzyme required for benzoate catabolism. Its substrate, *cis,cis*-muconate, is enzymatically produced from catechol by the *catA*-encoded catechol 1,2-dioxygenase. Muconate cycloisomerase was purified to

homogeneity from the wild type and the *catB* mutants. Each purified enzyme was active, although there were differences in the catalytic properties of wild-type and variant muconate cycloisomerases. Strains with a chromosomal *benA::lacZ* transcriptional fusion were constructed and used to investigate how *catB* mutations affected growth on benzoate. All the *catB* mutations increased *cis,cis*-muconate-activated *ben*-gene expression. A model was constructed in which the *catB* mutations reduce muconate cycloisomerase activity during growth on benzoate, thereby increasing intracellular *cis,cis*-muconate concentrations. This in turn may allow CatM, an activator similar to BenM in sequence and function, to activate *ben*-gene transcription. CatM normally responds to *cis,cis*-muconate to activate *cat*-gene expression. Consistent with this model, muconate cycloisomerase specific activities in cell-free extracts of benzoate-grown *catB* mutants were low relative to the wild type. Moreover, the catechol 1,2-dioxygenase activities of the mutants were elevated, which may result from CatM responding to the altered intracellular levels of *cis,cis*-muconate and increasing *catA* expression.

Collectively, these results support the important role of metabolite concentrations in controlling benzoate degradation via a complex transcriptional regulatory circuit.

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