

Report Title

Final Report for "The Structural Analysis and Bioengineering of Thermostable *Pyrococcus furiosus* Prolidase for Optimization of Organophosphorus Nerve Agent Detoxification"

ABSTRACT

The aims of this project were to structurally study and bioengineer thermostable prolidase from *Pyrococcus furiosus* to enable its use for organophosphorus nerve agent detoxification. Prolidase contains one dinuclear Co metal-center/monomer and has optimal activity at 100°C, exhibiting no activity in the absence of Co²⁺ or at temperatures <50°C. Requirement for metal ions is characteristic of all organophosphorus nerve agent hydrolases and results from these enzymes containing dinuclear metal-centers with one tight-binding metal atom and a second loose-binding metal atom. One primary objective of this study was to determine which of the metal sites is integral and which is labile, information that will be used to bioengineer prolidases. Another objective was to produce *P. furiosus* prolidase mutants that have increased catalytic activity over a lower range of temperatures using random mutation and a low-temperature selection method. Three mutant prolidases targeting metal-binding amino acids have been successfully produced and biochemical analysis has demonstrated that the Co1 metal-binding site is the high-affinity site and the Co2 site, the low-affinity site. Conditions for selection of mutant prolidases with increased activity at lower temperatures have been determined and mutant prolidases (G39E and E236V) isolated that have higher activity than wild type at 37 °C.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Du, X, Tove, S, Kast-Hutcherson, K, Grunden AM. 2005. Characterization of the dinuclear metal center of *Pyrococcus furiosus* prolidase by analysis of targeted mutants. *FEBS Lett.* 579:6140-6146.

Maher MJ, Ghosh M, Grunden AM, Menon AL, Adams MW, Freeman HC, Guss JM. 2004. Structure of the prolidase from *Pyrococcus furiosus*. *Biochem.* 43: 2771-83.

Number of Papers published in peer-reviewed journals: 2.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

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Grunden, AM. "Physiological analysis of the reactive oxygen species detoxification system in the hyperthermophilic archaeon *Pyrococcus furiosus*". Department of Microbiology. North Carolina State University. October, 2005.

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Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Du X, Grunden AM. 2004. Characterization of *Pyrococcus furiosus* Prolidase Metal Center by Targeted Mutant Analysis. North Carolina Chapter of the American Society for Microbiology, Raleigh, NC

Du X, Tove S, Grunden AM. 2005. Production of *Pyrococcus furiosus* Prolidase Mutants with Increased Catalytic Activity at Lower Temperatures. North Carolina Chapter of the American Society for Microbiology, Raleigh, NC.

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2

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Du X, Tove S, Grunden AM. 2004. Characterization of Pyrococcus furiosus prolidase metal center by targeted mutant analysis. 104th General Meeting of the American Society for Microbiology, New Orleans, LA.

Du X, Tove, S, Grunden AM. 2005. Production of Pyrococcus furiosus prolidase mutants with increased catalytic activity at lower temperatures. 105th General Meeting of the American Society for Microbiology, Atlanta GA.

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(d) Manuscripts

Du, X, Tove, S, Kast-Hutcheson, K, Grunden AM. 2006. Improving the catalytic activity of Pyrococcus furiosus prolidase at low temperatures.

Number of Manuscripts: 1.00

Number of Inventions:

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	
Xuelian Du	1.00	No
FTE Equivalent:	1.00	
Total Number:	1	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
Amy Grunden	0.00	No
FTE Equivalent:	0.00	
Total Number:	1	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Personnel receiving masters degrees

<u>NAME</u>
Total Number:

Names of personnel receiving PHDs

<u>NAME</u>	
Xuelian Du	No
Total Number:	1

Names of other research staff

NAME

PERCENT_SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

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Table of Contents

List of Tables and Figures	Page 2
Introduction	Page 4
Statement of Problem Studied	Page 13
Summary of the Most Significant Results	Page 14
Bibliography	Page 23

List of Tables and Figures

- Figure 1:** Chemical formulas of several G-type organophosphorus nerve agents used in chemical warfare (page 4)
- Figure 2.** Structure of the amino acid proline (page 6)
- Figure 3.** The effects of metal ions on the activities of native or recombinant *P. furiosus* prolidase (page 8)
- Figure 4.** Temperature profile for native and recombinant *P. furiosus* prolidase (page 9)
- Figure 5.** Alignment of *P. furiosus* prolidase with other prolidases, *Alteromonas* OPAA, and *E. coli* MAP (page 10)
- Figure 6.** Dinuclear Mn metal center from *E. coli* aminopeptidase. Modified from Wilce, 1998 (page 11)
- Figure 7.** Dinuclear Co center in *P. furiosus* prolidase based on analogy to *E. coli* MAP and aminopeptidase structures (page 11)
- Figure 8.** Proposed reaction mechanism for the hydrolysis of X-Pro dipeptides by prolidase based on analogy to the reaction mechanism predicted for *E. coli* MAP (page 12)
- Figure 9.** The structure of a monomer of *P. furiosus* prolidase (page 14)
- Figure 10.** Stereo-views of the dimer of *P. furiosus* prolidase (page 15)
- Figure 11.** Stereo-view showing the active site of *P. furiosus* prolidase (page 16)
- Figure 12.** Dinuclear metal center active site of *P. furiosus* prolidase (page 17)
- Table 1.** Specific activity and cobalt content of purified wild type and mutant *P. furiosus* prolidases (page 18)
- Figure 13.** Scheme for construction of *E. coli* strain JD1(λ DE3) that is used for selection of the *P. furiosus* prolidase mutants (page 20)
- Figure 14.** Strategy used for selection of low-temperature active *P. furiosus* prolidase mutants (page 21)
- Figure 15.** (A) Comparison of activities of wild type and G39E-prolidase at assay temperatures ranging from 10 °C to 100 °C. (B) Wild type and G39E-prolidase activities when assayed at temperatures ranging from 10 °C to 50 °C (page 22)

Introduction

1a. Biological Effects of Toxic Organophosphorus Compounds

For the past century, organophosphorus (OP) nerve agents have posed a significant battlefield threat as highly toxic chemical weapons. Today it is thought that these nerve agents have been stockpiled in the arsenals of a number of nations and several terrorist organizations. In the last decade, several incidences have demonstrated the devastating effects that these OP nerve agents can have when used as chemical weapons. For example, during the Iran-Iraq conflict, Iraqi forces exposed Iraqi soldiers and Kurdish civilians to toxic nerve gas, resulting in substantial numbers of casualties, and in 1995, the potent nerve agent, sarin, was used by a Japanese terrorist group to attack a civilian population in an underground subway, causing over 5,500 casualties.

Two major OP nerve agents that pose particular threat as chemical weapons are the G-type fluorine-containing organophosphorus nerve agents, sarin (GB, O-isopropyl methylphosphonofluoridate) and soman (GD; O-pinacolyl methylphosphonofluoridate), with soman having the greatest potency (see Figure 1) (Koelle 1994).

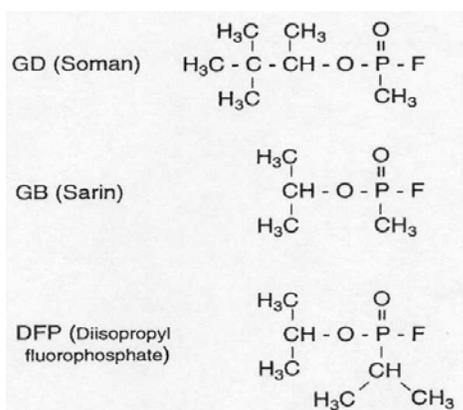


Figure 1. Chemical formulas of several G-type organophosphorus nerve agents used in chemical warfare (soman and sarin). DFP is an OP test compound commonly used as a model for OP nerve agent hydrolysis studies.

The toxicity of organophosphorus nerve agents is attributed to the ability of the OP compounds to irreversibly inhibit the enzyme acetylcholinesterase (AChE), thereby preventing hydrolysis of the neurotransmitter acetylcholine (ACh). The OP inhibition of AChE leads to excessive buildup of acetylcholine and over-stimulation of cholinergic neurons, resulting in convulsions, brain seizures, and neuronal death. Without proper treatment, exposure to OP nerve agents can result in death within minutes (Misulis et al. 1987; Solberg and Belkin 1997).

1b. Enzymatic Detoxification of Organophosphorus Compounds

Given the serious threat of OP nerve agents used in warfare and terrorist actions, the ability to quickly and safely decontaminate effected areas is paramount. Currently, decontamination of OP nerve agent-exposed sites and equipment involves treatment with decontamination solution #2 (DS2) or bleach (Richardson 1972). Although such treatment effectively degrades the OP compounds, both DS2 and bleach are highly

corrosive, resulting in damage to both the treated surfaces and the production of hazardous waste, which must then be removed. These complications have spurred interest in developing alternate approaches for the decontamination of OP-exposed sites. One alternate approach that has particular appeal is the use of enzymes to degrade OP compounds, a method that would not be corrosive and would not generate further hazardous waste.

Detoxification of OP compounds by enzymatic hydrolysis was first reported by Mazur, when it was determined that the P-F bond of the OP, diisopropylfluorophosphate (DFP), could be hydrolyzed using crude preparations of rabbit and human tissues (Mazur 1946). Since then a number of enzymes capable of degrading OP compounds have been identified from both eukaryotic and prokaryotic sources (Landis and DeFrank 1990). These enzymes that catalyze the hydrolysis of acetylcholinesterase-inhibiting compounds are classified as organophosphorus acid anhydrolases (OPAA, EC 3.1.8.2) and members of this class of enzyme are known to cleave OPs with P-F, P-O, P-CN, and P-S bonds (Cheng et al. 1996; Cheng et al. 1997).

The possibility of utilizing organophosphorus anhydrolases to detoxify OP nerve agents was initially considered upon the identification, cloning, and over-expression of the OP-hydrolyzing enzyme organophosphorus acid hydrolase (OPH) encoded by the plasmid-borne *opd* gene from the bacteria *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27751 (Mulbry et al. 1986; Harper et al. 1988; McDaniel et al. 1988). *Pseudomonas* OPH is a dimeric metalloenzyme (35 kDa per monomer) containing 1-2 Zn²⁺ or Co²⁺ ions per subunit (Dumas et al. 1989; Omburo et al. 1993). A second enzyme exhibiting high-level DFP-hydrolyzing activity, OPAA-2, was subsequently isolated from the bacterium *Alteromonas* sp. strain JD6.5 and is considered distinct from OPH since it bears little sequence identity to OPH [11% identity] (DeFrank and Cheng 1991). Nevertheless, OPAA-2 shares considerable functional similarity to OPH, as both OPAA-2 and OPH can hydrolyze DFP, sarin and the OP insecticide paraoxon. However, only OPAA-2 can degrade the nerve agent soman (Cheng, Harvey et al. 1996). Like OPH, OPAA-2 has been cloned and over-expressed and additionally has been successfully incorporated into foams used for the decontamination of OP-contaminated surfaces (Cheng et al. 1999).

Prolidases Can Hydrolyze Toxic Organophosphorus Compounds

Although the DFP-hydrolyzing enzymes identified from *Alteromonas* species were initially classified as OPAAAs, sequence and biochemical analyses revealed that these enzymes are in fact prolidases (E.C. 3.4.13.9), dipeptidases specific for hydrolyzing a dipeptide bond with a prolyl residue at the C-terminus [X-Pro] (Cheng, Harvey et al. 1996; Cheng, Liu et al. 1997). It is thought that the ability of the OPAA enzymes to hydrolyze P-F, P-O, P-CN, and P-S bonds in toxic OP compounds may arise from the fortuitous similarity of these compounds in shape, size and surface charges to the true prolidase X-Pro dipeptide substrates (Cheng, Harvey et al. 1996; Cheng, DeFrank et al. 1999)

2. General Properties of Prolidases (X-Pro Dipeptidases)

Proline residues are unique among the 20 common amino acids in that they impose a conformational constraint on peptide chains due to the cyclic nature of their pyrrolidine side group (see Figure 2), and as a result, only a few proteases are known which can hydrolyze bonds adjacent to proline (Walter et al. 1980; Cunningham and O'Connor 1997). One of these proteases is the proline dipeptidase prolidase, which preferentially catalyzes the hydrolysis of dipeptides with proline at the C-terminus (NH₂-X-/Pro-COOH) [Note X indicates any amino acid residue and the (/) indicates where hydrolytic cleavage occurs]. Proline-specific peptidases are thought to participate, in conjunction with other endo- and exopeptidases, in the terminal degradation of cytoplasmic proteins or peptides and may specifically aid in the recycling of proline in the cell.

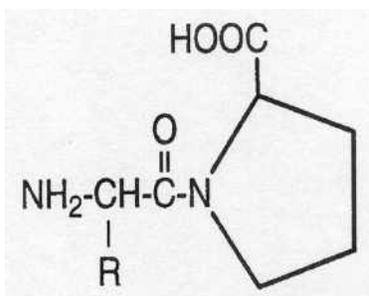


Figure 2. The amino acid proline bound through the imido N in the pyrrolidine ring to a second amino acid.

Prolidases are ubiquitous in nature and have been isolated from a variety of mammalian tissues (Sjostrom et al. 1973; Browne and O'Cuinn 1983; Endo et al. 1987; Myara et al. 1994), bacteria such as *Lactobacillus* (Booth et al. 1990; Fernandez-Esplá et al. 1997), *Aureobacterium* (Fujii et al. 1996) and *Xanthomonas* (Suga et al. 1995), and archaea such as *Pyrococcus* (Ghosh et al. 1998). Prolidases in bacteria and archaea are thought to participate in conjunction with other endo- and exo-peptidases in the degradation of intracellular proteins and in particular in proline recycling based on their enzymatic function (Cunningham and O'Connor 1997; Grunden and Adams 2004). However, in humans, prolidase is involved in the final stage of the degradation of endogenous and dietary protein and is particularly critical for collagen catabolism (Endo, Hata et al. 1987). Mutations in the gene coding for human prolidase can cause prolidase deficiency, an autosomal recessive disorder characterized by skin lesions, mental retardation, and recurrent infections (Scriver et al. 1983; Endo et al. 1989; Endo and Matsuda 1991; Forlino et al. 2002; Lopes et al. 2002; Perugini et al. 2005).

The majority of prolidases that have been studied to date exhibit metal-dependent activity, requiring divalent cations such as Zn²⁺, Mn²⁺, or Co²⁺ for maximal activity (Wilcox 1996; Ghosh, Grunden et al. 1998). Purified prolidases have been shown to exist either as monomers or dimers depending on the source (Cunningham and O'Connor 1997; Kobayashi and Shimizu 1999). Although, all characterized prolidases preferentially hydrolyze X-Pro dipeptides, some prolidases can cleave dipeptides with

proline as the N-terminal residue or can hydrolyze dipeptides that do not contain a prolyl residue (Browne and O'Cuinn 1983; Booth, Jennings et al. 1990; Fujii, Nagaoka et al. 1996; Fernandez-Espla, Martin-Hernandez et al. 1997). These differences in substrate specificities among the characterized prolidases are likely a function of the differences in the numbers of subunits present in the active enzyme as well as the nature of the metal ion occupying the prolidase active site.

Up until recently all characterized prolidases have been isolated from mesophilic sources and are only maximally active at temperatures up to 55 °C. However, the isolation of a prolidase from the hyperthermophilic archaeon *Pyrococcus furiosus* represents the first prolidase purified from either an archaeal or hyperthermophilic source (Ghosh, Grunden et al. 1998; Grunden et al. 2001; Grunden and Adams 2004). The extreme thermostability of *P. furiosus* prolidase has particularly engendered interest in investigating the possibility of utilizing this enzyme to detoxify OP nerve agents in high temperature environments that might arise in certain battlefield situations (in deserts and tropical areas), and as such, *P. furiosus* prolidase will serve as the focus of the studies proposed herein.

3. *Pyrococcus furiosus* Prolidase

3a. The Hyperthermophilic Archaeon *Pyrococcus furiosus*

Hyperthermophiles are organisms that have optimum growth temperatures of at least 80 °C and a maximum growth temperature of 90 °C and above and were first isolated in the early 1980s through the pioneering efforts of Stetter and coworkers (Stetter 1996). At present, more than 20 different genera of hyperthermophiles are known with two of these classified as Bacteria and the rest as Archaea (Adams 1999). Archaea which are known to constitute a third domain of life based on 16S rRNA analysis, are prokaryotes that have some unique features such as membrane architecture and some eukaryotic-like features such as a similar mechanism of transcription (Woese et al. 1990). The importance of the discovery of hyperthermophilic Archaea was immediately recognized by the scientific community and engendered research interest in a number of areas such as the potential biotechnological applications of purified thermostable enzymes from hyperthermophilic sources, the identification of thermoprotection mechanisms that enable growth at high temperatures, and the characterization of the basic biochemistry and physiology of hyperthermophiles and how it differs from mesophilic prokaryotes (Scholz et al. 1992; Aguilar 1996; Kengen et al. 1996; Martins et al. 1996; Sunna et al. 1997; Adams and Kelly 1998; Grunden et al. 2004).

The hyperthermophilic archaeon *Pyrococcus furiosus* is arguably the most studied of any of the hyperthermophilic Archaea (to date there are over 500 published research articles concerning *P. furiosus*). *P. furiosus* is a fermentative archaeon originally isolated from shallow marine hydrothermal vents which grows optimally at 100 °C (Fiala and Stetter 1986). *P. furiosus*, like many heterotrophic hyperthermophiles, utilizes proteins and peptides as growth substrates and fermentatively produces organic acids, CO₂, H₂S, and H₂. A number of enzymes from *P. furiosus* have been isolated and characterized

which are likely involved in the catabolism of amino acids in this organism including aminotransferases (Andreotti et al. 1995), 2-keto acid oxidoreductases (Mai and Adams 1994; Heider et al. 1996; Mai and Adams 1996), proteolytic enzymes that generate small peptides from protein-based growth substrates (Blumentals et al. 1990; Bauer et al. 1996), and the amino acid-yielding peptidase, prolidase (Ghosh, Grunden et al. 1998).

3b. Physical and Catalytic Properties

Both native and recombinant (produced in *E. coli* using a T7 RNA polymerase-driven expression plasmid) forms of *P. furiosus* prolidase have previously been purified and biochemically characterized (Ghosh, Grunden et al. 1998; Grunden, Ghosh et al. 2001). The native and recombinant *P. furiosus* prolidases exhibit essentially equivalent physical and catalytic properties, and therefore, the recombinant version of the protein can be used for structural and bioengineering studies (Ghosh, Grunden et al. 1998; Grunden, Ghosh et al. 2001). *P. furiosus* prolidase is active as a homodimer (39.4 kDa per subunit) and contains one Co^{2+} per subunit as purified. Catalytic activity requires the addition of Co^{2+} (K_d of 0.24 mM) to the assay indicating that the enzyme has at least a second Co^{2+} binding site. *P. furiosus* prolidase activity could also be supported by the presence of Mn^{2+} (75% activity compared to Co^{2+}) but could not be supported by the addition of other divalent (Mg^{2+} , Ca^{2+} , Fe^{2+} , Ni^{2+} , Cu^{2+} , or Zn^{2+}) or monovalent (Na^+ or K^+) ions (Figure 3) under aerobic assay conditions. In keeping with the observed metal dependence of *P. furiosus* prolidase activity, the treatment of prolidase with the metal chelator EDTA (1 mM) completely abolished activity.

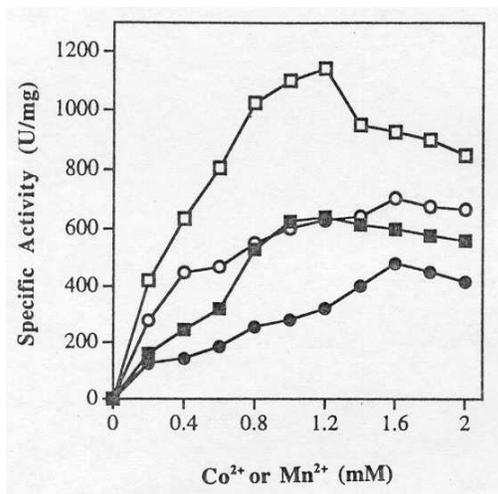


Figure 3. The effects of metal ions on the activities of native (open symbols) or recombinant (solid symbols) *P. furiosus* prolidase. Prolidase was assayed in the presence of Met-Pro (4 mM) and various concentrations of CoCl_2 (squares) or MnCl_2 (circles). Modified from Ghosh, 1998.

P. furiosus prolidase has a narrow substrate specificity, hydrolyzing only dipeptides with proline at the C-terminus and a non-polar amino acid (Met, Leu, Val, Phe, or Ala) in the N-terminal position. Maximal prolidase activity is observed using Met-Pro as the substrate and reaction conditions of pH 7.0 and an incubation temperature of 100 °C. In fact, little activity is seen when the assay is conducted at temperatures below 45 °C (Figure 4). Both the native and recombinant enzymes demonstrate considerable thermostability with no loss of activity observed for the native enzyme (0.3 mg/ml in 100 mM MOPS, (3-n-N-[morpholino]propanesulfonic acid) buffer, pH 7.0)

even after 12 h. of incubation at 100 °C and a loss of 50% activity of for the recombinant enzyme under comparable conditions after 3 h. at 100 °C.

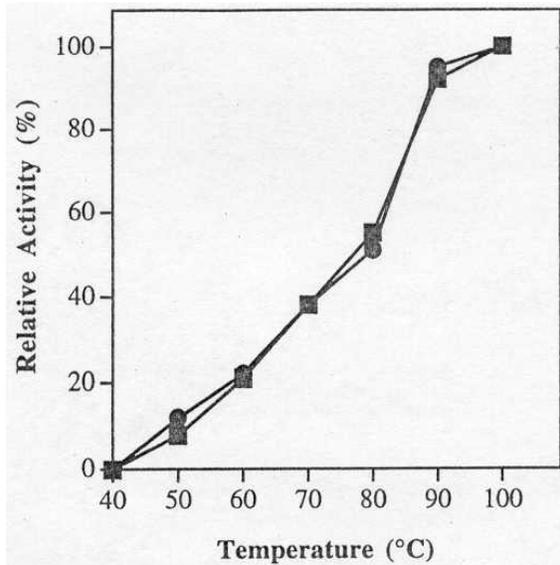


Figure 4. Temperature profile for native (squares) and recombinant (circles) *P. furiosus* prolidase. Assay mixtures contained prolidase (15 ng), Met-Pro (4 mM), and CoCl₂ (1.2 mM). Modified from Ghosh, 1998.

3c. Structural Properties

Metal content and kinetic analyses indicate that *P. furiosus* prolidase has at least two Co²⁺ binding sites per subunit. One of these sites appears to be tight-binding, as it is not removed during purification or by dialysis while the other site appears to be loosely bound with an association constant of 0.24 mM and, in addition to the tight binding site, is required for catalysis. The apparent metal-center properties of *P. furiosus* prolidase are reminiscent of a broad class of dinuclear metallohydrolases represented by the N-terminal exopeptidases, the active sites of which also contain two metal ions, generally with differing binding affinities (Wilcox 1996). Representative members of this family of enzymes include bovine leucine aminopeptidase (Burley 1992) and *Aeromonas proteolytica* aminopeptidase (Chevrier et al. 1994) both of which contain two Zn²⁺ ions in their active sites. The Zn atoms in the *A. proteolytica* aminopeptidase can be replaced *in vitro* with cobalt atoms, and like *P. furiosus* prolidase, is isolated with one metal ion per active site (Bennett and Holz 1997). Prior to the isolation of *P. furiosus* prolidase, the only other known naturally occurring Co-dependent enzymes from this family of enzymes were the methionine aminopeptidases (MAP), which also contain 2 Co²⁺ per active site (Arfin et al. 1995).

Methionine aminopeptidase from *Escherichia coli* has been crystallized and its structure solved (Roderick and Matthews 1993). Based on this structure, five Co-coordinating amino acid residues have been identified (Asp-97, Asp-108, His-171, Glu-204, and Glu-235), and all five amino acids are conserved among other characterized MAPs (Chang et al. 1992; Tsunasawa et al. 1997). Notably, although *P. furiosus* prolidase does not otherwise show significant sequence similarity with any known MAP, all five of the Co-binding amino acid residues are conserved in the *P. furiosus* enzyme (Asp-209, Asp-220, His-280, Glu-313, and Glu-327) (Figure 5). Furthermore, these five

amino acid residues are conserved among all of the prolidase enzymes as well as *Alteromonas* OPAA, and therefore were predicted to function as the metal-binding residues in prolidases, a prediction that has since been confirmed by X-ray crystal structure analysis of *P. furiosus* prolidase (Maher et al. 2004).

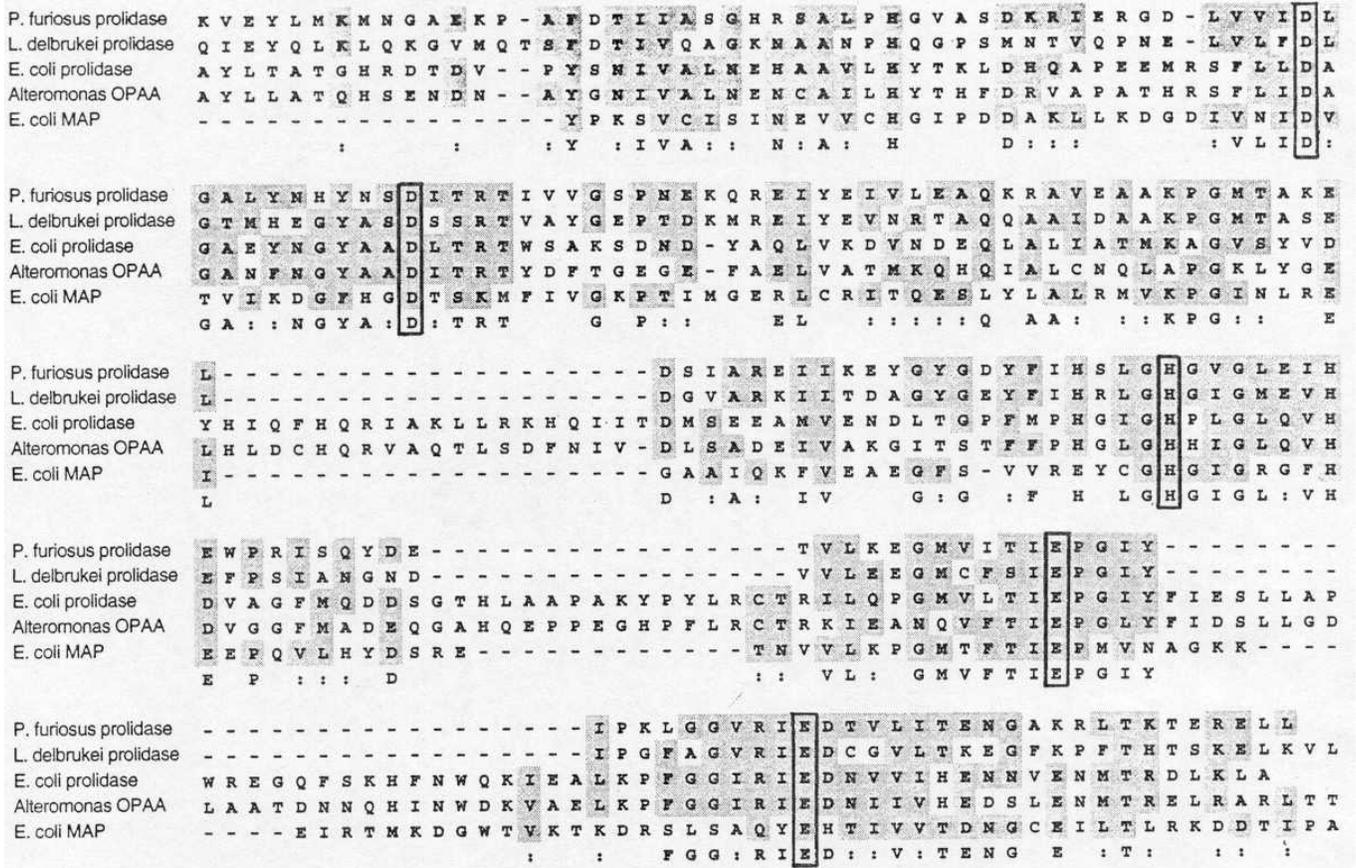


Figure 5. Alignment of *P. furiosus* prolidase with other prolidases, *Alteromonas* OPAA, and *E. coli* MAP. Identical residues are indicated by gray shading. The five amino acids that are ligands to the dinuclear cobalt site in *E. coli* MAP and the conserved residues present in the prolidases and OPAA are boxed.

An equivalent type metal center is also observed in the enzyme aminopeptidase from *E. coli*, which has a dinuclear metal center containing Mn^{2+} ions (Wilce et al. 1998) involving the five amino acids residues Asp-260, Asp-271, His-354, Glu-383, and Glu-406 (Figure 6). This comparison is also particularly relevant as a number of other characterized prolidases such as those isolated from *L. casei*, *X. maltophilia*, human, and *Alteromonas* OPAA are Mn^{2+} -dependent, rather than Co^{2+} -dependent, and thus likely contain a dinuclear Mn center instead.

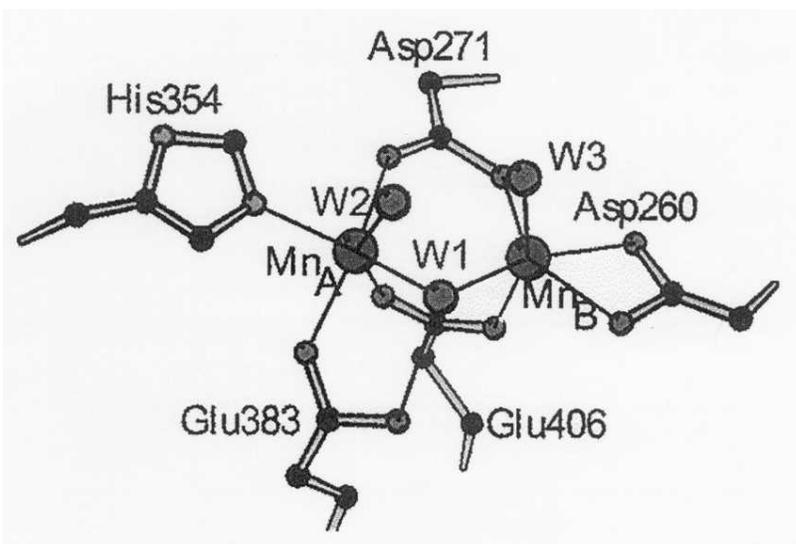


Figure 6. Dinuclear Mn metal center from *E. coli* aminopeptidase. Modified from Wilce, 1998.

As can be seen from the *E. coli* aminopeptidase structure, three of the amino acid residues participate solely in binding one of the metal atoms or the other (His-354 and Glu-383 bind to the Mn_A atom only and Asp-260 solely binds the second Mn center), while two of the amino acid residues serve as bidentate ligands to both Mn atoms (Asp-271 and Glu-406). By analogy, *P. furiosus* prolidase was predicted to have a similar conformation where His-280 and Glu-313 would solely bind to the first Co center (Co_1), Asp-209 to the second Co (Co_2), and Asp-220 and Glu-327 would ligand both cobalt atoms. This configuration for *P. furiosus* prolidase is depicted in Figure 7.

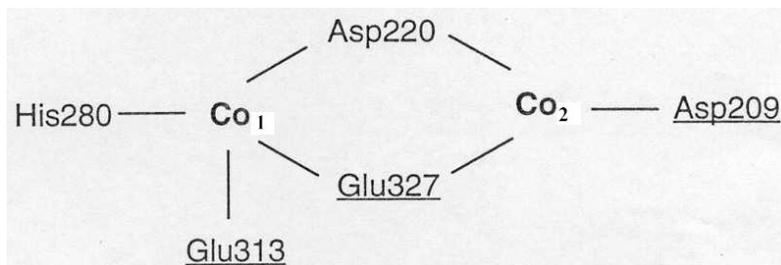


Figure 7. Dinuclear Co center in *P. furiosus* prolidase based on analogy to *E. coli* MAP and aminopeptidase structures.

Confirmation of the structure of the *P. furiosus* prolidase metal center by X-ray crystal structure analysis has been successfully completed and will be more fully discussed in Section A-4a (Willingham et al. 2001; Maher, Ghosh et al. 2004).

3d. Proposed Prolidase Reaction Mechanism Based on Analogy to *E. coli* MAP

From comparisons of inhibited and native forms of *E. coli* MAP, kinetic analyses of MAP mutants, and spectral analyses of MAP metal centers in response to substrate binding, a reaction mechanism for the cleavage of N-terminal methionine residues by *E. coli* MAP has been proposed (Lowther et al. 1999; Lowther and Matthews 2000; Lowther and Matthews 2002). Given the structural correspondence that exists between the *E. coli* MAP and *P. furiosus* prolidase metal centers as well as the similarity in MAP and prolidase activities with both involving hydrolysis of peptide bonds, it is reasonable to

presume that an analogous reaction mechanism can be ascribed to prolidase. Thus, from analogy, it is predicted that cleavage of the X-Pro peptide bond occurs as follows: (1) substrate binds to the active site and is thought to activate the nucleophile (O_N) and facilitate proton transfer to glutamate residue 313 (E-313), (2) the carboxy anion of the resultant tetrahedral intermediate, originating from the oxygen of the scissile bond (O_C) is stabilized by the expanded coordination sphere of Co1 and interactions with histidine-192 (H-192) and histidine-291 (H-291), (3) resolution of the intermediate to products returns the coordination of Co₁ to five, while the metal bridging and H-291 interactions are maintained, and (4) the active site is fully regenerated upon release of the proline and deprotonation of solvent molecules (Figure 8). The validity of this reaction mechanism for *P. furiosus* prolidase will be investigated in the future using structure and mutation analyses.

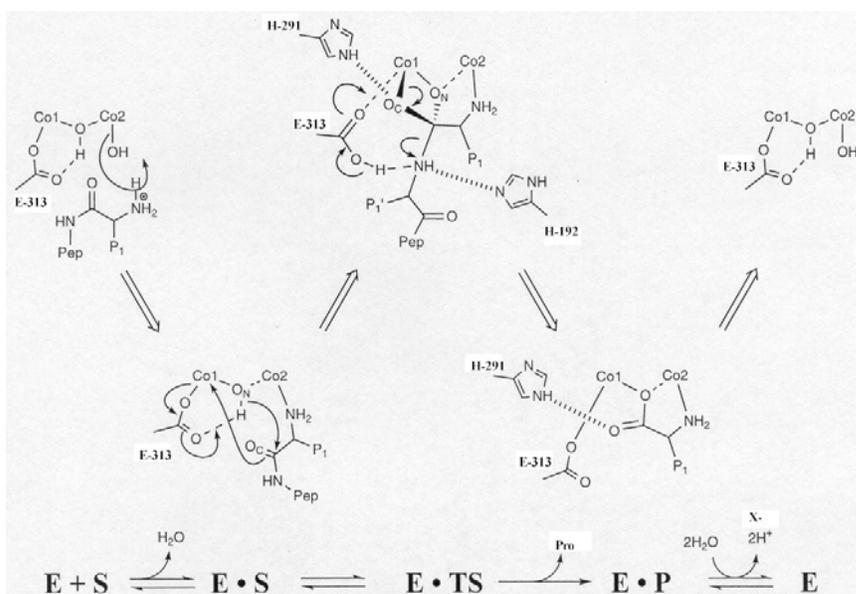


Figure 8. Proposed reaction mechanism for the hydrolysis of X-Pro dipeptides by prolidase based on analogy to the reaction mechanism predicted for *E. coli* MAP. (Figure modified from Lowther, 1999)

Statement of the Problem Studied

Based on work by Cheng and Defrank, the use of prolidases to detoxify OP nerve agent contamination has become feasible (Cheng et al. 1999). However, there remain questions in regards to the exact nature of the metal centers in prolidases, the identity of the amino acid residues that participate in substrate binding to these enzymes, as well as confirmation of the predicted reaction mechanism used by the enzyme. More specifically, it is currently unknown which of the two metal-binding sites is the tight-binding site and which is the labile site. Knowledge of the metal binding affinities of these sites will allow for targeted mutation of the metal binding sites with the ultimate goal of converting both sites to tight binding sites to potentially increase catalytic efficiency and relieve the requirement for exogenous metal for activity, which if prolidases are used for large scale decontaminations can represent a potential hazard of metal contamination of the treated site. Identification of the amino acid residues in prolidases which are specific for substrate binding will aid in targeting these amino acids for mutation to increase the affinity of prolidase for OP nerve agents. In short, answers to all of these questions will promote the design of more effective bioengineered versions of prolidases for use specifically in the degradation of OP nerve agents.

One long-term goal of this project is to fully determine the structure of *P. furiosus* prolidase particularly with respect to its metal centers and substrate binding regions. This structural information will provide insight into developing targeted bioengineering strategies to modify wild type *P. furiosus* prolidase to increase its hydrolytic cleavage of OP nerve agents, which is the ultimate long-term goal of this project. The specific objectives of this project, which are to be directly addressed during the funding period, are as follows:

- (1) To determine which of the two Co metal centers in *P. furiosus* prolidase is the tight-binding site and which is the loose-binding site
- (2) To identify amino acids in *P. furiosus* prolidase which participate in substrate binding and enzyme catalysis
- (3) To obtain *P. furiosus* prolidase variants which have increased catalytic activity at lower temperature ranges (ideally increased activity from 35 °C to 55 °C)

Summary of the Most Significant Results

(Advancements Made in Understanding *P. furiosus* Prolidase Structure As a Result of This ARO Funding)

1. Determination of the *P. furiosus* Prolidase Protein Structure

The structure of *P. furiosus* prolidase was recently solved by X-ray crystallographic analysis and refined at 2.0 Angstroms (Maher, Ghosh et al. 2004). *P. furiosus* prolidase is the first example of a solved prolidase structure. The *P. furiosus* prolidase crystals that were used for the structure analysis were determined to consist of homodimers of the enzyme, and that each of the two protein subunits has two domains (a C-terminal and N-terminal domain). The structure of a monomer of the *P. furiosus* prolidase is presented in Figure 9 and a stereo-view of the functional dimer is shown in figure 10.

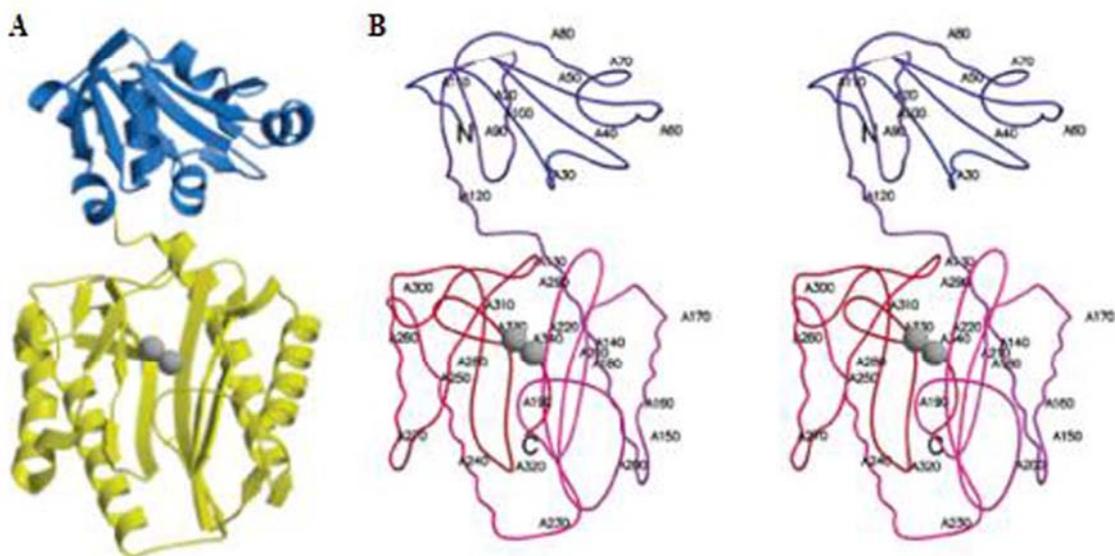


Figure 9. The structure of a monomer of *P. furiosus* prolidase. (A) Ribbon drawing of the monomer showing the domain structure; the N-terminal domain is in blue and the C-terminal domain is in yellow. The metal atoms are shown as the gray spheres. (B) A stereo-view presentation of the backbone trace of the monomer in the same orientation as shown in (A). The backbone is ramp-colored from blue to red with every tenth residue numbered. (Figure modified from Maher, 2004).

The subunit structure of *P. furiosus* prolidase contains the N-terminal domain (amino acid residues 1-112) an α -helical linker region (amino acids 113-123) and the C-terminal domain (amino acids 124-348). The N-terminal domain consists of a six-stranded mixed β -sheet flanked by five α -helices (three on one side of the β -sheet, two on the other). The C-terminal domain, which includes the catalytic site consisting of the Co-containing dinuclear metal cluster, is centered around a mixed six-stranded β -sheet with four α -helices on the outer surface. This β -sheet is strongly curved such that it has a “pita

bread” fold. The only direct contacts between the N- and C-terminal domains involve hydrogen bonds between the end of a small helix in the N-terminal domain (amino acid residues 24-32) and a β -turn in the C-terminal domain (amino acids residues 284-289). Additional hydrogen bonding contacts between the Arg-122 residue in the linker region and Gln-27 in the N-terminal domain and the Glu-289 and Glu-292 residues in the C-terminal domain also help to stabilize the prolidase tertiary structure.

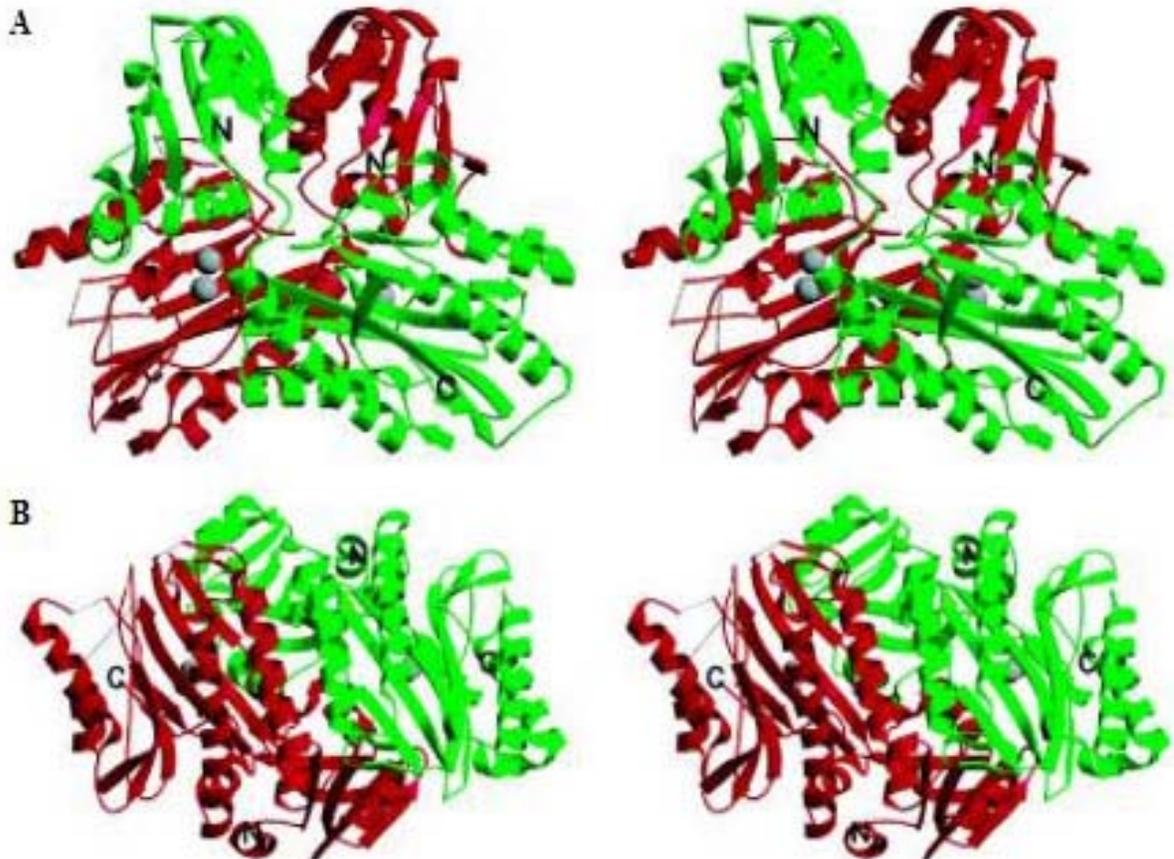


Figure 10. Stereo-views of the dimer of *P. furiosus* prolidase. Subunits 1 and 2 are colored green and red, respectively. Portions of the protein backbone that were not refined in the solved structure model are indicated by dashed gray lines. (A) Shows the view with the two-fold axis lying vertically in the plane of the paper. (B) Shows the view looking along the two-fold axis. (Figure modified from Maher, 2004).

The active site of the *P. furiosus* prolidase (Figure 11) is located in an oval depression formed on the inner surface of the curved β -sheet of the C-terminal domain of the protein. The primary feature of the active site is the presence of a dinuclear cobalt cluster, and as predicted by analogy to MAP and aminopeptidase metal centers, the amino acids Asp-209, Asp-220, His-284, Glu-313, and Glu-327 were shown to coordinate the metal ions. Specifically, it was shown that His-284 and Glu-313 are monodentate ligands binding solely to the first Co center (Co1), Asp-209 is a monodentate ligand binding solely to the second Co (Co2), and Asp-220 and Glu-327 serve as bidentate ligands of

both metal sites. The structure analysis also indicated that a water molecule identified as W176 functions as a bridging molecule for the metal center.

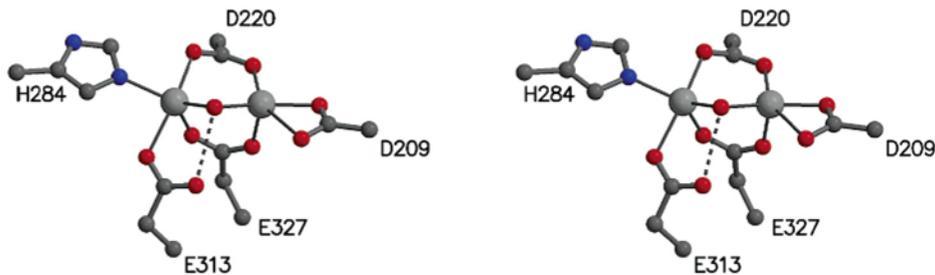


Figure 11. Stereo-view showing the active site of *P. furiosus* prolidase. The metal atoms are indicated as gray spheres. A hydrogen bond between Glu-313 and the bridging hydroxide ion is shown as a dotted line. (Figure modified from Maher, 2004).

A structure of *P. furiosus* prolidase bound with the potent inhibitor 3*R*-amino-2*S*-hydroxy-5-methyl-hexanoyl-proline (AHMH-Pro, $k_i = 16$ nM), which is a dipeptide with one β -amino acid (β 3-Leu-Pro) with a substituent on the 2- or α -position of Leu, was also solved. In the inhibitor-bound structure, it was demonstrated that the NH_2 -group of the inhibitor ligates the active site Co1 atom; that prolidase amino acid residues His-192 and His-291 form hydrogen bonds with the terminal carboxylate group and the O of the inhibitor, respectively; and that the $-\text{OH}$ group of the inhibitor bridges the two active site metal atoms. The first observation of the inhibitor-bound prolidase structure is consistent with the proposed reaction mechanism that predicts that the metal- NH_2 binding helps determine the specificity of the cleavage of the N-terminal residue of the substrate (Lowther and Matthews 2002). The second observation involving hydrogen bonding between the inhibitor and prolidase amino acid residues His-192 and His-291 was predicted based on the *E. coli* MAP reaction mechanism. The third observation involving the displacement of the hydroxide group is likely the greatest contributing factor to the inhibition since the same type of displacement has been observed in the inhibitor complexes of three other metalloproteases (Kim and Lipscomb 1993; Strater and Lipscomb 1995; Lowther et al. 1999).

Thus, in this funding period, in collaboration with Mitchell Guss at the School of Molecular and Microbial Biosciences at the University of Sydney and Michael W. W. Adams in the Department of Biochemistry and Molecular Biology at the University of Georgia, we have successfully solved the structure of *P. furiosus* prolidase. With the solved *P. furiosus* prolidase structure, we will now be able to make structure-informed decisions for specifically targeting regions of the *P. furiosus* prolidase enzyme to mutate in an effort to optimize its capabilities for OP nerve agent degradation.

2. Characterization of the Dinuclear Metal Center of *P. furiosus* Prolidase by Analysis of Targeted Mutants

Although the X-ray crystal structure analysis of *P. furiosus* prolidase as discussed above was able to confirm our predictions for the structure of the enzyme's metal center-

containing active site, the question as to which of the metal sites (Co1 or Co2) is the tightly-bound and which is loosely-bound (K_d 0.24 mM) site remained unresolved. Since an understanding of this is vital for potentially bioengineering prolidase to eliminate the requirement for the addition of exogenous Co for activity, we had undertaken studies to address this in particular in the previous funding period. Specifically to identify which Co site is tight-binding and which is loose-binding, site-directed mutagenesis was used to modify amino acid residues that participate in binding the Co1 (Glu-313 and His-284), the Co2 site (Asp-209) or the bidentate ligand (Glu-327). Metal-content, enzyme activity and CD-spectra analyses of the resulting D209A-, H284L, and E327L-prolidase mutants were then used to evaluate the mutants and determine which of the metal centers is tight-binding and which is loose-binding (Du et al. 2005). The specific amino acids in *P. furiosus* prolidase that were targeted for mutation are indicated in Figure 12.

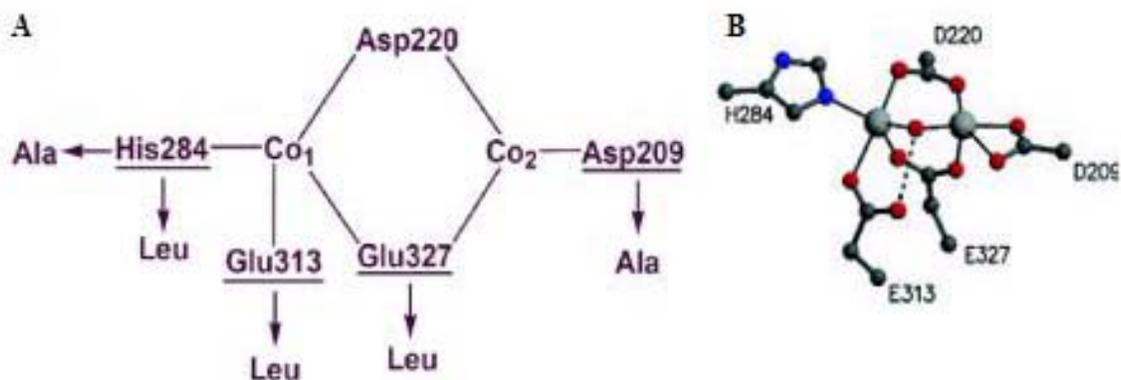


Figure 12. Dinuclear metal center active site of *P. furiosus* prolidase. (A) The amino acids that were targeted for mutation, and for comparison (B) the solved structure of the *P. furiosus* prolidase active site, where the Co atoms are indicated as gray spheres. (Figure modified from Du, 2005).

The D209A-, H284A-, H284L-, E313L-, and E327L-prolidase mutants were designed to systematically perturb the cobalt centers (as shown in Figure 12) in order to determine the resulting effect on metal content and enzymatic activity compared to wild type prolidase. Overexpression of the wild type and the five mutant *P. furiosus* prolidases was induced in the T7-RNA polymerase-producing *E. coli* strain BL21 (λ DE3). The wild type, D209A-, E327L-, H284A- and H284L-prolidases were each successfully purified by a multi-column chromatography strategy. However, we were unable to purify the E313L-prolidase. The E313L-prolidase protein was highly misfolded and remained aggregated and recalcitrant to purification techniques. Since the E313L-, H284A- and H284L-prolidases were each designed to affect the Co1 binding site, further analyses with the E313L-prolidase was not conducted.

As expected, mutations targeted to the either the Co1 or Co2 metal centers of *P. furiosus* prolidase significantly reduced prolidase activity. As shown in Table 1, the D209A-prolidase, which has a mutation affecting the Co2 metal center, had >1,300-fold reduction in activity compared to wild type prolidase. Single mutations affecting the Co1

metal center (H284A- or H284L-prolidase) had decreases of >2000-fold or 1,900-fold, respectively, compared to wild type prolidase activity. Mutation of the glutamate-327 residue (E327L-prolidase), which serves as a bidentate ligand for both the Co1 and Co2 metal-binding centers, resulted in no detectable activity. The activity data for the mutant prolidases confirm that occupation of both Co1 and Co2 is essential for prolidase activity, and that occupation of only the Co1 center provides the next highest activity, followed by occupation of only the Co2 binding site.

Table 1
Specific activity and cobalt content of purified wild-type and mutant *P. furiosus* prolidases

Prolidase	Specific activity ^a (U/mg)	Cobalt content (g-atom/subunit)
Wild type	1388 ± 220	0.76
D209A-prolidase (Co2) ^b	1.02 ± 0.21	0.70
H284A-prolidase (Co1)	0.67 ± 0.13	1.88
H284L-prolidase (Co1)	0.73 ± 0.18	0.28
E327L-prolidase (Co1/Co2)	N.D. ^c	0.03

^aThe specific activity is determined for purified protein and is reported as the average ± deviation of 9 values from 3 separate experiments.

^bThe Co-binding center that is disrupted in the mutant is indicated in parenthesis.

^cN.D., not detected.

To definitively determine which of the Co-binding centers is tight-binding and which is loose-binding, ICP emission spectrometry was used to evaluate the Co content of the purified wild type and mutant *P. furiosus* prolidases. From the Co-content data presented in Table 1, it was seen that D209A-prolidase (Co2 center mutant) contained 0.7 Co per subunit, a value close to that of the wild type enzyme (0.76 Co/subunit), suggesting that D209A-prolidase retains the tightly-bound Co like the wild type prolidase does. H284L-prolidase (Co1 center mutant) contained 0.28 Co/mol, which is 36% of the Co content of the wild type prolidase, indicating that the mutation targeted at the Co1 center disrupts binding of the normally tightly-bound Co. E327L-prolidase (Co1 and Co2 bidentate ligand mutant) contained only 4% of cobalt bound to the wild type (0.03 Co/ per subunit), confirming that disruption of both the Co1 and Co2 metal centers would prevent specific binding of Co to the enzyme. The loss of Co atoms found in the wild type and mutated enzymes is consistent with our expectations that Co²⁺ would be partially removed during the purification process, with the degree of loss depending on the tightness of the Co²⁺ binding sites. These metal content data, therefore, indicated that aspartate-209 participates in liganding the loose-binding Co (Co2), while the histidine-284 residue is involved in liganding the tight-binding Co (Co1).

The metal analysis data also indicated that the H284A-prolidase contained 1.88 Co/subunit, which is inconsistent with the determination that histidine-284 serves as a ligand for the tight-binding Co center, Co1. It was thought, therefore, that the anomalously high level of Co bound to the H284A-prolidase mutant was the result of

non-specific binding of Co to partially mis-folded protein. To determine whether this interpretation was correct, the secondary and tertiary structures of the wild type and all of the mutant *P. furiosus* prolidases were analyzed by circular dichroism in the far UV region (200 nm-250 nm) and near UV region (250 nm-320 nm) and it was shown that the H284A-prolidase mutant was highly unfolded. Apparently replacement of the amino acid histidine at position 284 with the small amino acid alanine dramatically changed the protein's folding order, while substitution of histidine with leucine at this position did not. The highly unfolded nature of the H284A-prolidase is undoubtedly responsible for the high levels of non-specifically bound Co that were observed for the H284A-prolidase mutant.

Thus, from the analysis of the *P. furiosus* prolidase metal-center mutants during this funding period, we were able to definitively establish that the Co1 atom occupies the tight-binding site and the Co2 atom occupies the loosely bound site. We are now in position to use this information to target amino acids participating in liganding the Co2 atom in an effort to improve the binding and potentially alleviate the requirement for the addition of cobalt for enzymatic activity.

3. Production of *P. furiosus* Mutants with Increased Catalytic Activity at Lower Temperatures Using Random Mutagenesis and a Positive Selection Method

Objective 3 was focused on broadening the temperature range at which *P. furiosus* prolidase has catalytic activity. Wild type *P. furiosus* prolidase is one of the most thermostable enzymes to have ever been studied (Adams et al. 1995; Grunden, Comfort et al. 2004) having maximal activity at 100 °C and little activity below 50 °C. While the considerable thermal stability exhibited by *P. furiosus* prolidase has prompted interest in possibly using the enzyme for OP nerve agent detoxification, its current limited activity at temperatures ranging from 30 °C to 50 °C would obviously restrict its utility in decontamination of exposed sites and equipment. Therefore, it was desirable to attempt to prepare and screen *P. furiosus* prolidase mutants that demonstrate increased activity at lower temperatures (30 °C to 50 °C). Besides the possibility of obtaining a better enzyme for OP nerve agent decontamination, isolation of such *P. furiosus* prolidase mutants would afford the important opportunity to investigate structural factors that influence protein thermostability, an area of study that remains woefully underdeveloped.

Random *P. furiosus* prolidase mutants will be prepared using standard, proven chemical and error-prone PCR mutagenesis procedures (Grunden et al. 1996; Samuelson et al. 2006) or by serial passage of the *P. furiosus* prolidase expression plasmid through the *E. coli* mutator strain XL1-Red (Bornscheuer et al. 1998). *P. furiosus* mutants with increased activity at lower temperatures will be selected for by using an *E. coli* strain that is auxotrophic for proline (has a *proA* deletion; *proA* encodes γ -glutamyl P reductase, the enzyme which catalyzes the second step in the conversion of L-glutamate to L-proline (Vogel and Davis 1952)) and has deletions in *pepQ* and *pepP*, dipeptidases with specificity for proline-containing dipeptides (Miller and Schwartz 1978).

This selection strain, JD1(λ DE3), was constructed in my laboratory using the following steps: 1) the *proA* mutant *E. coli* strain NK5525 (Nichols et al. 1998) (has a *proA::Tn10*, tetracycline resistance-encoding transposon disrupting *proA*) was obtained from the *E. coli* Genetic Stock Center (New Haven, CT). The *proA::Tn10* mutation was generated in the wild type *E. coli* strain BW454 via P1 phage infection using P1 phage lysates that had been harvested from P1-infected NK5525 cells. *E. coli* strain BW454 was chosen as the starting lineage for developing the *P. furiosus* prolidase mutant selection strain because it is considered a near wild type *E. coli* strain that is amenable to genetic manipulation and supports good protein overexpression; (2) Since *Tn10* insertions can be unstable under nutritional stress, resulting in their excision from the genome, fusaric acid selection was used to generate a permanent deletion in *proA* of the *E. coli* strain (Bochner et al. 1980); (3) A one-step inactivation of chromosomal genes method (Datsenko and Wanner 2000) was used to inactivate *E. coli pepP* by inserting a chloramphenicol resistance cassette within the *pepP* gene, and to inactivate *pepQ* by inserting a kanamycin resistance cassette into the *pepQ* gene; (4) Lambda infection was used to introduce λ DE3, which encodes the T7-RNA polymerase under control of the *lac* promoter, into the *E. coli* strain so that it could support high level protein expression from the prolidase expression plasmids (Figure 13).

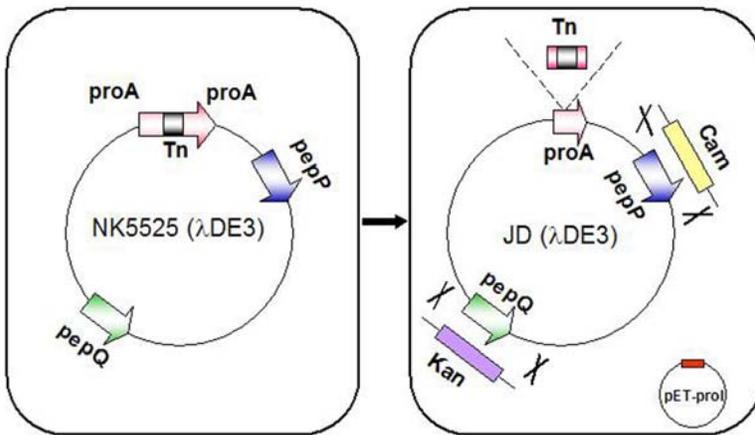


Figure 13. Scheme for construction of *E. coli* strain JD1(λ DE3) that is used for selection of the *P. furiosus* prolidase mutants.

Use of this *E. coli* proline auxotroph, JD1(λ DE3) strain will provide a powerful positive selection for recombinant *P. furiosus* prolidase mutants that have activity at temperatures required for *E. coli* growth (30 °C – 45 °C) when the *E. coli* strain is plated on M9 minimal media supplemented with 0.1 mM Met-Pro or Leu-Pro as the only source of proline. Only those *E. coli* cells expressing mutant *P. furiosus* prolidase that is active at the *E. coli* growth temperatures will be able to form colonies since active *P. furiosus* prolidase would be required to supply proline to the cells from the hydrolysis of Met-Pro or Leu-Pro as shown in Figure 14.

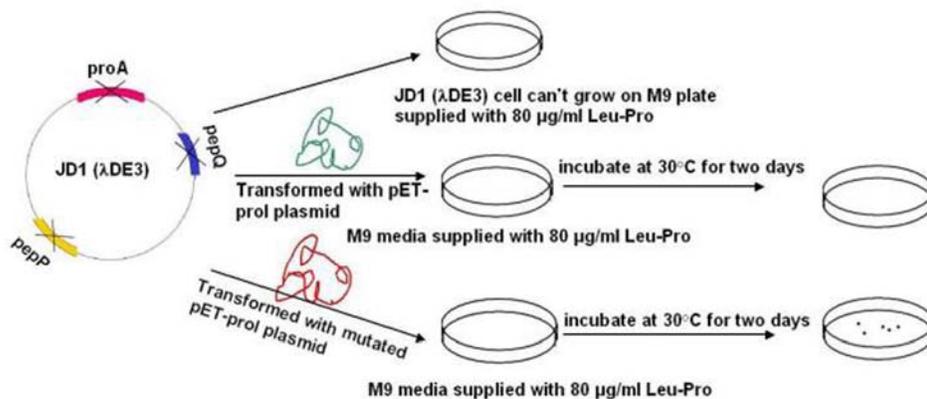


Figure 14. Strategy used for selection of low-temperature active *P. furiosus* prolidase mutants.

The strategy outlined above for generation and selection of the low-temperature active *P. furiosus* prolidase has recently been conducted in my laboratory to demonstrate its effectiveness for isolating prolidase enzymes with higher catalytic activity at lower temperatures ($< 50\text{ }^{\circ}\text{C}$) compared to wild type prolidase. Using this method, we have been able to isolate a *P. furiosus* prolidase expression plasmid that coded for a mutated prolidase with a change from glycine at amino acid position 39 to glutamate (G39E-prolidase). The G39E-prolidase was over-expressed, purified by multi-step chromatography and was evaluated for enzyme activity over a range of temperatures ($10\text{ }^{\circ}\text{C}$ to $100\text{ }^{\circ}\text{C}$). Enzyme activities demonstrated that the G39E-prolidase has ~2-fold higher activity compared to wild type when assayed at temperatures ranging from $30\text{ }^{\circ}\text{C}$ to $50\text{ }^{\circ}\text{C}$ (Figure 15). The finding that a change from glycine to glutamate at amino acid position 39 in *P. furiosus* prolidase increases activity at lower temperatures is supported by the *P. furiosus* structure data, which indicates that Gly-39 crowds the active site and is potentially involved with stabilizing N-terminal-C-terminal domain contacts (Maher, Ghosh et al. 2004). Presumably the change to Glu-39 provides increased flexibility of the prolidase enzyme, thereby supporting increased activity at lower temperature. Based on these initial positive results, we will now be extending our mutation generation/selection strategy by using the G39E-prolidase expression plasmid as the template for the next round of chemical or PCR-error prone mutagenesis in an effort to isolate a *P. furiosus* prolidase mutant with even greater activity at sub-optimal temperatures ($30\text{ }^{\circ}\text{C}$ to $50\text{ }^{\circ}\text{C}$).

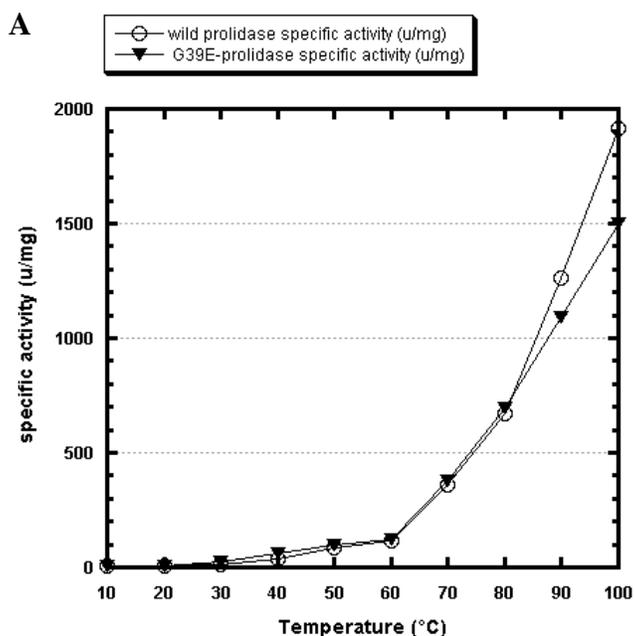
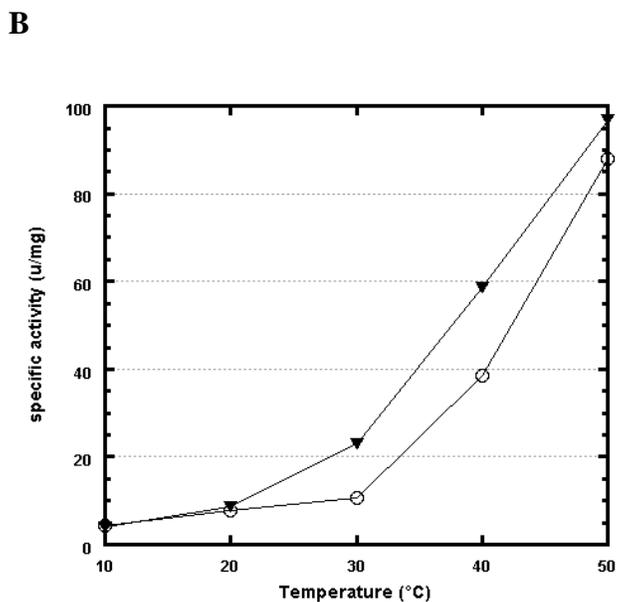


Figure 15. (A) Comparison of activities of wild type and G39E-prolidase at assay temperatures ranging from 10 °C to 100 °C. (B) Wild type and G39E-prolidase activities when assayed at temperatures ranging from 10 °C to 50 °C.



P. furiosus prolidase mutants selected using the described selection method will be subjected to activity temperature profiles, and mutants which demonstrate increased activity at lower temperatures compared to wild type will be sequenced to determine the nature of the mutation, purified for biochemical and X-ray structure analyses as well as screened for activity against OP nerve agents in collaboration with Dr. Joseph Defrank. Ultimately the mutants that are isolated from these experiments should prove very important both in producing a *P. furiosus* prolidase which is better suited for use in OP nerve agent detoxification and in potentially providing useful information as to structural determinants required for thermal stability in proteins.

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