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14. ABSTRACT The aims of this project were to structurally study and bioengineer thermostable prolidases from Pyrococcus furiosus (Pf) and horikoshii (Ph) to enable their use for organophosphorus nerve agent detoxification. Pf prolidase contains one dinuclear Co metal-center/monomer and has optimal activity at 100°C, exhibiting no activity without Co ²⁺ 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					
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Report Title

Final Report: Structural Analysis and Bioengineering of Thermostable Pyrococcus furiosus Prolidase for the Optimization of Organophosphorus Nerve Agent Detoxification

ABSTRACT

The aims of this project were to structurally study and bioengineer thermostable prolidases from Pyrococcus furiosus (Pf) and horikoshii (Ph) to enable their use for organophosphorus nerve agent detoxification. Pf prolidase contains one dinuclear Co metal-center/monomer and has optimal activity at 100°C, exhibiting no activity without 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. Our understanding of the metal cluster structures was used in this project to design a prolidase mutant with two integral metal sites, which will relieve the requirement for exogenous metal and improve its efficacy in OP nerve agent detoxification. In addition, a Ph prolidase homolog has been identified and has been biochemically characterized to determine its potential utility in OP nerve agent decontamination. Another objective was to produce Pf and Ph prolidase mutants that have increased catalytic activity over temperatures ranging from 20-60°C to increase their use for decontamination of OP nerve agents in the field. To this end, a random mutation and low-temperature selection method was successfully used.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

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

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

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

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Grunden AM. "Biotechnological Applications of Extremophiles". Invited Speaker. Sigma Xi Science at Starlight Seminar Series, March 21, 2011.

Grunden AM. "Thermostable Archaeal Prolidases and Their Use in Toxic Organosphosphates Degradation". NIH Proline Metabolism Conference, Lincoln, NE. November 8, 2010.

Grunden, AM. "Biotechnological Applications of Extremophiles" Invited NC Lecturer. NC American Society for Microbiology, October 2010.

Grunden AM. "Generation of Recombinant Pyrococcus Prolidases with Enhanced Activity Over a broad Temperature Range for Detoxification of Organophosphorus Nerve Agents". Chemical and Biological Defense Program Enzyme Colloquium. Falls Church, VA, September 22, 2010.

Theriot C, Tove S, Grunden AM. 2009. Generation of Recombinant Pyrococcus furiosus Prolidases with Enhanced Activity over a Broader Temperature Range for Detoxification of Organophosphorus Nerve Agents. 109th General Meeting of the American Society for Microbiology, Philadelphia, PA, May.

Theriot C, Tove S, Grunden AM. 2008. Generation of hyperthermophilic Pyrococcus furiosus prolidase mutants with higher catalytic activity at lower temperatures. 108th General Meeting of the American Society for Microbiology, Boston, MA, June.

Grunden, AM. "Biotechnological Applications of Extremophile Enzymes". Department of Microbiology. North Carolina State University. April, 2008.

Theriot CM, Quintero-Varca T, Joshi, P, Tove, S, Grunden AM. 2007. Expression and biochemical characterization of Pyrococcus horikoshii prolidase homolog 1 and 2 and Pyrococcus furiosus homology 2 and their potential for the detoxification of organophosphorus nerve agents. 107th General Meeting of the American Society for Microbiology, Toronto, CAN.

Theriot CM, Quintero-Varca T, Joshi, P, Tove, S, Grunden AM. 2007. Expression and biochemical characterization of Pyrococcus horikoshii prolidase homolog 1 and 2 and Pyrococcus furiosus homology 2 and their potential for the detoxification of organophosphorus nerve agents. 2007 Meeting North Carolina Branch of the American Society for Microbiology, Greensboro, NC.

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TOTAL:

Number of Manuscripts:

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Received Paper

TOTAL:

Patents Submitted

Invention Disclosure:

~~Microfluidic Device for the Analysis of Prolidase Activity~~

Patents Awarded

Awards

Named NASA Institute for Advanced Concepts Fellow

Named Park Faculty Scholar, 2006

Named North Carolina State University Outstanding Teacher, 2007

Named NSF ADVANCE Scholar Emerging Leader, 2009

Named DOE ARPA-E award recipient, 2011

Named NCSU College of Agriculture and Life Sciences Outstanding Graduate Instructor

Named as President of the North Carolina Chapter President of the American Society for Microbiology

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Xuelian Du	1.00	
Casey Theriot	1.00	
Rebecca Semcer	0.00	
FTE Equivalent:	2.00	
Total Number:	3	

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 M Grunden	0.00	
FTE Equivalent:	0.00	
Total Number:	1	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	Discipline
Prashant Joshi	0.00	Biochemistry
Carla Pistole	0.00	Microbiology
Tatiana Quintero-Varca	0.00	Biology
Julie Shelton	0.00	Biology
Eli Tiller	0.00	Biochemistry
FTE Equivalent:	0.00	
Total Number:	5	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

- The number of undergraduates funded by this agreement who graduated during this period: 5.00
- The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 5.00
- The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 3.00
- Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 5.00
- Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00
- The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00
- The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 2.00

Names of Personnel receiving masters degrees

<u>NAME</u>
Total Number:

Names of personnel receiving PHDs

<u>NAME</u>
Xuelian Du
Casey Theriot
Total Number:

Names of other research staff

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
Dr. Sherry Tove	0.20
FTE Equivalent:	0.20
Total Number:	1

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Inventions (DD882)

Scientific Progress

The aims of this project were to structurally study and bioengineer thermostable prolidases from *Pyrococcus furiosus* (Pf) and *horikoshii* (Ph) to enable their use for organophosphorus nerve agent detoxification. Pf prolidase contains one dinuclear Co metal-center/monomer and has optimal activity at 100°C, exhibiting no activity without 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. Our understanding of the metal cluster structures was used in this project to design a prolidase mutant with two integral metal sites, which will relieve the requirement for exogenous metal and improve its efficacy in OP nerve agent detoxification. In addition, a Ph prolidase homolog has been identified and has been biochemically characterized to determine its potential utility in OP nerve agent decontamination. Another objective was to produce Pf and Ph prolidase mutants that have increased catalytic activity over temperatures ranging from 20-60°C to increase their use for decontamination of OP nerve agents in the field. To this end, a random mutation and low-temperature selection method was successfully used.

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Statement of the Problem Studied

Certain prolidases have been shown to have activity against toxic organophosphorus (OP)-containing compounds such as diisopropylfluorophosphate (DFP), and the chemical warfare agents sarin, soman and tabun (DeFrank and Cheng, 1991). Prolidases from various bacteria and archaea, as well as human prolidase, are capable of hydrolyzing these compounds into their components, thus rendering them harmless (DeFrank and Cheng, 1991, Wang et al., 1998, Park et al., 2004, Theriot et al., 2010a). OP compounds are biodegradable, yet they are extremely toxic to mammals because they bind to acetyl cholinesterase and render it inactive leading to a buildup of the neurotransmitter acetylcholine in the synapse. Acetylcholine then produces a continuous signal resulting in hyper salivation, confusion, convulsions, respiratory complications, respiratory failure, coma and death. OP compounds exist mainly in the form of pesticides and chemical warfare agents (CWAs), and it is estimated that 38% of the pesticides used worldwide contain OP, and approximately 200,000 tons of OP nerve agents are stockpiled worldwide (Singh, 2009). Annually, there are an estimated 3 million poisonings and 300,000 human deaths owing to OP compounds (Singh, 2009). There is a need for OP detoxification strategies that are safe for humans and the environment. Enzymes from several microorganisms have been shown to degrade a range of OP compounds; however, an ideal enzyme or combination of enzymes to be used in a decontamination cocktail would be able to degrade all OP compounds while remaining stable for an extended period of time at a range of temperatures and pH.

Several microbial enzymes, such as organophosphorus acid anhydrolase (OPAA) and phosphotriesterase (PTE), are capable of degrading OP compounds and have been extensively studied for their high activity and specificity against G-type nerve agents (Singh, 2009). The stability of these enzymes, however, is limited when incorporated into formulations containing solvents and other denaturants. Additionally, high concentrations of metals must be added to formulations containing these enzymes in order to maximize their activity which poses further complications (Theriot et al., 2011). More recent studies have included recombinant prolidases from hyperthermophilic archaea (Vyas et al., 2010, Theriot and Grunden, 2010). Wild type and mutant prolidases from *Pyrococcus furiosus* and *Pyrococcus horikoshii* have been characterized and show promising enzymatic properties as candidates for OP decontamination in that they are highly thermostable with broad substrate specificity.

The long-term goal of this project was to utilize the structural information we now possess for *Pyrococcus furiosus* prolidase, particularly with respect to its metal centers and substrate binding regions, to improve the performance of *P. furiosus* prolidase

for degrading OP nerve agents. Specifically, this structural information has provided insight into developing informed targeted bioengineering strategies to modify wild type *P. furiosus* prolidase to increase its hydrolytic cleavage of OP nerve agents. In addition, with the recent completion of the genomic sequences of related *Pyrococcus* species, *Pyrococcus abyssi* and *Pyrococcus horikoshii*, other candidate prolidases have been identified, which were also shown to have utility for OP nerve agent degradation. Therefore, the specific objectives of this project, which were to be directly addressed during the three year funding period, were as follows:

- (1) Use a targeted mutation approach to specifically modify the *P. furiosus* prolidase in an effort to improve performance in OP nerve agent degradation.
- (2) Use a random mutagenesis approach to obtain *P. furiosus* prolidase mutants that have increased catalytic activity at lower temperature ranges (ideally increased activity from 35 °C to 55 °C).
- (3) Biochemically characterize other identified *Pyrococcus* prolidases to determine their potential use in OP nerve agent degradation.

Summary of Most Important Results

In order to accomplish the project goals described above specific experimental objectives had been proposed for the study. The results of the efforts targeted at these experimental objectives are described below.

Objective 1. Use targeted mutation strategies informed by the solved structure to improve *P. furiosus* prolidase function for OP nerve agent decontamination

Objective 1a. Use of a targeted mutation approach to convert the loose-binding metal center (Co2) to a tight-binding center

Based on studies by Du et al., it was shown that the Co1 atom is tightly bound and the Co2 atom loosely bound. Because the Co2 atom is only loosely bound in the prolidase active site (Kd 0.24 mM), additional cobalt is required for activity. Modification of the active site such that the Co2 atom is bound more tightly would significantly improve the potential of using *P. furiosus* prolidase for large-scale OP nerve agent detoxification. Thus, for Objective 1a, we had proposed to modify the following amino acid residues that are directly involved in liganding the Co2 atom: Asp-209 was to be changed to either a histidine or glutamate residue and Asp-220 was to be changed to a glutamate. In addition amino acid residues Phe-178 and Ile-290, which are located in close proximity to the Co2 binding site were changed to aspartate or glutamate in an effort to provide additional charge attraction to help bind the Co2 cobalt atom more tightly.

Objective 1a Research Results:

For this study, the following metal center prolidase mutants had been prepared: A209E-prolidase (change of aspartate at position 209 to glutamate), A209H-prolidase, and A220E-prolidase. The Phe-178 and Ile-290 positions were also modified to F178E and I290E. The genes encoding the metal center mutants that have been produced have been fully sequenced to verify the correct positioning of the targeted mutation and to confirm that no other mutations arose. Preliminary activity assays for the mutant proteins in crude extract revealed little activity. Protein gels had confirmed good production of soluble prolidases. Therefore, the poor activity associated with the mutants may be due to (1) failure of cobalt to bind to the modified Co2 binding site or (2) cobalt did in fact bind tightly to the Co2 site, but this tight binding interfered with the catalytic function of the enzyme. Given what we now know about the reaction mechanism used by prolidases (Alberto et al., 2011), in which the second (labile metal atom) is thought to enter the active site when the substrate is bound, it is most likely that having two tightly bound Co in the prolidase active site actually prevents substrate binding and catalysis. This is consistent with our findings for the metal center mutant prolidases.

Objective 1b. Use of a targeted mutation approach to modify amino acid residues important for substrate binding and enzyme catalysis in *P. furiosus* prolidase

Previous biochemical and structural analysis of the highly related enzyme *E. coli* methionine aminopeptidase had identified a number of amino acid residues that are required for substrate binding and enzyme catalysis. Among these, His-79 and Gln-238 are involved in productive binding of substrate and His-178 in stabilization of the enzyme-substrate transition state. By analogy, His-192, Arg-325, and His-291, respectively, were predicted to participate in cognate roles in *P. furiosus* prolidase, a prediction that was confirmed by the recent *P. furiosus* prolidase structure studies. Armed with this structure information, the *P. furiosus* prolidase amino acid residues His-192, His-291, and Arg-325 were initially targeted for mutation (H192L, H291L, and R325L).

Objective 1b Research Results:

After initial screening of the H192L, H291L-, and R325L-prolidase mutants for enzyme activity, mutant proteins, which exhibit differences in activity compared to wild type were to be purified in large scale and subjected to structural analysis and substrate/inhibitor binding studies. The *P. furiosus* H192L-, H291L-, and R325L-prolidase mutants have been generated and verified by DNA sequencing. Preliminary activity assays showed negligible activity, confirming our initial analysis that these residues are key for prolidase activity. Further insight derived from mutant structure will be used to further inform targeted mutations that may improve OP nerve agent binding to prolidase.

Objective 1c. Use of targeted mutation approaches to increase flexibility, and therefore, presumably activity of *P. furiosus* prolidase at lower temperatures

Studies of other high-temperature active enzymes have shown that removal of N-terminal regions or C-terminal regions of these proteins can in some cases increase the flexibility of the enzymes and increase their activities at low temperature. For *P. furiosus* prolidase, the N-terminal region of the protein is primarily involved in providing structural contacts for dimerization, whereas the C-terminal domain has the catalytic sites. Therefore, we focused on removing some of the N-terminal residues to determine whether the truncated form of the protein would have increased flexibility and higher activity at lower temperatures. Since there is a methionine residue at amino acid position 12 in the *P. furiosus* prolidase sequence, we designed PCR primers that would allow us to clone the *P. furiosus* prolidase into the pET expression system (pET-21b) such that Met-12 served as the start Met, resulting in a *P. furiosus* prolidase with a deletion of the first 11 residues. Another structure-informed targeted mutation that we wish to pursue involves the mutation of amino acid residue Gln-27. *P. furiosus* prolidase structure studies showed that Gln-27 is involved in hydrogen-bonding to Arg-122 that lies in the linker helix in domain II of the enzyme. This hydrogen bonding is considered to be important for constraining the flexibility of the enzyme. Thus, we specifically targeted Gln-27 and/or Arg-122 for mutation studies with the specific goal of switching the indicated amino acids to similarly sized amino acids that would not participate in hydrogen bonding.

Objective 1c Research Results:

We were able to successfully over-express this truncated form of the protein and evaluated its activity in comparison to the wild type *P. furiosus* enzyme at 37, 50 and 100°C. The activity results revealed that the N-terminal truncated protein had 12.4, 3.8, and 213.8-fold lower activity at 37, 50 and 100°C, respectively. Given that the N-terminal truncated *P. furiosus* enzyme did not appear to have greater activity at lower temperatures (37 and 50°C), and in fact, had impaired activity at all temperatures evaluated compared to wild type, studies of this particular mutant will not be continued.

Objective 2a. Production of *P. furiosus* Mutants with Increased Catalytic Activity at Lower Temperatures Using Random Mutagenesis and a Positive Selection Method

Wild type *P. furiosus* prolidase is one of the most thermostable enzymes to have ever been studied 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 is 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.

Objective 2a Research Results:

Random *P. furiosus* prolidase mutants had been prepared using standard, proven chemical and error-prone PCR mutagenesis procedures or by serial passage of the *P. furiosus* prolidase expression plasmid through the *E. coli* mutator strain XL1-Red. *P. furiosus* mutants with increased activity at lower temperatures were selected for by using an *E. coli* strain JD1(ΔDE3) that was developed in my laboratory that is auxotrophic for proline (has a proA deletion; proA encodes L-glutamyl P reductase, the enzyme which catalyzes the second step in the conversion of L-glutamate to L-proline) and has deletions in pepQ and pepP, dipeptidases with specificity for proline-containing dipeptides. The use of the *E. coli* proline auxotroph, JD1(ΔDE3) strain provides 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.

Using our mutagenesis and selection strategy, 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) and one with a change from glutamate at position 236 to valine (E236V-prolidase). The G39E-prolidase was over-expressed, purified by multi-step chromatography and was evaluated for enzyme activity over a range of temperatures (10 °C to 100 °C). Enzyme activities demonstrated that the G39E-prolidase has ~2-fold higher activity compared to wild type when assayed at temperatures ranging from 30°C to 50°C. 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. Presumably the change to Glu-39 provides increased flexibility of the prolidase enzyme, thereby supporting increased activity at lower temperature. The E236V-prolidase mutant also had ~2-fold increased activity compared to wild type prolidase when assayed at temperatures ranging from 30°C to 50°C. Based on the pf prolidase structure analysis, the glutamate-236 residue appears to be involved in dimerization and therefore, the change to a non-charged residue may be providing increased flexibility in the protein at low temperature.

Based on these initial positive results, we had extended our mutation generation/selection strategy by using the G39E-, E236V-, and G39E,E236V- Pf⁺ expression plasmids as the template for subsequent rounds 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°C to 50°C). We have completed three separate screenings for plasmids generated using error-prone PCR and passage through the mutator *E. coli* strain for the three different templates. For each screen over 100 colonies that grew up on the selection plates were analyzed for activity. Only one of the selected colonies expressed a mutant prolidase that had consistently higher activity (greater than two-fold compared to wildtype) at low temperature (30°C) when assayed in triplicate in three biological repeats. The DNA encoding for the prolidase was sequenced and it was found to have only the G36E mutation. In light of the poor performance of our initial strategy for obtaining useful Pf prolidase mutants, we reevaluated our mutagenesis and selection strategy and had determined the inefficient step of the initial process was the generation of the expression plasmids with actual mutations. Therefore, we switched to using the newly available Genemorph2 Random mutagenesis kit from Stratagene which features an error-prone Taq polymerase that can provide a low level, medium level or high-level mutation rate depending on the reaction conditions used. We were then able to generate pools of mutant prolidase expression plasmids at the low, medium and high mutation rates using the Genemorph2 Random mutagenesis kit and had plated approximately 200 selection plates and screened a total of 75 colonies that grew up on the plates. From this screen, one mutant, named Prolidase mutant 10, was shown to have increased activity at both 35°C and 100°C in comparison to the wild type *P. furiosus* enzyme. Sequence analysis of the mutant 10 revealed that the protein had the following amino acid substitutions: arginine at position 19 was changed to glycine and lysine at position 71 was changed to glutamate. Activity assays for wild type and mutant 10 were prepared using 35 and 100°C incubation temperatures, and activity comparisons indicated that mutant 10 had a 5.3-fold higher activity at 35°C and 3-fold higher activity at 100°C. This was an especially interesting result since relative activity increases were observed at both low (35°C) and high (100°C) temperatures for mutant 10, and often mutations that improve low temperature activity can compromise high temperature activity.

Since both the original G39E-prolidase and mutant 10 had shown increased activity at low temperature (35°C, 2-fold and 5.3-fold increase, respectively), a combined mutant was made in which the G39E mutation was introduced to mutant 10. This new mutant, G39E-mutant10, has been over-expressed and purified using multi-column chromatography. The purified G39E-mutant10 protein was evaluated for its activity over a range of temperatures and its activities were compared to the wild type, mutant10 and G39E-prolidase mutant activities.

The G39E-, G39E,E236V-, mutant10- and G39E-mutant10 *P. furiosus* prolidase mutants were completely biochemically characterized for their activity with the dipeptides Met-Pro and Leu-Pro over a broad range of temperatures and pH. In addition, these mutants were also tested at the Edgewood Chemical Biological Center at Aberdeen Proving Ground for their activities against the OP nerve agents DFP and p-nitrophenyl soman. The *P. furiosus* mutant prolidase studies were initially described in the publication (Theriot, C. M. & Grunden, A. M. 2010. Hydrolysis of organophosphorus compounds by microbial enzymes. *Appl Microbiol Biotechnol*, 89, 35-43). The full biochemical characterization of the *Pyrococcus* mutants was published in the journal *Applied Microbiology and Biotechnology* (Theriot CM, Tove SR, Grunden AM. 2011. Improving the Catalytic Activity of Hyperthermophilic *Pyrococcus* Prolidases for Detoxification of Organophosphorus Nerve Agents over a Broad Range of Temperatures. *Applied Microbiology and Biotechnology*, volume 87 pages 1715-1726).

Objective 2b. Production of *P. furiosus* Mutants with Increased Catalytic Activity at Lower Temperatures Using a Natural Evolution Approach

Another approach for improving the low temperature activity of *P. furiosus* prolidase is to allow it to evolve naturally over time under selective pressure. For this method, the wild type *P. furiosus* expression prolidase plasmid was transformed into the prolidase deficient, proline auxotrophic strain JD1 that was described above. These transformants were grown in 100 ml cultures using liquid selection media equivalent to the selection media described above (except that no agar is added). Two sets of cultures were grown (one set at 30°C and another at 37°C) on the selection media with Leu-Pro as the only source of

proline and with IPTG (0.1 mM) to induce prolidase expression and ampicillin (100 µg/ml) to maintain the expression plasmid. As a control, to see the growth rate of these cells, a set of similar cultures were grown with media that has proline in place of Leu-Pro (no IPTG). It was expected that it would take the cultures without proline a long time to grow. Cell growth of the cultures was evaluated and once cultures had shown growth, cells were serially transferred into fresh selection media. This process was repeated a number of times in hopes of isolating cells which may have naturally occurring mutations in the *P. furiosus* prolidase that enable efficient hydrolysis of Leu-Pro at low temperatures. Once fast-growing cells have been isolated from the liquid selection media cultures, expression plasmids were isolated from the cells and the plasmids were sequenced to determine what mutations occurred.

Objective 2b Research Results:

The *P. furiosus* prolidase expressing JD-1 cells were grown in the liquid selection media and the growth of the cultures was tracked. Transfers were made into fresh media (1% inoculum) once cells in the cultures had reached early stationary phase. Unfortunately this approach did not provide us with additional mutants to further characterize based on our sequencing of resulting isolates and we did not continue any further with this approach.

Objective 3. Biochemically characterize other identified *Pyrococcus* prolidases to determine their potential use in OP nerve agent degradation

Recently the genomic sequences of related *Pyrococcus* species, *Pyrococcus abyssi* and *Pyrococcus horikoshii* had been completed. An analysis of the three *Pyrococcus* genomes enabled the identification of three categories of prolidases or prolidase homologs: (1) prolidases annotated as a prolidase based on high-level identity to the biochemically characterized *P. furiosus* prolidase (>75% identity); (2) prolidase homologs that have significant levels of homology to *P. furiosus* prolidase (> 30% identity) and have all five matching conserved metal center-liganding amino acid residues (homolog-1 classification); and (3) prolidase homologs that share levels of identity greater than 25% but less than 30% and have only 2 of the 5 matching conserved metal-center liganding amino acids (homolog-2 classification). The prolidase homolog-2 proteins do have conserved amongst themselves all of the same types of amino acids present in the *P. furiosus* metal center-liganding amino acids but with alternate spacing (for example, in *P. furiosus* prolidase, the D-209 and D-220 amino acids have 10 intervening amino acids, whereas, the prolidase homolog-2 proteins have 15, and the spacing between the *P. furiosus* prolidase E-313 and E-327 residues is 13 amino acids, while the spacing between the prolidase homolog-2 proteins is five amino acids). From the homology analysis, it was determined that all three *Pyrococcus* have prolidase proteins, that only *P. abyssi* and *P. horikoshii* have prolidase homolog-1 proteins, and that all three organisms have prolidase homolog-2 proteins.

Objective 3 Research Results:

It is of considerable interest to determine how similar the prolidase homolog-1 and homolog-2 proteins in terms of enzyme activity, temperature profile, substrate preference, and metal preference are to *P. furiosus* prolidase and ultimately to determine whether any of these homologs would also have utility for OP nerve agent degradation. Therefore, we cloned, recombinantly expressed, purified, and biochemically characterized prolidase homolog-1 proteins from *P. horikoshii* and *P. abyssi* as well as prolidase homolog-2 proteins initially from *P. furiosus*. Since the nature of the metal-content for the prolidase homolog-1 and homolog-2 proteins is of prime importance to evaluating their potential efficacy for OP nerve agent degradation, the metal content has been evaluated for both the recombinant versions of the proteins using ICP emission spectroscopy as well as for the native enzymes.

From our biochemical analysis of Ph prolidase homolog-1 it was determined that the enzyme showed maximal activity with the addition of the metal ion Co^{2+} and with the substrate Met-Pro ($k_m = 4.0$) and 88% activity with Leu-Pro. Prolidase homolog-1 activity was also shown to be supported by Mn^{2+} (79% activity compared to Co^{2+}). As with Pf prolidase, Ph prolidase homolog-1 showed substrate specificity for dipeptides with non-polar amino acids (Met, Leu, Ala, or Arg) at the N-terminus and a proline at the C-terminus. It also showed maximal catalytic activity at pH 7.0 and at 100°C. Under temperatures of 80°C, Ph prolidase homolog-1 lost 60% activity and below 50°C it decreased to 10% activity. Thus, the characterization profile of the Ph prolidase homolog-1 described above is very similar to the profile of the previously characterized recombinant Pf prolidase. The only significant difference appeared to be the dissociation constants for the metals. Ph prolidase homolog-1 showed dissociation constants of 0.05 mM with Co^{2+} and greater than 0.8 mM for Mn^{2+} , whereas recombinant Pf prolidase displayed a dissociation constant of 0.5 mM for Co^{2+} and 0.66 mM for Mn^{2+} . The 10-fold lower dissociation constants for cobalt for Ph prolidase homolog-1 compared to Pf prolidase suggests that the *P. horikoshii* prolidase binds its metal more tightly than the *P. furiosus* enzyme and as such may be a better choice for OP nerve agent detoxification.

We sent the *P. horikoshii* prolidase homolog-1 to Dr. Joseph DeFrank, my collaborator at the U.S. Army Edgewood Research, Development and Engineering Center to test the enzyme for OP hydrolysis activity. Saumil Shah in Dr. DeFrank's laboratory performed OP hydrolysis assays using diisopropylfluorophosphate (DFP) as the OP substrate. Their activity data indicated that the *P. horikoshii* prolidase homolog-1 enzyme did have activity against DFP (5.3 µmoles/min/mg when assayed at 35°C), however, this was very low activity compared to that seen for *Alteromonas* OPAA (prolidase) which is 1820 µmoles/min/mg.

We have also cloned, expressed, and assayed *P. horikoshii* prolidase homolog-2. Although, Ph prolidase homolog-2 was successfully expressed as a soluble enzyme, it does not appear to have any prolidase activity when six different proline dipeptides were used as substrates. Based on sequence alignments with other metal-activated hydrolases, it appears that Ph-prolidase homolog-2 has sequence motifs similar to motifs found in methionine aminopeptidases. However, when we assayed it for aminopeptidase activity, no activity was detected, thus ruling out its function as an aminopeptidase.

The biochemical characterization studies of *P. horikoshii* prolidase (PH1149) and prolidase homolog1 (PH0974) were published in the journal *Applied Microbiology and Biotechnology* (Theriot CM, Tove SR, Grunden AM. 2010b. Characterization of Two Proline Dipeptidases (Prolidases) from the Hyperthermophilic Archaeon *Pyrococcus horikoshii*. *Appl. Microbiol. Biotechnol.* 86:177-188).

Because the *P. horikoshii* prolidase homolog-1 has properties that may be beneficial for applications involving detoxification of OP nerve agents such as having higher catalytic activity over a broader pH range, higher affinity for metal, and being more stable than *P. furiosus* prolidase, Pfp_{rol} (PF1343), we prepared randomly mutated *P. horikoshii* prolidases by using a mutazyme polymerase error prone PCR strategy. The selection strain JD1 (\square DE3) cells were transformed with mutated *P. horikoshii* prolidase homolog-1 expression plasmids, and transformants were plated on minimal media supplemented with 5 \square M Leu-Pro as the only source of proline and grown at 20°C. Four cold-adapted variants, *P. horikoshii* A195T/G306S-, Y301C/K342N-, E127G/E252D- and E36V-prolidase, were isolated. These mutants exhibited 2-3 times higher activity at 30°C than the WT-Ph1_{prol} in heat treated cell extract. Since the mutants appeared to be promising based on their activities in crude extract, large-scale expression of each mutant was accomplished using recombinant BL21 (\square DE3) *E. coli* cells grown in auto-induction media. The mutant Ph1_{prol} enzymes were purified using multi-column chromatography, and their activities against the OP nerve agent diisopropylfluorophosphate (DFP) were determined in collaboration with Dr. Joseph DeFrank (U.S. Army Edgewood Research, Development and Engineering Center) to test the enzyme for OP hydrolysis activity. Those mutants that show increased activity against DFP compared to wild type Ph1_{prol} or Pfp_{rol} were fully biochemically characterized and a paper describing the mutants was published in the journal *Archaea* (Theriot, C. M., Semcer, R. L., Shah, S. S. & Grunden, A. M. 2011. Improving the Catalytic Activity of Hyperthermophilic *Pyrococcus horikoshii* Prolidase for Detoxification of Organophosphorus Nerve Agents over a Broad Range of Temperatures. *Archaea*, 2011, 565127).

Objective 4. Biochemically characterize *Rhodospseudomonas palustris* prolidase to determine its potential use in OP nerve agent degradation

During discussion with Dr. DeFrank, it was suggested that prolidases from the photosynthetic mesophilic bacterium *Rhodospseudomonas palustris* would be of interest to the DOD since this particular bacterium is already being used in DOD and DOE funded projects for biohydrogen generation. In response to Dr. DeFrank's interest in the *R. palustris* prolidase, we have identified a good candidate prolidase gene in the *R. palustris* genome (annotated as PEP M24 in the *R. palustris* HAA2 genome; 40% similarity to *P. furiosus* prolidase).

Objective 4 Research Results:

To determine whether *R. palustris* expresses a functional prolidase, protein extracts were prepared from *R. palustris* cells and tested for prolidase activity. The assays were performed at both 30 and 37°C. Activity was observed (0.032 μ mol proline released/min/mg) when Met-Pro was used as the substrate. However, because the activity was low compared to activities observed in *P. furiosus* cell extracts (9.2 U/mg), other prolidase substrates (Gly-Pro, 0.015 U/mg; Ala-Pro, 0.014 U/mg; Ser-Pro, 0.012 U/mg) were used in the assay, but the highest activity was detected when Met-Pro was used as the substrate.

The putative *R. palustris* prolidase (PEP M24) gene was PCR amplified and cloned into the pBAD expression system. The resulting expression plasmid (pSRT1) was sequenced to ensure that no mutations were introduced into the candidate prolidase gene. After this sequence validation, the expression vector was transformed into strain JD1 for small scale expression in 30 ml cultures. JD1 cells were also transformed with the empty pBAD vector as an expression control. Cell extracts were prepared for PAGE analysis and for enzyme assays. Over-expression of the putative *R. palustris* prolidase was not detected by PAGE analysis nor was prolidase activity observed. Amino acid sequence analysis of the *R. palustris* protein indicated that there are a number (6) of rare arginine codons (AGG), which could lead to poor recombinant expression of the gene. Therefore, the over-expression experiment was repeated in Top-10 *E. coli* cells that had been transformed with both the *R. palustris* prolidase expression plasmid as well as the rare codon tRNA expression plasmid pRIL. This time an induced protein product was detected on an SDS PAGE at the appropriate size (~48 kDa) in the soluble fraction of the protein extract. However, again no activity was detected when Met-Pro, Gly-Pro, Ala-Pro, Arg-Pro or Phe-Pro was used as a substrate in the assays. This indicates that either the putative *R. palustris* prolidase is not a true prolidase or that the recombinant protein is not expressed in an active form. Because a significant time and effort investment would need to be made to work out alternate expression conditions that might result in an active protein, and it is not guaranteed that the enzyme does indeed function as a prolidase, work with this enzyme was not continued.

Summary of Project Accomplishments

As a result of the ARO 'Structural Analysis and Bioengineering of Thermostable *Pyrococcus furiosus* Prolidase for the Optimization of Organophosphorus Nerve Agent Detoxification' project funding the following research advances and information dissemination activities were accomplished:

- A group of *Pyrococcus furiosus* prolidase mutants were generated that have enhanced activity against proline-containing dipeptides and OP nerve agent analogs at lower temperatures (over a range of 75 to 20°C) while remaining thermostable.
- Two thermostable prolidases from the archaeal hyperthermophile *Pyrococcus horikoshii* (Phprol and Ph1prol) were biochemically characterized, and it was determined that the Ph1prol has some superior attributes (metal affinity, maximal activity) compared to *P. furiosus* prolidase.
- A group of *Pyrococcus horikoshii* prolidase homolog-1 mutants were generated that have enhanced activity against proline-containing dipeptides and OP nerve agent analogs at lower temperatures (over a range of 75 to 20°C) while remaining thermostable.
- Three research articles were published in high quality microbiology journals describing the research advances made as a result of this ARO funded study.
- Based on the research studies done in my laboratory focused on prolidases, I had been invited to submit (and have published) three reviews and a book chapter that discuss the biotechnological applications of prolidases.
- Based on the research studies done in my laboratory focused on prolidases, I had been invited to present my results at the Chemical and Biological Defense Program Enzyme Colloquium, Falls Church, VA, September 22, 2010. I gave a talk titled 'Generation of Recombinant *Pyrococcus* Prolidases with Enhanced Activity Over a broad Temperature Range for Detoxification of Organophosphorus Nerve Agents'.
- I was also asked to present my study findings at NIH Proline Metabolism Conference, Lincoln, NE, November 8, 2010. I presented the talk 'Thermostable Archaeal Prolidases and Their Use in Toxic Organosphosphates Degradation'.
- I was the invited keynote speaker for the North Carolina American Society for Microbiology meeting, October 2, 2010 and presented the talk 'Biotechnological Applications of Extremophile Enzymes'.

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Technology Transfer

Scientific Progress and Accomplishments — Forward

The aims of this project were to structurally study and bioengineer thermostable prolidases from *Pyrococcus furiosus* (*Pf*) and *horikoshii* (*Ph*) to enable their use for organophosphorus nerve agent detoxification. *Pf* prolidase contains one dinuclear Co metal-center/monomer and has optimal activity at 100°C, exhibiting no activity without 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. Our understanding of the metal cluster structures was used in this project to design a prolidase mutant with two integral metal sites, which will relieve the requirement for exogenous metal and improve its efficacy in OP nerve agent detoxification. In addition, a *Ph* prolidase homolog has been identified and has been biochemically characterized to determine its potential utility in OP nerve agent decontamination. Another objective was to produce *Pf* and *Ph* prolidase mutants that have increased catalytic activity over temperatures ranging from 20-60°C to increase their use for decontamination of OP nerve agents in the field. To this end, a random mutation and low-temperature selection method was successfully used.

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Statement of the Problem Studied

Certain prolidases have been shown to have activity against toxic organophosphorus (OP)-containing compounds such as diisopropylfluorophosphate (DFP), and the chemical warfare agents sarin, soman and tabun (DeFrank and Cheng, 1991). Prolidases from various bacteria and archaea, as well as human prolidase, are capable of hydrolyzing these compounds into their components, thus rendering them harmless (DeFrank and Cheng, 1991, Wang et al., 1998, Park et al., 2004, Theriot et al., 2010a). OP compounds are biodegradable, yet they are extremely toxic to mammals because they bind to acetyl cholinesterase and render it inactive leading to a buildup of the neurotransmitter acetylcholine in the synapse. Acetylcholine then produces a continuous signal resulting in hyper salivation, confusion, convulsions, respiratory complications, respiratory failure, coma and death. OP compounds exist mainly in the form of pesticides and chemical warfare agents (CWAs), and it is estimated that 38% of the pesticides used worldwide contain OP, and approximately 200,000 tons of OP nerve agents are stockpiled worldwide (Singh, 2009). Annually, there are an estimated 3 million poisonings and 300,000 human deaths owing to OP compounds (Singh, 2009). There is a need for OP detoxification strategies that are safe for humans and the environment. Enzymes from several microorganisms have been shown to degrade a range of OP compounds; however, an ideal enzyme or combination of enzymes to be used in a decontamination cocktail would be able to degrade all OP compounds while remaining stable for an extended period of time at a range of temperatures and pH.

Several microbial enzymes, such as organophosphorus acid anhydrolase (OPAA) and phosphotriesterase (PTE), are capable of degrading OP compounds and have been extensively studied for their high activity and specificity against G-type nerve agents (Singh, 2009). The stability of these enzymes, however, is limited when incorporated into formulations containing solvents and other denaturants. Additionally, high concentrations of metals must be added to formulations containing these enzymes in order to maximize their activity which poses further complications (Theriot et al., 2011). More recent studies have included recombinant prolidases from hyperthermophilic archaea (Vyas et al., 2010, Theriot and Grunden, 2010). Wild type and mutant prolidases from *Pyrococcus furiosus* and *Pyrococcus horikoshii* have been characterized and show promising enzymatic properties as candidates for OP decontamination in that they are highly thermostable with broad substrate specificity.

The long-term goal of this project was to utilize the structural information we now possess for *Pyrococcus furiosus* prolidase, particularly with respect to its metal centers and substrate binding regions, to improve the performance of *P. furiosus* prolidase for degrading OP nerve agents. Specifically, this structural information has provided insight into developing informed targeted bioengineering strategies to modify wild type *P. furiosus* prolidase to increase its hydrolytic cleavage of OP nerve agents. In addition, with the recent completion of the genomic sequences of related *Pyrococcus* species, *Pyrococcus abyssi* and *Pyrococcus horikoshii*, other candidate prolidases have been identified, which were also shown to have utility for OP nerve agent degradation.

Therefore, the specific objectives of this project, which were to be directly addressed during the three year funding period, were as follows:

- (1) Use a targeted mutation approach to specifically modify the *P. furiosus* prolidase in an effort to improve performance in OP nerve agent degradation.
- (2) Use a random mutagenesis approach to obtain *P. furiosus* prolidase mutants that have increased catalytic activity at lower temperature ranges (ideally increased activity from 35 °C to 55 °C).
- (3) Biochemically characterize other identified *Pyrococcus* prolidases to determine their potential use in OP nerve agent degradation.

Summary of Most Important Results

In order to accomplish the project goals described above specific experimental objectives had been proposed for the study. The results of the efforts targeted at these experimental objectives are described below.

Objective 1. Use targeted mutation strategies informed by the solved structure to improve *P. furiosus* prolidase function for OP nerve agent decontamination

Objective 1a. Use of a targeted mutation approach to convert the loose-binding metal center (Co₂) to a tight-binding center

Based on studies by Du *et al.*, it was shown that the Co₁ atom is tightly bound and the Co₂ atom loosely bound. Because the Co₂ atom is only loosely bound in the prolidase active site (K_d 0.24 mM), additional cobalt is required for activity. Modification of the active site such that the Co₂ atom is bound more tightly would significantly improve the potential of using *P. furiosus* prolidase for large-scale OP nerve agent detoxification. Thus, for Objective 1a, we had proposed to modify the following amino acid residues that are directly involved in liganding the Co₂ atom: Asp-209 was to be changed to either a histidine or glutamate residue and Asp-220 was to be changed to a glutamate. In addition amino acid residues Phe-178 and Ile-290, which are located in close proximity to the Co₂ binding site were changed to aspartate or glutamate in an effort to provide additional charge attraction to help bind the Co₂ cobalt atom more tightly.

Objective 1a Research Results:

For this study, the following metal center prolidase mutants had been prepared: A209E-prolidase (change of aspartate at position 209 to glutamate), A209H-prolidase, and A220E-prolidase. The Phe-178 and Ile-290 positions were also modified to F178E and I290E. The genes encoding the metal center mutants that have been produced have been fully sequenced to verify the correct positioning of the targeted mutation and to confirm that no other mutations arose. Preliminary activity assays for the mutant proteins in crude extract revealed little activity. Protein gels had confirmed good production of soluble prolidases. Therefore, the poor activity associated with the mutants may be due to (1) failure of cobalt to bind to the modified Co₂ binding site or (2) cobalt did in fact bind tightly to the Co₂ site, but this tight binding interfered with the catalytic function of the enzyme. Given what we now know about the reaction mechanism used by prolidases (Alberto *et al.*, 2011), in which the second (labile metal atom) is thought to enter the active site when the substrate is bound, it is most likely that having two tightly bound Co in the prolidase active site actually prevents substrate binding and catalysis. This is consistent with our findings for the metal center mutant prolidases.

Objective 1b. Use of a targeted mutation approach to modify amino acid residues important for substrate binding and enzyme catalysis in *P. furiosus* prolidase

Previous biochemical and structural analysis of the highly related enzyme *E. coli* methionine aminopeptidase had identified a number of amino acid residues that are required for substrate binding and enzyme catalysis. Among these, His-79 and Gln-238 are involved in productive binding of substrate and His-178 in stabilization of the enzyme-substrate transition state. By analogy, His-192, Arg-325, and His-291, respectively, were predicted to participate in cognate roles in *P. furiosus* prolidase, a prediction that was confirmed by the recent *P. furiosus* prolidase structure studies. Armed with this structure information, the *P. furiosus* prolidase amino acid residues His-192, His-291, and Arg-325 were initially targeted for mutation (H192L, H291L, and R325L).

Objective 1b Research Results:

After initial screening of the H192L, H291L-, and R325L-prolidase mutants for enzyme activity, mutant proteins, which exhibit differences in activity compared to wild type were to be purified in large scale and subjected to structural analysis and substrate/inhibitor binding studies. The *P. furiosus* H192L-, H291L-, and R325L-prolidase mutants have been generated and verified by DNA sequencing. Preliminary activity assays showed negligible activity, confirming our initial analysis that these residues are key for prolidase activity. Further insight derived from mutant structure will be used to further inform targeted mutations that may improve OP nerve agent binding to prolidase.

Objective 1c. Use of targeted mutation approaches to increase flexibility, and therefore, presumably activity of *P. furiosus* prolidase at lower temperatures

Studies of other high-temperature active enzymes have shown that removal of N-terminal regions or C-terminal regions of these proteins can in some cases increase the flexibility of the enzymes and increase their activities at low temperature. For *P. furiosus* prolidase, the N-terminal region of the protein is primarily involved in providing structural contacts for dimerization, whereas the C-terminal domain has the catalytic sites. Therefore, we focused on removing some of the N-terminal residues to determine whether the truncated form of the protein would have increased flexibility and higher activity at lower temperatures. Since there is a methionine residue at amino acid position 12 in the *P. furiosus* prolidase sequence, we designed PCR primers that would allow us to clone the *P. furiosus* prolidase into the pET expression system (pET-21b) such that Met-12 served as the start Met, resulting in a *P. furiosus* prolidase with a deletion of the first 11 residues. Another structure-informed targeted mutation that we wish to pursue involves the mutation of amino acid residue Gln-27. *P. furiosus* prolidase structure studies showed that Gln-27 is involved in hydrogen-bonding to Arg-122 that lies in the linker helix in domain II of the enzyme. This hydrogen bonding is considered to be important for constraining the flexibility of the enzyme. Thus, we specifically targeted Gln-27 and/or Arg-122 for mutation studies with the specific goal of switching the indicated amino acids to similarly sized amino acids that would not participate in hydrogen bonding.

Objective 1c Research Results:

We were able to successfully over-express this truncated form of the protein and evaluated its activity in comparison to the wild type *P. furiosus* enzyme at 37, 50 and 100°C. The activity results revealed that the N-terminal truncated protein had 12.4, 3.8, and 213.8-fold lower activity at 37, 50 and 100°C, respectively. Given that the N-terminal truncated *P. furiosus* enzyme did not appear to have greater activity at lower temperatures (37 and 50°C), and in fact, had impaired activity at all temperatures evaluated compared to wild type, studies of this particular mutant will not be continued. .

Objective 2a. Production of *P. furiosus* Mutants with Increased Catalytic Activity at Lower Temperatures Using Random Mutagenesis and a Positive Selection Method

Wild type *P. furiosus* prolidase is one of the most thermostable enzymes to have ever been studied 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 is 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.

Objective 2a Research Results:

Random *P. furiosus* prolidase mutants had been prepared using standard, proven chemical and error-prone PCR mutagenesis procedures or by serial passage of the *P. furiosus* prolidase expression plasmid through the *E. coli* mutator strain XL1-Red. *P. furiosus* mutants with increased activity at lower temperatures were selected for by using an *E. coli* strain JD1(λ DE3) that was developed in my laboratory 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) and has deletions in *pepQ* and *pepP*, dipeptidases with specificity for proline-containing dipeptides. The use of the *E. coli* proline auxotroph, JD1(λ DE3) strain provides 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.

Using our mutagenesis and selection strategy, 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) and one with a change from glutamate at position 236 to valine (E236V-prolidase). The G39E-prolidase was over-expressed, purified by multi-step chromatography and was evaluated for enzyme activity over a range of temperatures (10 °C to 100 °C). Enzyme activities demonstrated that the G39E-prolidase has ~2-fold higher activity compared to wild type when assayed at temperatures ranging from 30°C to 50°C. 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. Presumably the change to Glu-39 provides increased flexibility of the prolidase enzyme, thereby supporting increased activity at lower temperature. The E236V-prolidase mutant also had ~2-fold increased activity compared to wild type prolidase when assayed at temperatures ranging from 30°C to 50°C. Based on the *pf* prolidase structure analysis, the glutamate-236 residue appears to be involved in dimerization and therefore, the change to a non-charged residue may be providing increased flexibility in the protein at low temperature.

Based on these initial positive results, we had extended our mutation generation/selection strategy by using the G39E-, E236V-, and G39E,E236V- *Pf* expression plasmids as the template for subsequent rounds 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°C to 50°C). We have completed three separate screenings for plasmids generated using error-prone PCR and passage through the mutator *E. coli* strain for the three different templates. For each screen over 100 colonies that grew up on the selection plates were analyzed for activity. Only one of the selected colonies expressed a mutant prolidase that had consistently higher activity (greater than two-fold compared to wildtype) at low temperature (30°C) when assayed in triplicate in three biological repeats. The DNA encoding for the prolidase was sequenced and it was found to have only the G36E mutation. In light of the poor performance of our initial strategy for obtaining useful *Pf* prolidase mutants, we reevaluated our mutagenesis and selection strategy and had determined the inefficient step of the initial process was the generation of the expression plasmids with actual mutations. Therefore, we switched to using the newly available Genemorph2 Random mutagenesis kit from Stratagene which features an error-prone Taq polymerase that can provide a low level, medium level or high-level mutation rate depending on the reaction conditions used. We were then able to generate pools of mutant prolidase expression plasmids at the low, medium and high mutation rates using the Genemorph2 Random mutagenesis kit and had plated approximately 200 selection plates and screened a total of 75 colonies that grew up on the plates. From this screen, one mutant, named Prolidase mutant 10, was shown to have increased activity at both 35°C and 100°C in comparison to the wild type *P. furiosus* enzyme. Sequence analysis of the mutant 10 revealed that the protein had the following amino acid substitutions: arginine at position 19 was changed to glycine and lysine at position 71 was changed to glutamate. Activity assays for wild type and mutant 10 were prepared using 35 and 100°C incubation temperatures, and activity comparisons indicated that mutant 10 had a 5.3-fold higher activity at 35°C and 3-fold higher activity at 100°C. This was an especially interesting result since relative activity increases were observed at both low

(35°C) and high (100°C) temperatures for mutant 10, and often mutations that improve low temperature activity can compromise high temperature activity.

Since both the original G39E-prolidase and mutant 10 had shown increased activity at low temperature (35°C, 2-fold and 5.3-fold increase, respectively), a combined mutant was made in which the G39E mutation was introduced to mutant 10. This new mutant, G39E-mutant10, has been over-expressed and purified using multi-column chromatography. The purified G39E-mutant10 protein was evaluated for its activity over a range of temperatures and its activities were compared to the wild type, mutant10 and G39E-prolidase mutant activities.

The G39E-, G39E,E236V-, mutant10- and G39E-mutant10 *P. furiosus* prolidase mutants were completely biochemically characterized for their activity with the dipeptides Met-Pro and Leu-Pro over a broad range of temperatures and pH. In addition, these mutants were also tested at the Edgewood Chemical Biological Center at Aberdeen Proving Ground for their activities against the OP nerve agents DFP and *p*-nitrophenyl soman. The *P. furiosus* mutant prolidase studies were initially described in the publication (Theriot, C. M. & Grunden, A. M. 2010. Hydrolysis of organophosphorus compounds by microbial enzymes. *Appl Microbiol Biotechnol*, 89, 35-43). The full biochemical characterization of the *Pyrococcus* mutants was published in the journal Applied Microbiology and Biotechnology (Theriot CM, Tove SR, Grunden AM. 2011. Improving the Catalytic Activity of Hyperthermophilic *Pyrococcus* Prolidases for Detoxification of Organophosphorus Nerve Agents over a Broad Range of Temperatures. *Applied Microbiology and Biotechnology*, volume 87 pages 1715-1726).

Objective 2b. Production of *P. furiosus* Mutants with Increased Catalytic Activity at Lower Temperatures Using a Natural Evolution Approach

Another approach for improving the low temperature activity of *P. furiosus* prolidase is to allow it to evolve naturally over time under selective pressure. For this method, the wild type *P. furiosus* expression prolidase plasmid was transformed into the prolidase deficient, proline auxotrophic strain JD1 that was described above. These transformants were grown in 100 ml cultures using liquid selection media equivalent to the selection media described above (except that no agar is added). Two sets of cultures were grown (one set at 30°C and another at 37°C) on the selection media with Leu-Pro as the only source of proline and with IPTG (0.1 mM) to induce prolidase expression and ampicillin (100 µg/ml) to maintain the expression plasmid. As a control, to see the growth rate of these cells, a set of similar cultures were grown with media that has proline in place of Leu-Pro (no IPTG). It was expected that it would take the cultures without proline a long time to grow. Cell growth of the cultures was evaluated and once cultures had shown growth, cells were serially transferred into fresh selection media. This process was repeated a number of times in hopes of isolating cells which may have naturally occurring mutations in the *P. furiosus* prolidase that enable efficient hydrolysis of Leu-Pro at low temperatures. Once fast-growing cells have been isolated from the liquid selection media cultures, expression plasmids were isolated from the cells and the plasmids were sequenced to determine what mutations occurred.

Objective 2b Research Results:

The *P. furiosus* prolidase expressing JD-1 cells were grown in the liquid selection media and the growth of the cultures was tracked. Transfers were made into fresh media (1% inoculum) once cells in the cultures had reached early stationary phase. Unfortunately this approach did not provide us with additional mutants to further characterize based on our sequencing of resulting isolates and we did not continue any further with this approach.

Objective 3. Biochemically characterize other identified *Pyrococcus* prolidases to determine their potential use in OP nerve agent degradation

Recently the genomic sequences of related *Pyrococcus* species, *Pyrococcus abyssi* and *Pyrococcus horikoshii* had been completed. An analysis of the three *Pyrococcus* genomes enabled the identification of three categories of prolidases or prolidase homologs: (1) prolidases annotated as a prolidase based on high-level identity to the biochemically characterized *P. furiosus* prolidase (>75% identity); (2) prolidase homologs that have significant levels of homology to *P. furiosus* prolidase (> 30% identity) and have all five matching conserved metal center-liganding amino acid residues (homolog-1 classification); and (3) prolidase homologs that share levels of identity greater than 25% but less than 30% and have only 2 of the 5 matching conserved metal-center liganding amino acids (homolog-2 classification). The prolidase homolog-2 proteins do have conserved amongst themselves all of the same types of amino acids present in the *P. furiosus* metal center-liganding amino acids but with alternate spacing (for example, in *P. furiosus* prolidase, the D-209 and D-220 amino acids have 10 intervening amino acids, whereas, the prolidase homolog-2 proteins have 15, and the spacing between the *P. furiosus* prolidase E-313 and E-327 residues is 13 amino acids, while the spacing between the prolidase homolog-2 proteins is five amino acids). From the homology analysis, it was determined that all three *Pyrococci* have prolidase proteins, that only *P. abyssi* and *P. horikoshii* have prolidase homolog-1 proteins, and that all three organisms have prolidase homolog-2 proteins.

Objective 3 Research Results:

It is of considerable interest to determine how similar the prolidase homolog-1 and homolog-2 proteins in terms of enzyme activity, temperature profile, substrate preference, and metal preference are to *P. furiosus* prolidase and ultimately to determine whether any of these homologs would also have utility for OP nerve agent degradation. Therefore, we cloned, recombinantly expressed, purified, and biochemically characterized prolidase homolog-1 proteins from *P. horikoshii* and *P. abyssi* as well as prolidase homolog-2 proteins initially from *P. furiosus*. Since the nature of the metal-content for the prolidase homolog-1 and homolog-2 proteins is of prime importance to evaluating their potential efficacy for OP nerve agent degradation, the metal content has been evaluated for both the recombinant versions of the proteins using ICP emission spectroscopy as well as for the native enzymes.

From our biochemical analysis of *Ph* prolidase homolog-1 it was determined that the enzyme showed maximal activity with the addition of the metal ion Co^{2+} and with the substrate Met-Pro ($k_m= 4.0$) and 88% activity with Leu-Pro. Prolidase homolog-1 activity was also shown to be supported by Mn^{2+} (79% activity compared to Co^{2+}). As with *Pf* prolidase, *Ph* prolidase homolog-1 showed substrate specificity for dipeptides with non-polar amino acids (Met, Leu, Ala, or Arg) at the N-terminus and a proline at the C-terminus. It also showed maximal catalytic activity at pH 7.0 and at 100°C. Under temperatures of 80°C, *Ph* prolidase homolog-1 lost 60% activity and below 50°C it decreased to 10% activity. Thus, the characterization profile of the *ph* prolidase homolog-1 described above is very similar to the profile of the previously characterized recombinant *Pf* prolidase. The only significant difference appeared to be the dissociation constants for the metals. *Ph* prolidase homolog-1 showed dissociation constants of 0.05 mM with Co^{2+} and greater than 0.8 mM for Mn^{2+} , whereas recombinant *Pf* prolidase displayed a dissociation constant of 0.5 mM for Co^{2+} and 0.66 mM for Mn^{2+} . The 10-fold lower dissociation constants for cobalt for *Ph* prolidase homolog-1 compared to *Pf* prolidase suggests that the *P. horikoshii* prolidase binds its metal more tightly than the *P. furiosus* enzyme and as such may be a better choice for OP nerve agent detoxification.

We sent the *P. horikoshii* prolidase homolog-1 to Dr. Joseph DeFrank, my collaborator at the U.S. Army Edgewood Research, Development and Engineering Center to test the enzyme for OP hydrolysis activity. Saumil Shah in Dr. Defrank's laboratory performed OP hydrolysis assays using diisopropylfluorophosphate (DFP) as the OP substrate. Their activity data indicated that the *P. horikoshii* prolidase homolog-1 enzyme did have activity against DFP (5.3 $\mu\text{moles}/\text{min}/\text{mg}$ when assayed at 35°C), however, this was very low activity compared to that seen for *Alteromonas* OPAA (prolidase) which is 1820 $\mu\text{moles}/\text{min}/\text{mg}$.

We have also cloned, expressed, and assayed *P. horikoshii* prolidase homolog-2. Although, *Ph* prolidase homolog-2 was successfully expressed as a soluble enzyme, it does not appear to have any prolidase activity when six different proline dipeptides were used as substrates. Based on sequence alignments with other metal-activated hydrolases, it appears that *Ph* prolidase homolog-2 has sequence motifs similar to motifs found in methionine aminopeptidases. However, when we assayed it for aminopeptidase activity, no activity was detected, thus ruling out its function as an aminopeptidase.

The biochemical characterization studies of *P. horikoshii* prolidase (PH1149) and prolidase homolog1 (PH0974) were published in the journal *Applied Microbiology and Biotechnology* (Theriot CM, Tove SR, Grunden AM. 2010b. Characterization of Two Proline Dipeptidases (Prolidases) from the Hyperthermophilic Archaeon *Pyrococcus horikoshii*. Appl. Microbiol. Biotechnol. 86:177-188).

Because the *P. horikoshii* prolidase homolog-1 has properties that may be beneficial for applications involving detoxification of OP nerve agents such as having higher catalytic activity over a broader pH range, higher affinity for metal, and being more stable than *P. furiosus* prolidase, *Pf*prol (PF1343), we prepared randomly mutated *P. horikoshii* prolidases by using a mutazyme polymerase error prone PCR strategy. The selection

strain JD1 (λ DE3) cells were transformed with mutated *P. horikoshii* prolidase homolog-1 expression plasmids, and transformants were plated on minimal media supplemented with 5 μ M Leu-Pro as the only source of proline and grown at 20°C. Four cold-adapted variants, *P. horikoshii* A195T/G306S-, Y301C/K342N-, E127G/E252D- and E36V-prolidase, were isolated. These mutants exhibited 2-3 times higher activity at 30°C than the WT-*PhI*prol in heat treated cell extract. Since the mutants appeared to be promising based on their activities in crude extract, large-scale expression of each mutant was accomplished using recombinant BL21 (λ DE3) *E. coli* cells grown in auto-induction media. The mutant *PhI*prol enzymes were purified using multi-column chromatography, and their activities against the OP nerve agent diisopropylfluorophosphate (DFP) were determined in collaboration with Dr. Joseph DeFrank (U.S. Army Edgewood Research, Development and Engineering Center) to test the enzyme for OP hydrolysis activity. Those mutants that show increased activity against DFP compared to wild type *PhI*prol or *Pf*prol were fully biochemically characterized and a paper describing the mutants was published in the journal *Archaea* (Theriot, C. M., Semcer, R. L., Shah, S. S. & Grunden, A. M. 2011. Improving the Catalytic Activity of Hyperthermophilic *Pyrococcus horikoshii* Prolidase for Detoxification of Organophosphorus Nerve Agents over a Broad Range of Temperatures. *Archaea*, 2011, 565127).

Objective 4. Biochemically characterize *Rhodopseudomonas palustris* prolidase to determine its potential use in OP nerve agent degradation

During discussion with Dr. DeFrank, it was suggested that prolidases from the photosynthetic mesophilic bacterium *Rhodopseudomonas palustris* would be of interest to the DOD since this particular bacterium is already being used in DOD and DOE funded projects for biohydrogen generation. In response to Dr. DeFrank's interest in the *R. palustris* prolidase, we have identified a good candidate prolidase gene in the *R. palustris* genome (annotated as PEP M24 in the *R. palustris* HAA2 genome; 40% similarity to *P. furiosus* prolidase).

Objective 4 Research Results:

To determine whether *R. palustris* expresses a functional prolidase, protein extracts were prepared from *R. palustris* cells and tested for prolidase activity. The assays were performed at both 30 and 37°C. Activity was observed (0.032 μ mol proline released/min/mg) when Met-Pro was used as the substrate. However, because the activity was low compared to activities observed in *P. furiosus* cell extracts (9.2 U/mg), other prolidase substrates (Gly-Pro, 0.015 U/mg; Ala-Pro, 0.014 U/mg; Ser-Pro, 0.012 U/mg) were used in the assay, but the highest activity was detected when Met-Pro was used as the substrate.

The putative *R. palustris* prolidase (PEP M24) gene was PCR amplified and cloned into the pBAD expression system. The resulting expression plasmid (pSRT1) was sequenced to ensure that no mutations were introduced into the candidate prolidase gene. After this sequence validation, the expression vector was transformed into strain JD1 for small scale expression in 30 ml cultures. JD1 cells were also transformed with the empty

pBAD vector as an expression control. Cell extracts were prepared for PAGE analysis and for enzyme assays. Over-expression of the putative *R. palustris* prolidase was not detected by PAGE analysis nor was prolidase activity observed. Amino acid sequence analysis of the *R. palustris* protein indicated that there are a number (6) of rare arginine codons (AGG), which could lead to poor recombinant expression of the gene. Therefore, the over-expression experiment was repeated in Top-10 *E. coli* cells that had been transformed with both the *R. palustris* prolidase expression plasmid as well as the rare codon tRNA expression plasmid pRIL. This time an induced protein product was detected on an SDS PAGE at the appropriate size (~48 kDa) in the soluble fraction of the protein extract. However, again no activity was detected when Met-Pro, Gly-Pro, Ala-Pro, Arg-Pro or Phe-Pro was used as a substrate in the assays. This indicates that either the putative *R. palustris* prolidase is not a true prolidase or that the recombinant protein is not expressed in an active form. Because a significant time and effort investment would need to be made to work out alternate expression conditions that might result in an active protein, and it is not guaranteed that the enzyme does indeed function as a prolidase, work with this enzyme was not continued.

Summary of Project Accomplishments

As a result of the ARO 'Structural Analysis and Bioengineering of Thermostable *Pyrococcus furiosus* Prolidase for the Optimization of Organophosphorus Nerve Agent Detoxification' project funding the following research advances and information dissemination activities were accomplished:

- A group of *Pyrococcus furiosus* prolidase mutants were generated that have enhanced activity against proline-containing dipeptides and OP nerve agent analogs at lower temperatures (over a range of 75 to 20°C) while remaining thermostable.
- Two thermostable prolidases from the archaeal hyperthermophile *Pyrococcus horikoshii* (*Phprol* and *PhIprol*) were biochemically characterized, and it was determined that the *PhIprol* has some superior attributes (metal affinity, maximal activity) compared to *P. furiosus* prolidase.
- A group of *Pyrococcus horikoshii* prolidase homolog-1 mutants were generated that have enhanced activity against proline-containing dipeptides and OP nerve agent analogs at lower temperatures (over a range of 75 to 20°C) while remaining thermostable.
- Three research articles were published in high quality microbiology journals describing the research advances made as a result of this ARO funded study.
- Based on the research studies done in my laboratory focused on prolidases, I had been invited to submit (and have published) three reviews and a book chapter that discuss the biotechnological applications of prolidases.
- Based on the research studies done in my laboratory focused on prolidases, I had been invited to present my results at the Chemical and Biological Defense Program Enzyme Colloquium, Falls Church, VA, September 22, 2010. I gave a talk titled 'Generation of Recombinant *Pyrococcus* Prolidases with Enhanced Activity Over a broad Temperature Range for Detoxification of Organophosphorus Nerve Agents'.
- I was also asked to present my study findings at NIH Proline Metabolism Conference, Lincoln, NE, November 8, 2010. I presented the talk 'Thermostable Archaeal Prolidases and Their Use in Toxic Organosphosphates Degradation'.
- I was the invited keynote speaker for the North Carolina American Society for Microbiology meeting, October 2, 2010 and presented the talk 'Biotechnological Applications of Extremophile Enzymes'.

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