Research Article

Improving the Catalytic Activity of Hyperthermophilic *Pyrococcus horikoshii* Prolidase for Detoxification of Organophosphorus Nerve Agents over a Broad Range of Temperatures

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Prolidases hydrolyze Xaa-Pro dipeptides and can also cleave the P-F and P-O bonds found in organophosphorus (OP) compounds, including the nerve agents soman and sarin. *Ph1* prol (PH0974) has previously been isolated and characterized from *Pyrococcus horikoshii* and was shown to have higher catalytic activity over a broader pH range, higher affinity for metal, and increased thermostability compared to *P. furiosus* prolidase, *Pf* prol (PF1343). To obtain a better enzyme for OP nerve agent decontamination and to investigate the structural factors that may influence protein thermostability and thermoactivity, randomly mutated *Ph1* prol enzymes were prepared. Four *Ph1* prol mutants (A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Ph1 prol) were isolated which had greater thermostability and improved activity over a broader range of temperatures against Xaa-Pro dipeptides and OP nerve agents compared to wild type *Pyrococcus* prolidases.

1. Introduction

*Pyrococcus horikoshii* and *Pyrococcus furiosus* are both hyperthermophilic archaea, growing optimally at 98–100°C that were isolated from a deep hydrothermal vent in the Okinawa Trough in the northeastern Pacific Ocean and from a shallow marine sulfataras at Vulcano Island off the coast of Italy, respectively [1, 2]. *Pyrococcus* spp. are some of the most studied hyperthermophilic archaea to date owing in part to their utility for a variety of biotechnological applications [3–7]. For example, recombinant prolidases from *Pyrococcus* spp. are being studied for their potential use in bio-decontamination applications [8].

Prolidases function *in vivo* to hydrolyze dipeptides with proline in the C-terminus, Xaa-Pro, and a non-polar amino acid in the N-terminus [9]. However, studies have demonstrated that prolidases can also hydrolyze and detoxify organophosphate (OP) compounds such as chemical warfare agents (CWA) [8]. Two enzymes that have been characterized for potential field detoxification of nerve agents are organophosphorus acid anhydrolase (OPAA) and phosphotriesterase (PTE) [10, 11]. Recently, the crystal structure of OPAA from *Alteromonas* sp. JD6.5 strain has been solved, and it has been determined to be a prolidase [12]. While OPAA does have the capability to degrade OP nerve agents, its activity can be limited by exposure to high temperatures and solvents during use in field situations [13, 14].

In 2008, the Defense Threat Reduction Agency (DTRA), under the auspices of the Department of Defense recognized the importance of developing enzyme-based OP nerve agent detoxification systems and created an initiative calling for new enzymes and biocatalysts that are stable over a broad temperature and pH range, in the presence of salts and surfactants, and that do not pose an environmental hazard.
In response to the need to develop stable OP nerve agent degrading enzyme systems, thermostable prolidases from *Pyrococcus* spp. were studied [16–18]. Specifically, *Pyrococcus* prolidases from *P. furiosus* (PF1343 or Pfprol) and *P. horikoshii* (PH1149 or Ph prol and PH0974 or Ph1 prol) were characterized both structurally and enzymatically [16, 18, 19]. The *Pyrococcus* prolidases were determined to be very similar, with Pf prol showing 88% amino acid similarity to Ph prol and 55% similarity to Ph1 prol. Although they have high similarity to each other, the kinetic properties of Ph1 prol appeared to be more favorable for application purposes than those of Pf prol and Ph prol. Ph1 prol has demonstrated higher activity at lower temperatures and over a broader pH range. It has long-term stability, higher affinity for substrates, and a lower metal requirement for catalysis [16]. Therefore, it was deemed to be advantageous to use Ph1 prol in mutagenesis studies in order to develop a better enzyme for OP nerve agent detoxification and to further investigate factors that influence the catalysis of thermophilic metalloenzymes. To this end, a random mutagenesis Ph1 prol gene library was constructed and screened for production of mutants that showed increased prolidase activity at 30°C compared to wild type Ph1 prol. Four Ph1 prol mutants were isolated and subsequently characterized to determine their substrate catalysis over a broad range of temperatures and their performance against OP nerve agent analogs in comparison to Ph1 prol and the previously characterized Pf prol.

**2. Materials and Methods**

**2.1. Bacterial Strains, Media, and Materials.** The *E. coli* K-12 derivative NK5525 (proA::Tn10) was used to construct the selection strain JD1 (ADE3) as described in [20] for screening of cold-adapted *P. horikoshii* prolidase variants. The *P. horikoshii* prolidase expression plasmid pET-Ph1 prol was previously constructed as described in [16]. The *E. coli* strains were cultured either in Luria-Bertani (LB) broth or M9 selective minimal medium supplemented with 0.2% glucose, 1 mM MgSO4, 0.05% VitB1, 20 μM IPTG, 20 μM Leu-Pro. Ampicillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (34 μg/mL), and tetracycline (6 μg/mL) were added into the medium when required.

**2.2. Construction of a Pool of pET-Ph1 prol Plasmids Encoding Randomly Mutated *P. horikoshii* Prolidase Genes.** Error-prone PCR mutagenesis using the Genemorph II Random mutagenesis kit (Stratagene, La Jolla, Calif) was used to amplify and insert mutations into the *P. horikoshii* prolidase gene (PH0974). PCR amplification was carried out for 30 cycles: (60 sec at 95°C, 60 sec at 55°C, 120 sec at 72°C), with a 10-minute final extension at 72°C. Reactions contained Mutazyme II reaction buffer, 125 ng/μL of each primer, 40 mM dNTP mix, and 2.5 U of Mutazyme II DNA polymerase. Initial DNA template amounts used were 250 ng and 750 ng in order to select for medium-to-low mutation rates, respectively. The Genemorph II EZClone (Stratagene, La Jolla, Calif) reaction was employed to clone the mutated prolidase gene into the expression vector pET-21b.

The EZClone reaction included EZClone enzyme mix, 50 ng of template plasmid (pET-prol), 500 ng of megaprimer (mutated prolidase PCR product), and EZClone solution. The reactions were amplified for 25 cycles: (50 sec at 95°C, 50 sec at 60°C, 14 min at 68°C). Amplified products were digested with *Dpn I* for 2 hours at 37°C to remove template plasmid. XL1-Gold super competent *E. coli* cells were transformed with the mutant plasmid mixture.

**2.3. Screening for Increased Activity at Low Temperature.** pET-Ph1 prol plasmids from the mutant *P. horikoshii* library were transformed into the selective strain JD1 (ADE3) and were plated on M9 selective agar plates. Colonies that grew after being incubated for 3–7 d at 20°C were isolated on LB plates and then grown in 10 mL LB medium at 37°C with shaking (200 rpm) until an optical density of 0.6–0.8 was reached. IPTG was then added to the cell culture to a final concentration of 1 mM. The induced culture was shaken at 37°C for 3 h before harvesting the cells. These cell pellets were lysed using 300 μL of B-per buffer (Thermo Scientific, Rockford, Ill), and the resulting cell extracts were used for enzyme activity assays conducted at 30°C and at 100°C. Heat-treated soluble protein samples were heated at 80°C for 20 min. Four mutant colonies that exhibited at least 2–3-fold higher activities compared to the cells expressing the wild type *P. horikoshii* prolidase were selected for characterization, and their plasmids were isolated. The prolidase genes present in those isolated plasmids were sequenced using the T7 promoter and T7 terminator primers (MWG Biotech, Huntsville, AL).

**2.4. Large-Scale Expression of Recombinant *P. horikoshii* Prolidase Mutants.** Production of *P. horikoshii* prolidase variants (A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Ph1 prol) was carried out in transformed *E. coli* BL21 (ADE3) cells with the appropriate pET-Ph1 prol plasmid and pRIL vector. The transformants were grown in 1 L cultures of autoinduction media [21] incubated at 37°C with shaking (200 RPM) overnight.

**2.5. Purification of Recombinant *P. horikoshii* Prolidase Mutants.** Cell pellets from the four Ph1 prol variants (A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Ph1 prol) were suspended in 50 mM Tris–HCl, pH 8.0 (3 mL Tris per 1 gram of cell paste), with 1 mM benzamidine and 1 mM DTT. For each variant, diluted cell slurry was passed through a French pressure cell (20,000 lb/in²) three times. Cell lysates were centrifuged at 38,720 x g for 30 min at 4°C, and then the supernatants were heated to 80°C for 30 min anaerobically to denature any proteins not stable at that temperature. Heat-treated supernatants were centrifuged at 38,720 x g to remove the denatured proteins. Supernatants were sampled both before and after heat treatment for activity analysis. (NH₄)₂SO₄ was added gradually to the supernatants to make a final concentration of 1.5 M prior to loading onto a 5 mL Phenyl-Sepharose hydrophobic
2.6. Prolidase Enzyme Activity Assay. The enzyme activity assay protocol is based on a method previously described by [17, 20]. The reaction mixture (500 μL) contained 50 mM MOPS buffer (3-[N-morpholino]propanesulfonic acid) pH 7.0, 200 mM NaCl, water, 5% (vol/vol) glycerol, 100 μg/mL BSA (bovine serum albumin) protein, 0.2 mM CoCl₂, and the enzyme. The reaction mixture was heated at 100°C for 5 min allowing time for the metal and enzyme to interact. The reaction was initiated by the addition of substrate (Xaa-Pro, 4 mM final concentration) and allowed to proceed for 10 min at 100°C. The reaction was then cooled to 23°C. Prolidase samples were assayed in triplicate and specific activity was calculated using the absorbance value at 515 nm and an extinction coefficient of 4,570 M⁻¹ cm⁻¹ for the ninyhdrin-proline complex.

For assays evaluating the temperature profile, WT-Ph₁ prol and the four prolidase mutants were assayed in triplicate for activity with 4 mM Met-Pro at 10°C, 20°C, 35°C, 50°C, 70°C, and 100°C. Experiments were performed in duplicate.

2.7. Substrate Specificity and Kinetics Experiments. In order to study substrate specificity, the following Xaa-Pro dipeptides were used as substrates (4 mM final concentration) in the enzyme activity assays for WT-Ph₁ prol and the four prolidase mutants: Val-Pro, Met-Pro, Phe-Pro, Ala-Pro, and Gly-Pro. Prolidase samples were assayed with two additional substrates, Pro-Ala and Val-Leu-Pro, to further illustrate prolidase preference of Xaa-Pro dipeptides [20]. Kinetic parameters of the Ph₁ prol mutants were determined at 70°C using a range of Leu-Pro concentrations (0.25–12 mM).

2.8. Thermostability and Pot-Life Experiments. Thermostability experiments were performed in duplicate on WT-Ph₁ prol and the four mutants. Each enzyme was diluted to a concentration of 0.04 mg/mL in 50 mM MOPS, pH 7.0, and 200 mM NaCl and incubated in an anaerobic sealed vial at 90°C. An initial sample was taken to represent Time = 0 h, and additional samples were taken at Time = 24, 48, and 72 h. Samples were diluted to 0.4 μg/mL in 50 mM MOPS, pH 7.0, and 200 mM NaCl and were then assayed in triplicate in accordance with the enzyme activity assay protocol described in Section 2.6. In all cases, the substrate used in the activity assay was 4 mM Met-Pro.

Pot-life experiments were also performed in duplicate. Each enzyme was diluted to a concentration of 0.04 mg/mL in 50 mM MOPS, pH 7.0, and 200 mM NaCl and incubated anaerobically in a sealed vial at 70°C. An initial sample was taken to represent Time = 0 days, and additional samples were taken at Time = 1, 7, 14, 16, and 21 days. Samples were diluted to 0.4 μg/mL in 50 mM MOPS, pH 7.0, and 200 mM NaCl and were then assayed in triplicate to determine specific activity.

2.9. DSC Experiment. Differential scanning calorimetry was performed using a MicroCal VP-DSC scanning calorimeter. The calorimetric samples contained ~1 mg/mL protein in 50 mM MOPS, 200 mM NaCl, 0.2 mM CoCl₂, pH 7.0. Protein samples were dialyzed 15 h against this buffer before the experiment. Samples were degassed before loading into the chamber cell. The calorimetric experiment was performed by heating the samples at a scan rate of 100°C/hr.

2.10. DFP (diisopropylfluorophosphate) Assay. The hydrolysis of DFP by prolidases was measured by monitoring fluoride release with a fluoride-specific electrode as previously described [22]. Assays were performed at 35°C and 50°C, with continuous stirring in 2.5 mL of buffer (50 mM MOPS, 200 mM NaCl, pH 7.0), 0.2 mM CoCl₂ and 3 mM DFP. The enzyme and metal were incubated at the reaction temperature 5 min prior to the start of the reaction. The background of DFP hydrolysis was measured by running a reaction without enzyme present at 35°C and 50°C. The background hydrolysis of DFP was subtracted from enzymatic hydrolysis to determine specific activity of the enzyme.

2.11. p-Nitrophenyl Soman Assay (O-Pinacolyl p-Nitrophenyl Methylphosphonate Activity). Prolidase hydrolysis of p-nitrophenyl soman was monitored by accumulation of p-nitrophenolate [10, 22]. The p-Nitrophenyl Soman was synthesized at Edgewood Chemical Biological Center in Aberdeen Proving Ground, Md. and contained a racemic mixture of all four stereoisomers. The purity of the soman analog was greater than 90% based on gas chromatography analysis [12]. Two mL reaction assays contained buffer (50 mM MOPS, 200 mM NaCl, pH 7.0), 0.2 mM CoCl₂, and 0.3 mM p-nitrophenyl soman. The reactions were conducted at three different temperatures (35°C, 50°C and 70°C). The enzyme and metal were incubated at the specified reaction temperature 5 min prior to the start of the reaction. Absorbance of the product p-nitrophenolate was measured at 405 nm over a 5 min range. To calculate activity, the extinction coefficient for p-nitrophenolate of 10,101 M⁻¹ cm⁻¹ was used.
3. Results and Discussion

3.1. P. furiosus and P. horikoshii Prolidase-Specific Activities with OP Nerve Agents: DFP and Soman Analog. Previous studies characterizing P. horikoshii prolidase homolog 1 (Ph1prol, PH0974) demonstrated that it has higher catalytic activity over a broader pH range, higher affinity for metal, and is more thermostable than either P. furiosus prolidase (Pfprol, PF1343) or P. horikoshii prolidase (Ph1prol, PH11149) when assayed with the dipeptide substrate Met-Pro [20]. Based on these favorable attributes for Ph1prol when reacting with its natural substrates, Xaa-Pro dipeptides, there was interest in determining the relative activity of recombinant Ph1prol compared to Pfprol and Ph1prol against G-type nerve agent simulants DFP and soman analog, p-nitrophenylsoman. As indicated in Figure 1, DFP exhibited the greatest hydrolysis with Ph1prol. Ph1prol had a relative activity that was 843% higher than Pfprol and 817% higher than Ph1prol at 35 °C and 1870% higher than both Pfprol and Ph1prol at 50 °C (Figure 1). In contrast, the trends with the soman analog were very different as shown in Figure 2. The relative activity of Ph1prol was only 70%, 63%, and 68% of the Pfprol activity at 35 °C, 50 °C, and 70 °C, respectively (Figure 2). These results indicate that Ph1prol has a preference for DFP and does not exhibit high activity with the soman analog. Differences in the protein structures likely play a role in the substrate preference since Pfprol and Ph1prol share only 55% amino acid residue similarity [16]. By altering the Ph1prol structure further using a random mutagenesis approach, it would be possible to isolate Ph1prol variants that show even greater hydrolysis of DFP and/or improved activity against the soman analog.

3.2. Screening and Isolation of the Ph1prol Mutant Population Using Proline Auxotrophic Strain JD1 (ADE3). Since Ph1prol showed the most favorable properties including higher activity with DFP, it was selected for further mutagenesis using an error-prone PCR strategy, which employs a mutated polymerase. Transformed E. coli JD1 (λDE3) cells were used to select for Ph1prol variants on minimal media plates that were supplemented with 20 μM Leu-Pro and grown at 20 °C. Colonies that were visible in 3–7 days were plated on minimal and rich (LB) media. Ph1prol variants were screened using small-scale expression cultures (10 mL) induced with IPTG. Four Ph1prol variants out of over 200 screened were selected for sequencing after showing two-fold higher activity with Leu-Pro at 30 °C and somewhat reduced activity at 100 °C as compared to wild type. The increased activity at the lower temperature of 30 °C and variation at the higher temperature of 100 °C is indicative of a mutation in a thermophilic protein, which can compromise activity or stability at higher temperatures but could create more flexibility and increased catalysis at the lower temperatures.

3.3. Sequencing of Ph1prol Mutants. Prior to sequencing, the four Ph1prol mutants were numbered (10, 19, 35, and 72) based solely on the order in which they had been isolated. Based on these favorable attributes for Ph1prol when reacting with its natural substrates, Xaa-Pro dipeptides, there was interest in determining the relative activity of recombinant Ph1prol compared to Pfprol and Ph1prol against G-type nerve agent simulants DFP and soman analog, p-nitrophenylsoman. As indicated in Figure 1, DFP exhibited the greatest hydrolysis with Ph1prol. Ph1prol had a relative activity that was 843% higher than Pfprol and 817% higher than Ph1prol at 35 °C and 1870% higher than both Pfprol and Ph1prol at 50 °C (Figure 1). In contrast, the trends with the soman analog were very different as shown in Figure 2. The relative activity of Ph1prol was only 70%, 63%, and 68% of the Pfprol activity at 35 °C, 50 °C, and 70 °C, respectively (Figure 2). These results indicate that Ph1prol has a preference for DFP and does not exhibit high activity with the soman analog. Differences in the protein structures likely play a role in the substrate preference since Pfprol and Ph1prol share only 55% amino acid residue similarity [16]. By altering the Ph1prol structure further using a random mutagenesis approach, it would be possible to isolate Ph1prol variants that show even greater hydrolysis of DFP and/or improved activity against the soman analog.

Sequencing of the variants revealed the locations of the amino acid substitutions for each mutant (Figure 3). Mutant no.10 has two mutations: one at position 195 in which there is a change from alanine to threonine (A195T) and the second at amino acid residue 306 in which glycine is changed to serine (G306S). Both of these mutations reside...
in the C-terminal region of *Ph1* prol. Mutant no.19 has two mutations: one at position 301 in which there is a change from tyrosine to cysteine (Y301C) and the second at amino acid residue 342 which has a substitution of lysine with an asparagine (K342N). Both of these mutations are in the C-terminal region of the enzyme. Mutant no.35 contains two mutations: one at position 127 in the α-helical linker region in which there is a change from glutamate to glycine (E127G) and the second in the C-terminal region of position 252 with a substitution of glutamate for aspartate (E252D). Mutant no.72 is the only mutation in the N-terminal region at position 36 with a change from glutamate to valine (E36V) in *Ph1* prol. The mutations are remote from the active site pocket, which is shown in Figure 3 as being located between two 3_10 helices (red helices, residues 191–195, and 281–284). Therefore, the mutations are not likely directly changing the active site chemistry. Rather the mutations such as E36V, E127G, and Y301C may be affecting prolidase dimerization as those residues are located along the dimerization interface. Furthermore, the mutations may be affecting the conformational dynamics of the enzymes since some of the mutations are located in the loop and linker regions. The change in conformation dynamics is giving the variants better activity over a broader range of temperatures as indicated in Section 3.4.

3.4. Effects of Mutagenesis on the Temperature Profile of *P. horikoshii* Prolidase Variants. Both the wild type *Ph1* prol and the four variants were most active at 100 °C (Figure 4). WT-*Ph1* prol and E36V-*Ph1* prol had very high specific activities (3,955 U/mg and 4094 U/mg, resp.) with 4 mM Met-Pro, both of which are twice as high as that of *Pf* prol at 100 °C (2,154 U/mg) [21]. A195T/G306S- and E127G/E252D-*Ph1* prol were slightly less active (2,307 U/mg and 2,831 U/mg, resp.) than WT *Ph1* prol, and Y301C/K342N-*Ph1* prol showed the lowest level of activity in comparison to the other mutants at 1,842 U/mg. Activity was reduced by more than half at 70 °C for all of the variants; however, WT-*Ph1* prol, A195T/G306S-, and E36V-*Ph1* prol all had specific activities close to 1,000 U/mg (973 U/mg, 1000 U/mg, and 913 U/mg, resp.), whereas the specific activity of *Pf* prol at 70 °C was 806 U/mg [21]. At 50 °C, Y301C/K342N-*Ph1* prol had a higher specific activity than any of the other variants and the wild type (450 U/mg) and was roughly three times more active than *Pf* prol at 50 °C [21].

At the lower temperatures, 35 °C, 20 °C and 10 °C, Y301C/K342N-*Ph1* prol out-performs the other *Ph1* prol variants, the WT-*Ph1* prol, the WT-*Pf* prol and R19G/G39E/K71E/S229T-*Pf* prol (the highest performing *Pf* prol mutant at lower temperatures) [21]. At 35 °C, Y301C/K342N-*Ph1* prol (298 U/mg) has relative activity that is 121% that of WT-*Ph1* prol, 489% that of WT-*Pf* prol, and 244% that of R19G/G39E/K71E/S229T-*Pf* prol (246 U/mg, 61 U/mg, and 122 U/mg, resp.). At 20 °C, Y301C/K342N-*Ph1* prol (241 U/mg) has a relative activity 184% that of WT-*Ph1* prol, 964% higher than WT-*Pf* prol, and 482% higher than R19G/G39E/K71E/S229T-*Pf* prol (specific activities of 131 U/mg for WT-*Ph1* prol, 25 U/mg for WT-*Pf* prol and 2,831 U/mg, resp.) than WT-*Ph1* prol, 964% higher than WT-*Pf* prol, and 482% higher than R19G/G39E/K71E/S229T-*Pf* prol (specific activities of 131 U/mg at 10 °C, 131 U/mg at 20 °C, 246 U/mg at 35 °C, 340 U/mg at 50 °C, 973 U/mg at 70 °C, and 3,955 U/mg at 100 °C.

Figure 4: Temperature profile of WT-*Ph1* prol and the four variants. Relative activities are shown as a percentage of the WT-*Ph1* prol. Prolidase assays were performed in triplicate at 10 °C, 20 °C, 35 °C, 50 °C, 70 °C, and 100 °C and contained 0.2 mM CoCl2 and 4 mM Met-Pro. WT-*Ph1* prol-specific activity was 66 U/mg at 10 °C, 131 U/mg at 20 °C, 246 U/mg at 35 °C, 340 U/mg at 50 °C, 973 U/mg at 70 °C, and 3,955 U/mg at 100 °C.
50 U/mg for R19G/G39E/K71E/S229T-Pf prol) [21]. The greatest improvement in performance for the Ph1 prol mutants is seen when assayed at 10°C. Y301C/K342N-Ph1 prol (109 U/mg) has a relative activity that is 166% higher than that of WT-Ph1 prol, 1,982% higher than that of WT-Ph prol, and 396% higher than R19G/G39E/K71E/S229T-Pf prol (specific activities of 66 U/mg for WT-Ph1 prol, 5.5 U/mg for WT-Ph prol, and 27.5 U/mg for R19G/G39E/K71E/S229T-Pf prol) [21].

3.5. Effects of Mutagenesis on Substrate Specificity and Kinetics of P. horikoshii Prolidase Variants. Substrate specificity of WT-Ph1 prol is shown in Table 1 along with the specific activities of A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Ph1 prol, which are reported as a percentage relative to the activity of the wild type. WT-Ph1 prol was most active with the dipeptide Val-Pro (4,602 U/mg), while mutants A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Ph1 prol had much lower activity at 33%, 36%, 46%, and 56% of the WT Ph1 prol, respectively. A195T/G306S-Ph1 prol showed the highest activity with Met-Pro at 143% that of the wild type (2,809 U/mg), while Y301C/K342N-, E127G/E252D-, and E36V-Ph1 prol preferred Ala-Pro with specific activities of 183%, 324%, and 556% that of WT-Ph1 prol (1,452 U/mg). WT-Ph1 prol seems to prefer the most hydrophobic amino acids, while the four variants have the highest activity with a less hydrophobic amino acid in the N-terminal position of the dipeptide substrate. While alanine is considered to be a hydrophobic amino acid, it is less hydrophobic than valine, methionine, phenylalanine, and leucine and is most similar in structure to glycine. WT-Ph1 prol has much lower activity with Gly-Pro than with Ala-Pro (369 compared to 1,452 U/mg). A195T/G306S-, Y301C/K342N-, and E127G/E252D-Ph1 prol have less or similar activity with Gly-Pro as compared to WT-Ph1 prol (369 U/mg) while E36V-Ph1 prol has 444% higher activity.

Specific activities were consistently low with both Pro-Ala and Val-Leu-Pro (2 U/mg, WT-Ph1 prol) for the wild type Ph1 prol and the four mutants. Due to the nature of the prolidase enzyme and its unique ability to cleave the bond between Xaa-Pro dipeptides, it was expected that the enzyme would not show any notable activity with a Pro-Xaa dipeptide or a tripeptide. While two of the four variants (mutants A195T/G306S- and E36V-Ph1 prol) show increased activity with Pro-Ala when compared to wild type, it must be noted that specific activity with Pro-Ala for WT-Ph1 prol was extremely low, at only 14 U/mg. Y301C/K342N-Ph1 prol had 88% WT activity with Val-Leu-Pro; however, WT-Ph1 prol-specific activity was only 2 U/mg.

The catalytic activities of WT-Ph1 prol and its mutants were tested at 70°C with Leu-Pro (Table 2). All Ph1 prol mutants had higher $k_{cat}$ values than the WT-Ph1 prol suggesting that the amino acid changes in the mutant enzymes did have an effect on structure and ultimately the catalytic activity of the prolidase with the substrate Leu-Pro. Although the $k_{cat}$ values were higher, the overall enzyme turnover rates were not for some of the mutants compared to WT-Ph1 prol.

### Table 1: Substrate specificity of recombinant wild type and variant P. horikoshii prolidases with different proline dipeptides and a single-proline tripeptide.

| Substrate | Relative Activity (%) of WT-Ph1 prol-specific activity |
|-----------|-------------------------------------------------------|
| Val-Pro   | 100 (4,602)                                           |
| Met-Pro   | 100 (2,809)                                           |
| Phe-Pro   | 100 (2,199)                                           |
| Leu-Pro   | 100 (2,132)                                           |
| Ala-Pro   | 100 (1,452)                                           |
| Gly-Pro   | 100 (369)                                             |
| Pro-Ala   | 100 (14)                                              |
| Val-Leu-Pro| 100 (2)                                                |

Prolidase assays were performed at 100°C and contained 0.2 mM CoCl$_2$ and 4 mM of each substrate. One hundred percent specific activity is reported for WT-Ph1 prol and correlates to U/mg in parentheses below the 100% relative activity.

### Table 2: Kinetic parameters of wild type Pyrococcus horikoshii prolidase, Ph1 prol, and prolidase variants with Leu-Pro at 70°C.

| Prolidase   | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$) |
|-------------|------------|-------------------------|----------------------|----------------------------------|
| WT-Ph1 prol | 0.92 ± 0.16| 809 ± 55                | 1079                 | 1172                             |
| A195T/G306S | 0.81 ± 0.26| 1245 ± 149              | 1660                 | 2049                             |
| Y301C/K342N | 2.92 ± 0.40| 2146 ± 177              | 2861                 | 980                              |
| E127G/E252D | 0.98 ± 0.24| 1119 ± 101              | 1492                 | 1522                             |
| E36V       | 1.6 ± 0.37 | 1597 ± 174              | 2129                 | 1331                             |

Enzyme assays were done using a range of Leu-Pro concentrations (0.25–12 mM). Enzyme kinetics were calculated using nonlinear regression (curve fit) and analyzed using the Michaelis-Menten equation utilizing software from Prism 5 (GraphPad La Jolla, CA). All Ph1 prol assays contained 0.2 mM CoCl$_2$.

All the Ph1 prol mutants showed an increased turnover rate, $k_{cat}/K_m$, with Leu-Pro except for Y301C/K342N-Ph1 prol. This could be due to the increase in the $K_m$ of this mutant, which is almost three times higher than WT-Ph1 prol.

3.6. Effects of Amino Acid Substitutions on the Thermostability and Pot-Life Activity of P. horikoshii Prolidase Mutants. To determine whether the amino acid substitutions in the four Ph1 prol variants had any effect on thermostability, the mutants were incubated in sealed vials at 90°C, anaerobically, for 72 h. Samples were taken every 24 h to measure
catalytic activity with Met-Pro (4 mM) as the substrate. In Theriot et al. 2010, it was shown that WT-Pf prol was more thermostable than its mutants and had lost 50% activity with Met-Pro by 21 hours at 90°C [20]. After 40 h at 90°C, WT-Pf prol had lost 50% activity. Mutants Y301C/K342N-Pf prol and E127G/E252D-Pf prol both demonstrated increased thermostability at 90°C compared to wild type and had lost 50% activity after 58 hours of incubation. Mutants A195T/G306S-Pf prol and E36V-Pf prol displayed a 50% loss of activity after 32 and 35 hours at 90°C, respectively. Both WT-Pf prol and the four Ph prol mutants were stable at 90°C for a significantly longer time period than Pf prol and its mutants [20].

Pot-life activity was monitored over the course of 21 days with samples taken every seven days until day 14 and then again on days 16 and 21 while the samples were incubated anaerobically at 70°C (Figure 5). Pot-life experiments with WT-Pf prol and its mutants as reported in Theriot et al., 2010 were conducted over a 48 h period at 70°C [20]. In preliminary pot-life experiments with WT-Pf prol and its mutants, the specific activities with Met-Pro were still remarkably high after 48 h (no obvious decrease in specific activity was detected; data not shown), so the experiments were continued until the enzymes were considered to be no longer active (21 days). Initial specific activities for WT-Pf prol, A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Pf prol were 3,150 U/mg, 3,400 U/mg, 1,250 U/mg, 2,200 U/mg, and 3,600 U/mg, respectively, with 4 mM Met-Pro at 70°C. Mutants Y301C/K342N-, E127G/E252D-, and E36V-Pf prol had 50% activity remaining by days 12, 13, and 14, respectively. A195T/G306S-Pf prol was at 50% activity after 10 days at 70°C. By 21 days at 70°C, all five prolidases were at or below 25% of the initial activity.

As reported in Theriot et al., 2010, the specific activities of WT-Pf prol and its three mutants (G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-Pf prol) were 1,083 U/mg, 599 U/mg, 722 U/mg, and 4,496 U/mg, respectively, with 4 mM Met-Pro as the substrate. WT-Pf prol had lost 50% activity by day 12 of incubation at 70°C. Mutants Y301C/K342N-, E127G/E252D-, and E36V-Pf prol had 50% activity remaining by days 12, 13, and 14, respectively. A195T/G306S-Pf prol was at 50% activity after 10 days at 70°C. By 21 days at 70°C, all five prolidases were at or below 25% of the initial activity.

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3.7. DSC Results. The thermal stability of wild type Ph prol and variants was determined by differential scanning calorimetry (DSC) experiments. Table 3 shows the denaturation temperature of the wild type and variant enzymes. The mutations that improved catalytic activity of the Ph prol at lower temperatures did not adversely affect the temperature stability of the enzymes.

3.8. Effects of Amino Acid Substitutions on Substrate Specificity with OP Nerve Agents DFP and Soman Analog, p-Nitrophenyl Soman. Substrate specificity of WT-Pf prol with DFP is shown in Figure 6 along with the specific activities of A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Pf prol, which are reported as a percentage relative to the activity of the wild type. WT-Pf prol was most active with DFP at 35°C and 50°C with a specific activity of 4 U/mg and 10 U/mg, respectively. The mutants A195T/G306S-, Y301C/K342N-, E127G/E252D-, and E36V-Pf prol had significantly lower activity with DFP; even at 50°C the activity was only 59%, 25%, and 55% of WT-Pf prol activity. However, it should be noted that the Ph prol mutants have 808%, 183%, and 402% (A195T/G306S, Y301C/K342N-, E127G/E252D-, and E36V-Pf prol, resp.) of the DFP activity compared to WT P. furiosus prolidase and also compared favorably to the highest DFP activity reported

![Figure 5: Pot-life activity of WT-Pf prol and prolidase mutants incubated anaerobically at 70°C. Pot-life experiments were performed in duplicate and samples were taken at 0, 1, 7, 14, 16, and 21 days to assess reactivity. Prolidase assays were performed in triplicate at 100°C and contained 0.2 mM CoCl₂ and 4 mM Met-Pro. Error bars in Figure 5 represent standard deviation of specific activity across duplicate runs.](image-url)
for the R19/G39E/K71E/S229T Pf prol mutant, which was shown to have 398% higher activity than WT Pf prol [21].

Figure 7 reveals a different trend, where the mutations in WT Pf prol increased the specific activity with the soman analog, p-nitrophenyl soman. WT Pf prol showed the highest activity with the soman analog at 70°C, with a specific activity of 0.56 U/mg. The mutant A19ST/G306S-Pf prol had a similar specific activity to WT Pf prol when incubated at 35°C, 50°C, and 70°C. Mutants Y301C/K342N-, E127G/E252D-, and E36V-Pf prol showed increased activity with the soman analog over WT Pf prol at each assay temperature. The most significant specific activities with the soman analog were seen with Y301C/K342N-, E127G/E252D-, and E36V-Pf prol at 70°C, which correlated to 125%, 186%, and 157% increase over WT Pf prol. Furthermore, the activities for the Pf prol mutants against p-nitrophenyl soman (0.7, 1.0, and 0.9 U/mg for Y301C/K342N-, E127G/E252D-, and E36V-Pf prol, resp.) compare favorably to the improved soman analog activities reported for the P. furiosus prolidase mutants (0.86, 1.02, and 1.7 U/mg for G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-Pf prol, resp.) [20]. When looking at the substrate specificity of the WT Pf prol and variants with proline dipeptides, it was noticed that there was a shift in preference from more hydrophobic to less hydrophobic amino acids among the mutants. This is also seen with the OP nerve agents, where there is a shift in substrate specificity from DFP to the soman analog. The WT Pf prol prefers DFP as a substrate over the soman analog, while the Pf prol variants show decreased activity with DFP and increased activity with the soman analog.

4. Conclusion

Current biodecontamination formulations for degradation of OP nerve agents that incorporate Alteromonas prolidases (OPAA) and PTE have limitations when used in the field [12]. Long-term stability of the enzyme is not attainable in a formulation mixture that includes other solvents and denaturing solutions, and the need to add excess metal to reach maximum activity poses further complications for an enzyme-based detoxification system. A highly active enzyme that is stable over the long term and requires very little metal addition would be best suited for this application. The wild type and mutant prolidases characterized from P. horikoshii show promising enzymatic properties that make them potential candidates for future optimization studies for OP nerve agent degradation. Compared to Pf prol, Pf prol and the four Pf prol mutants show higher activity, higher affinity for the substrate, and significantly lower metal requirement for catalysis. Two of the variants, Y301C/K342N- and E127G/E252D-Pf prol are thermostable for nearly three times as long as Pf prol and double the time of Pf prol. A19ST/G306S-Pf prol has 808% of the DFP activity compared to wild type P. furiosus prolidase and is superior to any of the improved P. furiosus prolidase mutants [21]. Furthermore, Y301C/K342N-, E127G/E252D-, and E36V-Pf prol all have improved activities against p-nitrophenyl soman relative to WT Pf prol and also compare favorably to the best performing P. furiosus prolidase mutants [20]. The Pf prol variants have the potential to significantly improve upon current biodecontamination strategies. Their increased thermostability and pot life and activities against

![Figure 6: Relative activity with WT Pf prol and prolidase mutants with OP nerve agent DFP.](image1)

![Figure 7: Relative activity with WT Pf prol and prolidase mutants with OP nerve agent analog, p-nitrophenyl soman.](image2)
OP nerve agent analogs warrant further study into large-scale production and purification of these prolidases.

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