

Improving the catalytic activity of hyperthermophilic *Pyrococcus* prolidases for detoxification of organophosphorus nerve agents over a broad range of temperatures

Casey M. Theriot · Xuelian Du · Sherry R. Tove · Amy M. Grunden

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Abstract Prolidase isolated from the hyperthermophilic archaeon *Pyrococcus furiosus* has potential for application for decontamination of organophosphorus compounds in certain pesticides and chemical warfare agents under harsh conditions. However, current applications that use an enzyme-based cocktail are limited by poor long-term enzyme stability and low reactivity over a broad range of temperatures. To obtain a better enzyme for OP nerve agent decontamination and to investigate structural factors that influence protein thermostability and thermoactivity, randomly mutated *P. furiosus* prolidases were prepared by using XL1-red-based mutagenesis and error-prone PCR. An *Escherichia coli* strain JD1 (Δ DE3) (auxotrophic for proline [Δ *proA*] and having deletions in *pepQ* and *pepP* dipeptidases with specificity for proline-containing dipeptides) was constructed for screening mutant *P. furiosus* prolidase expression plasmids. JD1 (Δ DE3) cells were transformed with mutated prolidase expression plasmids and plated on minimal media supplemented with 50 μ M Leu-Pro as the only source of proline. By using this positive selection, *Pyrococcus* prolidase mutants with improved activity over a broader range of temperatures were isolated. The activities of the mutants over a broad temperature range were

measured for both Xaa-Pro dipeptides and OP nerve agents, and the thermoactivity and thermostability of the mutants were determined.

Keywords Prolidase · *Pyrococcus furiosus* · OP nerve agents · Mutagenesis · Directed evolution

Introduction

Organophosphorus compounds (OPs), one of the most frequently occurring components in pesticides and chemical warfare agents, are highly toxic and often hard to degrade. Examples of these OPs include the serine protease inhibitor diisopropylfluorophosphates (DFP) and G-type nerve agents such as soman (GD; *O*-pinacolyl methylphosphonofluoridate), sarin (GB; *O*-isopropyl methylphosphonofluoridate), and GF (*O*-cyclohexyl methylphosphonofluoridate). Exposure to OP compounds can irreversibly inhibit peripheral and central nervous system acetylcholinesterase (AChE). AChE is responsible for terminating the action of the neurotransmitter acetylcholine. Inhibition of this enzyme results in an accumulation of acetylcholine, which causes an over stimulation of muscarinic and nicotinic receptors, and even produces serious nerve agent poisoning including hypersecretion, convulsions, respiratory distress, coma, and death (Wetherell et al. 2006).

Currently, decontamination solutions such as DS2 and bleach are used to inactivate toxic OP compounds (Cheng et al. 1998). Despite their effective decontamination of OP compounds, both DS2 and bleach are very corrosive and result in hazardous waste production. While enzyme-based decontamination cocktails are being considered, the Defense

C. M. Theriot · X. Du · S. R. Tove
Department of Microbiology, North Carolina State University,
4548 Gardner Hall, Campus Box 7615, Raleigh,
NC 27695-7615, USA

A. M. Grunden (✉)
Department of Microbiology, North Carolina State University,
4550A Gardner Hall, Campus Box 7615, Raleigh,
NC 27695-7615, USA
e-mail: amy_grunden@ncsu.edu

Threat Reduction Agency (DTRA) recommends that enzymes to be used in these cocktails must have at least a 12-h pot-life reactivity over a range of conditions including different temperatures, pH, and in the presence of salts and other surfactants (DTRA 2008). Previously, organophosphorus acid anhydrolase (OPAA) isolated from *Alteromonas* sp. strain JD6.5, a halophilic organism, has been used for detoxification of DFP, sarin, soman, and tabun; however, OPAA demonstrates low hydrolysis of P–O and P–C bonds (DeFrank and Cheng 1991). Recently, the crystal structure of OPAA has been solved, and it was determined to be a prolidase (Vyas et al. 2010).

Prolidase is a ubiquitous dipeptidase that specifically cleaves dipeptides with proline at the C-terminus (NH₂-X-/Pro-COOH). This enzyme has been isolated and characterized from a variety of sources, such as from mammalian tissues (Browne and O’Cuinn 1983; Endo et al. 1987; Fukasawa et al. 2001; Myara et al. 1994; Sjostrom et al. 1973) as well as from bacterial and archaeal sources (Booth et al. 1990; Fernandez-Esplá et al. 1997; Fujii et al. 1996; Ghosh et al. 1998; Jalving et al. 2002; Morel et al. 1999; Suga et al. 1995). The majority of the characterized prolidases contain a common “pita-bread” fold with a dinuclear metal center, which is generated by five conserved amino acid residues (Lowther and Matthews 2002). The OPAA crystal structure shares the same “pita-bread” fold with the characteristic catalytic dinuclear metal site. These findings indicate that prolidases can and are currently being used to decontaminate and detoxify OP exposed surfaces.

Among all prolidases that have been characterized so far, the ones that have been isolated from mesophilic sources are only maximally active at temperatures up to 55°C. However, prolidases that have been isolated from the hyperthermophilic archaea *Pyrococcus furiosus* and *Pyrococcus horikoshii* have maximum activity at 100°C. In addition, both recombinant *Pyrococcus* prolidases produced in *Escherichia coli* possess long-term thermostability. When incubated at 100°C, *P. furiosus* and *P. horikoshii* prolidases show no activity loss after 12 and 8 h, respectively (Ghosh et al. 1998; Theriot et al. 2009). *P. horikoshii* prolidases have a 50% activity loss after 21 h at 90°C (Theriot et al. 2009). The ability to maintain reactivity at high temperatures over long periods of time (pot-life activity) makes *Pyrococcus* prolidases ideal candidates for OP decontamination, where harsh conditions, such as high temperatures, organic solvents, and denaturants, are often required (Adams et al. 1995).

Despite the advantage *P. furiosus* prolidase exhibits under high temperature conditions, the use of *P. furiosus* prolidase for OP nerve agent decontamination is restricted by the fact that this enzyme displays a narrow functional temperature range. Like many other enzymes isolated from

hyperthermophiles, *P. furiosus* prolidase has only 50% activity at 80°C and displays little activity at temperatures below 50°C (Ghosh et al. 1998). Therefore, it was desirable to attempt to prepare and screen *P. furiosus* prolidase mutants that demonstrate increased activity against OP compounds at low temperature while maintaining thermostability.

For these reasons, we constructed a random-mutated *P. furiosus* prolidase gene library and screened it for production of mutants with increased activity at room temperature. *P. furiosus* mutant prolidases were purified and characterized to determine their substrate catalysis over a range of temperature as well as their thermoactivity and thermostability compared to the wild-type enzyme.

Materials and methods

Bacterial strains and media

E. coli K-12 derivative NK5525 (*proA*::Tn10) was used to construct the selective strain JD1(λDE3) for screening of low-temperature active *P. furiosus* prolidase mutants. *E. coli* strains used in this study were cultured either in Luria–Bertani (LB) broth or M9 minimal medium supplemented with 0.2% glucose, 1 mM MgSO₄, 0.05% thiamin, 20 μM IPTG, and 50 μM Leu-Pro. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (34 μg/ml), and tetracycline (6 μg/ml) were added as required.

Construction of the *E. coli* selection strain JD1 (λDE3)

Selection strain JD1 (λDE3) was constructed as follows: (1) integration of λDE3 prophage into NK5525 chromosome by using a λDE3 lysogenization Kit (Novagen) according to the supplier’s protocol; (2) permanent disruption of *proA* in NK5525(λDE3) by removal of the chromosomal *proA*:Tn10 transposon using positive selection on fusaric acid media as described by Maloy and Nunn (1981); and (3) disruption of *pepP* and *pepQ* in NK5525(λDE3) by introducing chloramphenicol resistance (Cm^r) and kanamycin resistance (Km^r) cassettes within each gene, respectively, using the λ-Red system (Datsenko and Wanner 2000). The Cm^r cassette was amplified from the pKD3 plasmid using primer Ec PepP F and primer Ec PepP R (Table 1). The 5′ terminus of primer Ec PepP F and Ec PepP R had 50 and 52 nucleotides identical to sequences on the 5′-end and 3′-end of *pepP*, respectively; the Km^r cassette was amplified from the pKD4 plasmid using primer Ec PepQ F and primer Ec PepQ R (Table 1). The 5′ terminus of primer Ec PepQ F had 51 nucleotides identical to sequence upstream of *pepQ*, and the 5′ terminus of primer Ec PepQ R had 50 nucleotides identical to downstream sequence of *pepQ*.

Table 1 PCR and sequencing primers used in this study

Name	Sequence (5'–3')	Function of PCR product
Ec PepP F	GTGAGATATCCCGCAAGAGTTTCAGCGTCGCCGT CAGGCCCTGGTGGAGGTAGGCTGGAGCTGCTTCG	For replacement of <i>pepP</i> with Cm cassette
Ec PepP R	GACGGATAGCGCGCACCGTGCGGTTAAATTCGTG GTGAATTCGCCTTCCATATGAATATCCTCCTTAG	For replacement of <i>pepP</i> with Cm cassette
Ec PepQ F	AAATCATATAGCTACCTTACAAGAACGGACTCGCG ATGCGCTGGCGCGCTTGTAGGCTGGAGCTGCTTCG	For replacement of <i>pepQ</i> with Km cassette
Ec PepQ R	TCAGCCACTTTCGCCGCCGGAAGCGCCACCCGCAG CAGAACGAATGCCTGCATATGAATATCCTCCTTAG	For replacement of <i>pepQ</i> with Km cassette
PepP-Up	GGTTGGGTCAATCACTTCCTG	For PCR verification of <i>pepP</i> deletion
PepP-Dn	AGCATGTGACTCTGGCGCAGT	For PCR verification of <i>pepP</i> deletion
PepQ-Up	GCCATCTCCAGCCAGTCAA	For PCR verification of <i>pepQ</i> deletion
PepQ-Dn	TTGCGGTTACGCCACCACAGC	For PCR verification of <i>pepQ</i> deletion
Prol-1	ATAGGATCCGGTGAGGAGTTGTATGAAAGAAAG ACTTGAA	Cloning of prolidase gene into vector
Prol-2	ATAGCGGCCGCCTACATTAATCAGAAAGGCTGAA GTTGTTA	Cloning of prolidase gene into vector
Prol-3	TGATGACGTGATAAAGGA	For prolidase gene sequencing
Prol-4	TGATATGAAAGCAGCTCT	For prolidase gene sequencing
Prol-5	GCCCCTCTCTATCCTCTGTG	For prolidase gene sequencing
Prol G39E F	TCTGGA ^{ACTTCTCCCTGGGAGAGGG} GATACATAA TAGTTGACGGT (The mutation site of Gly to Glu is underlined)	For generation of R19G/G39E/K71E/S229T pET-prol plasmid
Prol G39E R	ACCGTCAACTATTATGTATCC ^{CTCTCC} CAGGGGA GAAGTTCCAGA (The mutation site of Gly to Glu is underlined)	For generation of R19G/G39E/K71E/S229T pET-prol plasmid

PCR-amplified products were purified using a commercial purification kit (Qiagen Germantown, MD, USA) and were integrated into the *E. coli* NK5525(λDE3) strain chromosome using the method developed by Datsenko and Wanner. The *pepP* and *pepQ* disruptions in NK5525 (λDE3) were confirmed by PCR using primers PepP-Up and PepP-Dn and PepQ-Up and PepQ-Dn, respectively (Table 1).

Construction of a pool of pET-prol plasmids carrying randomly mutated *P. furiosus* prolidase genes

To avoid biased results generated from a single mutagenesis method, random mutations were introduced into the gene encoding *P. furiosus* prolidase by three independent mutagenesis methods: error-prone PCR mutagenesis, hydroxylamine mutagenesis, and passage through mutation-prone *E. coli* XL1-red cells.

Error-prone PCR mutagenesis was done using the Genemorph II Random mutagenesis kit. Mutazyme II DNA polymerase (Stratagene, La Jolla, CA, USA) was used to amplify the *P. furiosus* prolidase gene with the pET-prol expression plasmid serving as template (Ghosh et al. 1998). PCR amplification was carried out for 30

cycles (60 s at 95°C, 60 s at 55°C, 120 s at 72°C), with a 10-min 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 50, 250, and 750 ng in order to select for high, medium, and low mutation rates, respectively. The Genemorph II EZClone (Stratagene) reaction was used to clone mutated prolidase genes into expression vector pET-21b according to the supplier's protocol.

Hydroxylamine mutagenesis was conducted based on a previously described method (Grunden et al. 1996). *P. furiosus* prolidase gene (7.5 μg) was subjected to hydroxylamine mutagenesis. Hydroxylamine was removed using the PCR purification kit (Qiagen). Mutated prolidase gene fragments were digested with *Bam*HI and *Not*I and were religated into pET-21b.

For XL1-red-based mutagenesis, mutation was directed against the intact pET-prol plasmid by transforming a pool of pET-prol plasmids into XL1-red cells seven times (Stratagene) according to the supplier's protocol. The mutagenized prolidase genes were then isolated from the passaged pET-prol plasmids using *Bam*HI and *Not*I restriction followed by religation into pET-21b vector.

Generation of the R19G/G39E/K71E/S229T mutant pET-prol plasmid

Production of the R19G/G39E/K71E/S229T pET-prol plasmid was carried out using the Site-Directed Mutagenesis kit (Stratagene). Two complementary oligonucleotide primers (Prol G39E F and Prol G39E R, Table 1) containing a mutation from glycine to glutamate at amino acid position 39 of R19G/K71E/S229T pET-prol plasmid were designed. These primers were used in a PCR reaction to amplify G39E pET-prol plasmid templates for 16 cycles (30 s at 95°C, 60 s at 55°C, and 7 min at 68°C). The PCR products were digested with *DpnI* prior to transformation into *E. coli* to remove un-mutated template DNA.

Screening for increased *P. furiosus* prolidase activity at low temperature (30°C)

pET-prol plasmids from the mutant *P. furiosus* library were transformed into the selection strain, JD1(ΔDE3), and were plated on M9 selective agar plates. Colonies that grew after being incubated for 3–7 days at room temperature were isolated and grown in 10 ml of LB medium at 37°C with shaking (200 rpm) until an optical density of 0.6–0.8 was reached. IPTG (1 mM) was then added to the cultures. Induced cultures were shaken at 37°C for 3 h before cell harvest. Cell pellets were lysed using 300 μl of B-PER reagent (Thermo Scientific, Rockford, IL, USA), and the resulting cell extracts were used for enzyme activity assays conducted at 30°C and 100°C. Mutant colonies that exhibited at least 2–3-fold higher activities compared to wild-type *P. furiosus* prolidase-expressing cells were selected and their plasmids were isolated. Prolidase genes from selected clones were sequenced using T7 promoter, prol-3, prol-4, and prol-5 and T7 terminator primers (Table 1) (MWG Biotech, Highpoint, NC, USA).

Purification of recombinant *P. furiosus* prolidase mutants

Production of *P. furiosus* prolidase and its variants G39E P_fprol, R19G/K71E/S229T P_fprol, and R19G/G39E/K71E/S229T P_fprol were carried out in *E. coli* BL21 (ΔDE3) that had been transformed with the appropriate pET-prol plasmid and pRIL vector. The transformants were grown in 1-L cultures in LB media and/or autoinduction media (Studier 2005) by incubation of the cultures at 37°C with shaking (200 rpm) until an optical density of 0.6–0.8 was reached. Expression of mutant prolidases was initiated when IPTG (1 mM) was added to the cell culture. Induced cultures were incubated at 37°C for 3 h prior to cell harvest. Autoinduction cultures were incubated overnight with shaking.

To purify G39E-, R19G/K71E/S229-, and R19G/G39E/K71E/S229T-prolidase, cell pellets were suspended in 50 mM Tris-HCl, pH 8.0, containing 1 mM benzamidine and 1 mM DTT (each 1 g wet wt of cell paste was suspended in 3 ml Tris-HCl buffer). Cell suspensions were passed through a French pressure cell (20,000 lb/in.²) twice. Lysed cell suspensions were centrifuged at 38,720×g for 30 min to remove any cell debris, and supernatants were heat-treated at 80°C for 30 min anaerobically. Denatured protein was removed by centrifugation at 38,720×g for 30 min. (NH₄)₂SO₄ was slowly added to each heat-treated mutant prolidase extract to a final concentration of 1.5 M, and extracts were kept on ice until applied to a 20-ml phenyl-sepharose column. Fractions containing the prolidase mutants were further purified by passage through a Q column. In the case of purification of the G39E-prolidase mutant, an additional purification step through a gel-filtration column was also required. Buffers used for the phenyl-sepharose and Q column chromatography are previously described in Theriot et al. (2009). For the gel-filtration column, the equilibration buffer was 50 mM Tris-HCl, 400 mM NaCl, pH 8.0. All fractions were visualized on 12.5% SDS-polyacrylamide gels and were assayed for prolidase activity.

Enzyme activity assay

The enzyme activity assay used was based on a previously described method (Du et al. 2005; Theriot et al. 2009) with slight modification indicated below. Assay mixtures (500 μl) contained 50 mM MOPS buffer (3-[*N*-morpholino]propanesulfonic acid), 200 mM NaCl pH 7.0, 4 mM Xaa-Pro (substrate), 5% (vol/vol) glycerol, 100 μg/ml BSA protein, and 1.2 mM CoCl₂. Assays were done in at least triplicate, and specific activities were calculated using the extinction coefficient of 4,570 M⁻¹ cm⁻¹ for the ninhydrin-proline complex (Theriot et al. 2009).

For assays conducted over a pH range, the reactions contained 200 mM NaCl and 50 mM of the following buffers: pH 4.0–5.0, sodium acetate; pH 6.0–8.0, MOPS; pH 9.0, CHES; pH 10.0, CAPS. When determining substrate specificity of the prolidases, the following dipeptides were used at a final concentration of 4 mM: Met-Pro, Leu-Pro, Phe-Pro, Ala-Pro, Gly-Pro, Arg-Pro, and Pro-Ala. To evaluate the enzyme kinetic parameters, enzyme assays were conducted at 35°C, 70°C, and 100°C with increasing concentrations of Met-Pro and a final metal concentration of 1.2 mM CoCl₂.

Thermostability and pot-life activity assays

To measure the thermostability of wild-type *P. furiosus* prolidase and the mutant prolidases, each enzyme (0.04 mg/

ml in 50 mM MOPS 200 mM NaCl, pH 7.0) was incubated in an anaerobic sealed vial at 90°C. Samples, taken at select time points, were used for enzyme activity assays that were conducted at 100°C and contained 4 mM Met-Pro and 1.2 mM CoCl₂. To measure the pot-life reactivity of wild-type *P. furiosus* prolidase and the mutant prolidases, each enzyme (0.04 mg/ml in 50 mM MOPS 200 mM NaCl, pH 7.0) was incubated in an anaerobic sealed vial at 70°C. Samples were taken at 0, 6, 12, 24, 28, 32, and 48 h time points to assess the pot-life activity in reactions containing 4 mM Met-Pro and 1.2 mM CoCl₂ at 100°C.

Organophosphorus nerve agent enzymatic assays

DFP assay

The method used was previously described in DeFrank and Cheng (1991). The hydrolysis of DFP by prolidases was measured by monitoring fluoride release with a fluoride specific electrode. 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.

p-Nitrophenyl soman assay (*O*-pinacolyl *p*-nitrophenyl methylphosphonate activity)

Prolidase hydrolysis of *p*-nitrophenyl soman was monitored by accumulation of *p*-nitrophenyl (Hill et al. 2001; Vyas et al. 2010). Two-milliliter reaction assays contained buffer (50 mM MOPS, 200 mM NaCl, pH 7.0), 0.2 mM CoCl₂, and 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 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.

Results

Isolation of *P. furiosus* prolidase mutants with increased activity over a broader range of temperature

P. furiosus prolidase loses 50% of its activity at 80°C and has limited activity at temperatures below 50°C (Ghosh et

al. 1998). Therefore, its gene sequence must be altered in order to obtain variants of *P. furiosus* prolidase that exhibit higher catalytic activity at lower temperatures (10°C to 70°C). To achieve this goal, the gene encoding *P. furiosus* prolidase was randomly mutagenized and subsequently cloned back into expression vector pET-21b to generate a pool of mutant pET-prol plasmids. Clones of the desired phenotype were positively selected by genetic complementation of an *E. coli* selective strain JD1(λDE3) that has deletions of *proA*, *pepP*, and *pepQ* genes (Δ*proA*, Δ*pepP*, and Δ*pepQ*). The *proA* gene encodes γ-glutamylphosphate reductase, the enzyme responsible for the third step in proline biosynthesis, conversion of L-γ-glutamyl phosphate to γ-glutamic semialdehyde, and *pepP* and *pepQ* are the only two genes in the *E. coli* genome that code for dipeptidases with specificity for proline-containing dipeptides. As a consequence of these deletions, JD1(λDE3) is incapable of growing on minimal media supplied with Leu-Pro as the only proline source.

JD1(λDE3) cells were transformed with plasmids carrying mutant *P. furiosus* prolidase genes. The transformed cells were plated on minimal media supplemented with Leu-Pro, and the plates were incubated at room temperature. Because wild-type *P. furiosus* prolidase could not support growth of the JD1(λDE3) cells at the temperatures that permit growth of *E. coli*, colonies that survived and grew on the selective plates would have to have been able to produce cold-adapted variants that had higher activity than the wild-type enzyme at these lower temperatures. Using this positive selection, approximately 500 colonies were isolated and screened. To avoid false results that could come from variation in enzyme production levels, the mutant prolidase expression plasmids were isolated from colonies on the original selection plates and were transformed into both JD1(λDE3) and BL21(λDE3). The resultant transformants were screened for prolidase activity, and those isolates that exhibited at least 2–3-fold higher activity at 30°C compared to cells expressing wild-type prolidase were retained for further evaluation. Results of this study led to the identification of two mutants with increased activity at low temperature, with one containing a single mutation at amino residue 39 (G39E) and the other containing a triple mutation at amino residues R19G, K71E, and S229T, a change from arginine to glycine at position 19, a change from lysine to glutamate at position 71, and serine to threonine at position 229.

To avoid biased results that may be generated from a single mutagenesis method and to construct a library of *P. furiosus* prolidase expression plasmid mutants with a good variety of mutation types, three mutagenesis methods were employed, which included error-prone PCR using GeneMorph mutazyme polymerase, hydroxylamine mutagenesis, and passage of the prolidase expression plasmid through

mutation-prone *E. coli* strain XL1-red. This strategy proved to be successful with the G39E-prolidase mutant arising from XL1-red cell mutagenesis and the R19G/K71E/S229T-prolidase mutant being generated using the Genomorph II random mutagenesis method.

Construction and purification of a third prolidase mutant: R19G/G39E/K71E/S229T-prolidase

To gain further insight into the effects of cold-adapted substitutions on the *P. furiosus* prolidase, another variant R19G/G39E/K71E/S229T-prolidase (a combination of all four mutations) was produced. Specifically, the G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-prolidases were over-expressed by large-scale expression in BL21(λ DE3) cells and were purified through a multi-column chromatography strategy. Each protein was successfully purified and was shown to have a molecular weight of approximately 39.4 kDa. The purified prolidases were used for subsequent enzyme assays.

Locations of amino acid substitutions in the mutants

The three-dimensional model of *P. furiosus* prolidase had previously been solved and its structural characteristics well evaluated (Maher et al. 2004). This structure was used to analyze the position and possible interactions of each of the amino acid substitutions found in the G39E- and R19G/K71E/S229T- and R19G/G39E/K71E/S229T-prolidase mutants (Fig. 1a/b).

Effects of the amino acid substitutions on the catalytic activity of *P. furiosus* prolidase at different temperatures

To study whether the substitutions affect the activity of *P. furiosus* prolidases over a range of temperatures, the kinetic parameters of G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-prolidase were measured at 35°C, 70°C, and 100°C (Table 2). All mutants performed better at 35°C compared to wild-type *Pf*prol, as each mutant exhibited higher V_{\max} and k_{cat} values than wild-type prolidase. Although these values were higher, so was the K_m suggesting changes or increases in flexibility of the binding pocket. A similar trend was seen at the higher assay temperatures. At 70°C, the K_m 's were observed to be 1.6-fold higher for R19G/K71E/S229T-prolidase than WT-*Pf*prol, and the K_m for R19G/G39E/K71E/S229T-prolidase was 1.3-fold higher than WT-*Pf*prol. The V_{\max} and the k_{cat} values for R19G/G39E/K71E/S229T-prolidase were both 3.2-fold higher than WT at 70°C. The k_{cat}/K_m value for R19G/G39E/K71E/S229T-prolidase showed the highest value at 2.4-fold higher than the wild-type prolidase. This trend continued at 100°C, where the K_m , V_{\max} , k_{cat} , and k_{cat}/K_m

for the mutant prolidases were all higher than WT-*Pf*prol. At higher temperatures, as the number of mutations increase, so did the enzyme reaction rate. At 100°C, the k_{cat}/K_m were 1.2-, 1.8-, and 2.5-fold higher than WT for G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-prolidase, respectively. Mutated prolidases showed overall improved enzyme activity and reaction rates at all three temperatures when compared to WT-*Pf*prol.

When examining the relative activity of prolidases with the substrate Met-Pro (4 mM) (Fig. 2), there were differences observed between WT-*Pf*prol and the mutants at temperatures ranging from 10°C to 100°C. The most dramatic differences were seen in the improved activity of G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-prolidase mutants at 10°C with 2.3-, 3.5-, and 5.1-fold increases in relative activity, respectively, compared to wild-type activity. The improved activity of R19G/G39E/K71E/S229T-prolidase was seen at all temperatures, but was especially evident at 50°C, in which it showed a 2.9-fold increase in activity relative to wild-type prolidase. It is intriguing to find that the increased activities of the G39E mutation at the low-temperature range came at the expense of decreased activity at the high temperature range. Specifically, G39E-prolidase had lower activity at 70°C, exhibiting 83% of the activity of wild type; however, it did not seem to have compromised activity when assayed at 100°C, since it was observed to have 103% of the activity detected for the wild-type prolidase. This dip in activity at the higher temperatures was not seen with the R19G/K71E/S229T- and R19G/G39E/K71E/S229T-prolidase mutants. The R19G, K71E and S229T mutations appeared to significantly increase the activity and improve the rate of catalysis of the *P. furiosus* prolidase.

Effect of the amino acid substitutions on the pH optimum of *P. furiosus* prolidase

Effects of the substitutions on the pH optima of the wild-type and mutant enzymes were analyzed by measuring activities of G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-prolidase mutants at 100°C from pH 4 to 10 with the wild-type enzyme serving as the reference. As shown in Fig. 3, the catalytic activities of G39E- and R19G/G39E/K71E/S229T-prolidase have similar responses to changes in pH, as both showed optimal activities at pH 6.0 and pH 7.0, although the activity was 60% and 89% of wild-type activity, respectively. These proteins both include the G39E mutation, which strongly suggests that Gly39 plays an important role in maintaining the integrity of the active site, and the substitution of this residue with glutamate greatly reduces enzyme activity at optimal pH and high temperature.

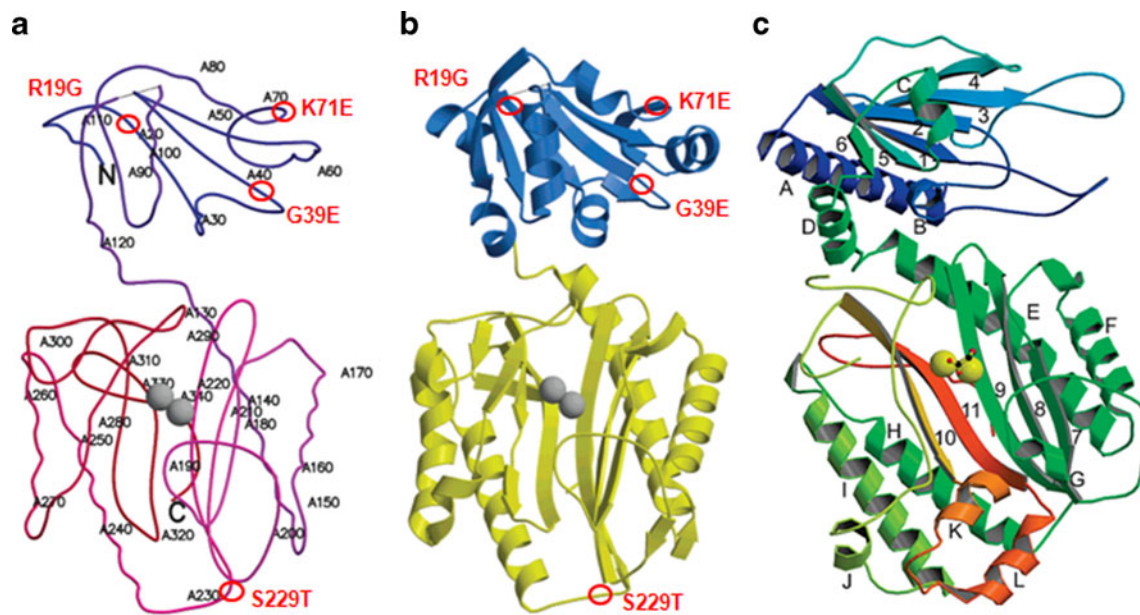


Fig. 1 Mapping of the mutations in the monomer structure of *P. furiosus* prolidase and OPAA/prolidase from *Alteromonas* sp. strain JD6.5. Mutations made in the *Pf*prol are indicated by red circles and include R19G, G39E, K71E, and S229T. **a** Stereoview of the backbone trace of the domain structure. **b** Ribbon drawing of the

domain structure in the same orientation as **a**. N-terminal domain is in blue, and the C-terminal domain is in yellow. The metal atoms are depicted as gray spheres (modified from Maher et al. 2004). **c** Ribbon drawing of the *Alteromonas* sp. strain JD6.5 OPAA/prolidase monomer (modified from Vyas et al. 2010)

In contrast, R19G/K71E/S229T-prolidase showed increased activity over the pH range of 5–7. At pH 5.0, the mutant activity was 1.6-fold higher than wild-type activity, and at pH 7.0, it was 1.2-fold higher than wild type. In addition, the activity was somewhat less affected than the

other mutants at pH 6.0 and pH 8.0, showing similar activities to wild type. It was noticed that the optimal pH for the activity of *P. furiosus* prolidase presented in our study is pH 6.0, which is different from the previous report of pH 7.0 (Ghosh et al. 1998). The reason for the pH

Table 2 Kinetic parameters of wild-type and mutated prolidases from *Pyrococcus furiosus*

Temperature	Prolidase	K_m (mM)	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \text{s}^{-1}$)
35°C	WT- <i>Pf</i> prol	6.4±1.5	187±22	123	19
	G39E	13±1.3	254±15	167	13
	R19G/K71E/S229T	11.3±1.7	261±20	171	15
	R19G/G39E/K71E/S229T	7.9±1.5	263±26	173	22
70°C	WT- <i>Pf</i> prol	5.7±1.4	1,168±140	767	135
	G39E	6.8±1.2	1,549±118	1,017	150
	R19G/K71E/S229T	9.2±1.7	1,853±162	1,217	132
	R19G/G39E/K71E/S229T	7.5±1.4	3,679±344	2,416	322
100°C	WT- <i>Pf</i> prol	7.95±1.3	3,194±247	2,104	266
	G39E	14.5±4.7	6,995±1,416	4,594	317
	R19G/K71E/S229T	13.7±3.4	9,740±1,490	6,396	467
	R19G/G39E/K71E/S229T	11.1±2.4	11,594±1,427	7,481	674

Enzyme assays were done using a range of Met-Pro (1–16 mM) with at least five points and in duplicate. Enzyme kinetic parameters were plotted using nonlinear regression (r^2 values >0.9) and analyzed using the Michaelis–Menton equation utilizing software from Prism 5 (GraphPad La Jolla, CA, USA). All enzyme assays contained 1.2 mM of CoCl_2

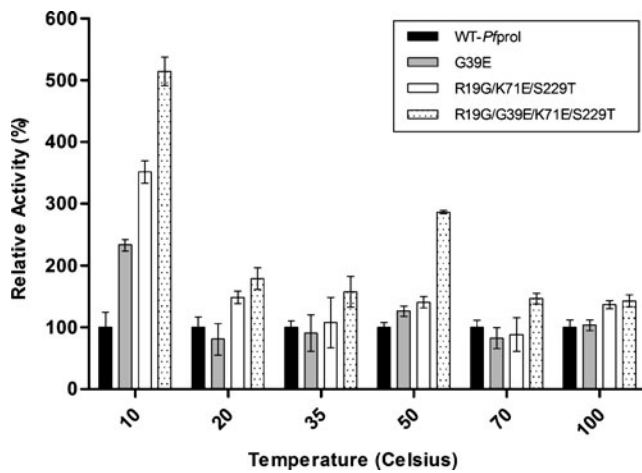


Fig. 2 Relative activity of WT-*Pfprol* and the prolidase mutants with Met-Pro (4 mM) and CoCl_2 (1.2 mM) at temperatures ranging from 10°C to 100°C. One hundred percent relative activity corresponds to WT-*Pfprol* specific activities of 5.5 U/mg at 10°C, 25 U/mg at 20°C, 61 U/mg at 35°C, 138 U/mg at 50°C, 806 U/mg at 70°C, and 2,154 U/mg at 100°C

optimum shifting is not known; however, it might be the result of the use of different protein expression and purification methods.

Effect of the amino acid substitutions on the thermostability and pot-life activity of *P. furiosus* prolidase

To study whether the substitutions affected enzyme thermostability, the mutated prolidases were incubated at 90°C under anaerobic conditions, and their catalytic activities were measured at specific time points. For these activity assays, Met-Pro (4 mM) was used as the substrate (see “Materials and methods”). Although the R19G/K71E/

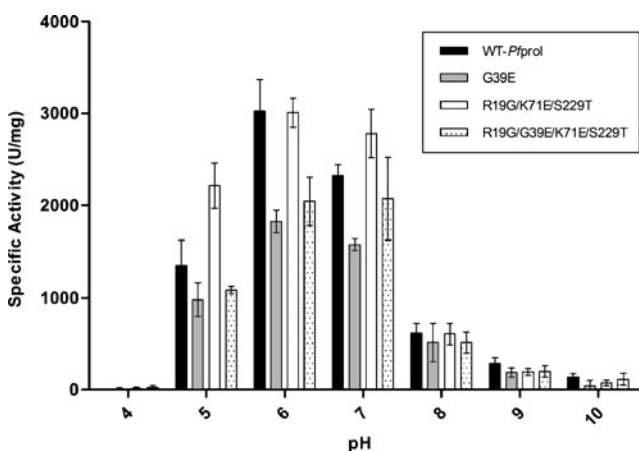


Fig. 3 Specific activity of WT-*Pfprol* and prolidase mutants over a pH range of 4.0–10.0. Prolidase assays were done at 100°C and contained Met-Pro (4 mM), CoCl_2 (1.2 mM), and a final concentration of 200 mM NaCl and 50 mM of the following buffers: pH 4.0–5.0, sodium acetate; pH 6.0–8.0, MOPS; pH 9.0, CHES; pH 10.0, CAPS

S229T- and R19G/G39E/K71E/S229T-prolidase showed higher activity over a broad range of temperatures, the thermostability of these enzymes was less compared to wild type. R19G/K71E/S229T- and R19G/G39E/K71E/S229T-prolidase lost 50% activity after 4 and 3 h at 90°C, respectively, whereas G39E-prolidase did not show this loss of activity until 14 h, and WT-*Pfprol* did not lose 50% activity until 21 h had elapsed. The increased number of substitutions resulted in decreases in long-term stability of prolidases at the physiologically relevant temperature of 90°C.

The pot-life activity was assessed over a 3-day period with constant incubation at 70°C (Fig. 4). After 12 h, all the prolidases were capable of degrading Met-Pro (4 mM) with specific activities of 1,563, 1,768, 843, and 1,366 for WT-, G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-prolidase, respectively. After 48 h, the prolidases were still functional and had activities of 1,083, 599, 722, and 496 U/mg for WT-, G39E-, R19G/K71E/S229T-, and R19G/G39E/K71E/S229T-prolidase, respectively

Effect of the amino acid substitutions on the substrate specificity of *P. furiosus* prolidase

Table 3 shows the relative activity of mutants compared to WT-*Pfprol* with other prolidase specific dipeptides. For the substrate Met-Pro, there was little difference between the activity observed for the wild type when compared to G39E-prolidase, but an increase in activity occurred with R19G/K71E/S229T- and R19G/G39E/K71E/S229T-prolidase mutants, showing 137% (2,886 U/mg) and 143% (3,080 U/mg) activity of wild type with Met-Pro, respectively. There was a more significant increase seen for R19G/K71E/S229T-prolidase when assayed with Leu-Pro, as it had 169% (2,674 U/mg) of the wild-type prolidase

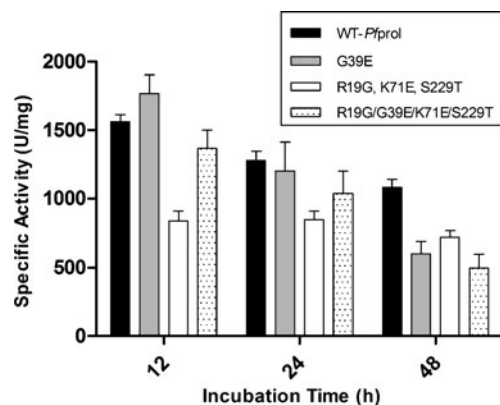


Fig. 4 Pot-life activity of WT-*Pfprol* and prolidase mutants at 70°C. Prolidase assays were performed at 100°C and contained 1.2 mM CoCl_2 and 4 mM of Met-Pro. *P. furiosus* prolidases were incubated anaerobically at 70°C over a 48-h period. Enzyme samples were taken after 12, 24, and 48 h to assess reactivity

Table 3 Substrate specificity of recombinant wild-type and mutant *P. furiosus* prolidase with different proline dipeptides

Substrate	Relative Activity (%) of WT- <i>Pfp</i> rol specific activity			
	WT- <i>Pfp</i> rol	G39E	R19G/K71E/S229T	R19G/G39E/K71E/S229T
Met-Pro	100 (2,154)	103	137	143
Leu-Pro	100 (1,582)	85	169	79
Phe-Pro	100 (395)	60	97	122
Ala-Pro	100 (392)	35	95	38
Gly-Pro	100 (347)	37	47	10
Arg-Pro	100 (164)	132	101	112
Pro-Ala	100 (24)	125	117	13

Prolidase assays were performed at 100°C and contained 1.2 mM CoCl₂ and 4 mM of each substrate. One hundred percent specific activity is reported for WT-*Pfp*rol and correlates to U/mg in parentheses next to the 100% relative activity

activity with Leu-Pro. This surpasses the activity of WT with Met-Pro, which has a specific activity of 2,154 U/mg. This higher activity for the R19G/K71E/S229T-prolidase mutant could be due to the addition of Leu-Pro in the positive selection method used to isolate the low-temperature active mutants, in effect selecting an enzyme that prefers and is more active with Leu-Pro. When the G39E substitution was made in the prolidase, the enzyme activity decreased when Leu-Pro was used as the substrate, suggesting that this particular mutation could be hindering substrate specificity with Leu-Pro. The dipeptide Phe-Pro showed the highest activity with R19G/G39E/K71E/S229T-prolidase at 122% activity of wild type, whereas G39E-prolidase had the lowest detected activity at 60% of wild type. The mutants all showed decreased activity with Ala-Pro and Gly-Pro. The incorporation of mutation G39E seemed to substantially reduce activity with both substrates, showing 35% and 38% activity of wild type for Ala-Pro and 37% and 10% for Gly-Pro for G39E- and R19G/G39E/K71E/S229T-prolidase, respectively. Activity for R19G/K71E/S229T-prolidase with Gly-Pro was 47% of wild type, but was fairly unaffected when Ala-Pro was used as the substrate, maintaining 95% of wild-type activity. Relative activities with Arg-Pro all went up for the mutants relative to the activity of wild type, which was reported as 164 U/mg. As for Pro-Ala, a substrate that is not usually hydrolyzed by prolidases, even though mutant prolidase activities increased relative to WT, it is important to note that the starting activity of WT was very low to begin with at 24 U/mg.

Effect of the substitutions on substrate specificity with OP nerve agents DFP and soman analog, *p*-nitrophenyl soman

Increases in activity at lower temperatures with Xaa-Pro dipeptides, which is thought to be the physiologically relevant substrate of prolidases, were also observed for the *P. furiosus* prolidase mutants when OP nerve agents, DFP and *p*-nitrophenyl soman, served as the substrate. In Fig. 5, the relative activity of the prolidase mutants and WT-*Pfp*rol

with DFP as the substrate can be seen. The G39E substituted prolidase exhibited increases in relative activities that were 2- and 2.2-fold higher than wild-type activity with DFP at temperatures 35°C and 50°C. R19G/G39E/K71E/S229T-prolidase showed the highest activity at 50°C with 7.5-fold higher activity than wild type. The addition of the G39E mutation to the R19G/K71E/S229T prolidase substitutions had in this case increased the activity with DFP as the substrate, whereas R19G/K71E/S229T-prolidase had decreased activity with DFP (71% activity compared to wild type).

Activity with *p*-nitrophenyl soman is even more promising with all three mutant prolidases showing significant activity at 35°C, 50°C, and 70°C (Fig. 6). As the temperature increases, there was increased hydrolysis of *p*-nitrophenyl soman, which is characteristic of a hyperthermophilic enzyme. The highest activity can be seen with R19G/G39E/K71E/S229T-prolidase, with a 3.4-fold increase in activity over WT at 70°C. It is interesting to note that *P. furiosus* prolidase mutants all showed increased activity compared to WT-*Pfp*rol with *p*-nitrophenyl soman at all temperatures that were evaluated.

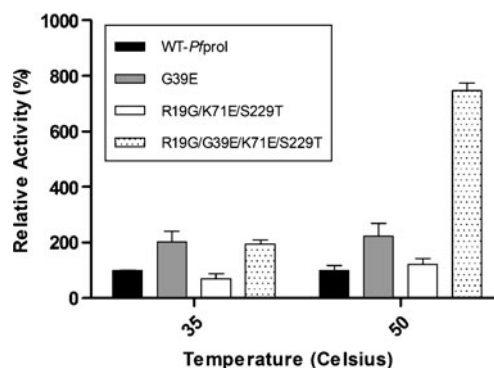


Fig. 5 Relative activity with WT-*Pfp*rol and prolidase mutants with OP nerve agent DFP. All prolidase assays contained 50 mM MOPS 200 mM NaCl pH 7.0, 0.2 mM CoCl₂, and 3 mM DFP. One hundred percent relative activity corresponds to WT-*Pfp*rol specific activity for DFP of 0.42 μmol product formed per minute per milligram at 35°C and 0.73 μmol product formed per minute per milligram at 50°C

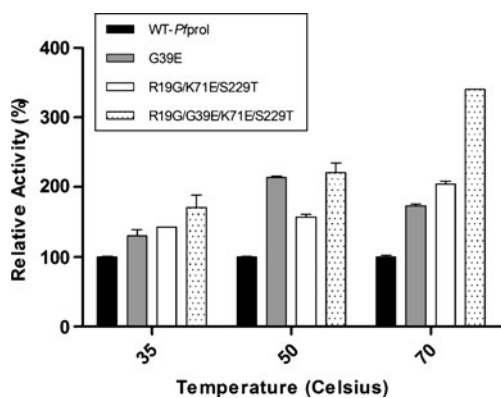


Fig. 6 Relative activity with WT-*Pfprol* and prolidase mutants with the OP nerve agent analog, *p*-nitrophenyl soman. All prolidase assays contained 50 mM MOPS 200 mM NaCl pH 7.0, 0.2 mM CoCl₂, and 3 mM *p*-nitrophenyl soman. One hundred percent relative activity corresponds to WT-*Pfprol* specific activity of *p*-nitrophenyl soman of 0.23 μmol product formed per minute per milligram at 35°C, 0.3 μmol product formed per minute per milligram at 50°C, and 0.5 μmol product formed per minute per milligram at 70°C

Table 4 shows a comparison of the specific activities of wild-type and mutant *P. furiosus* prolidases with *Alteromonas* sp. strain JD6.5 OPAA/prolidase, an enzyme presently being studied for its use in enzyme decontamination cocktails. R19G/G39E/K71E/S229T-prolidase showed the highest activity with substrates DFP and soman analog, but a 90% decrease with Gly-Pro compared to WT-*Pfprol*. When comparing these activities to OPAA/prolidase (Vyas et al. 2010), R19G/G39E/K71E/S229T-prolidase exhibited 1%, 267%, and 35% the total activity of OPAA/prolidase for substrates DFP, Gly-Pro, and *p*-nitrophenyl soman, respectively.

Discussion

Using a positive selection strategy (directed evolution), three cold-adapted *P. furiosus* prolidase variants were

screened, purified, and characterized. Because the substitutions occurred at different locations in the protein structure, G39E- and R19G/K71E/S229T- and R19G/G39E/K71E/S229T-prolidase each exhibit different responses toward the changes in temperature, pH, substrate specificity, and thermostability.

It has been previously reported by Lebbink et al. (2000) and Suzuki et al. (2001) that increased activity at low temperature is accompanied by lower activity at high temperature in the screen for cold-adapted hyperthermophilic enzymes. This can also be referred to as the activity–stability trade off because it too is accompanied by a decrease in thermostability (Zecchinon et al. 2001). Our study supports this theory since the R19G/K71E/S229T- and R19G/G39E/K71E/S229T-prolidase mutants demonstrated not only an increased activity at lower temperatures but also a decrease in thermostability at 90°C, in which enzyme activity fell by half in the first 3–4 h of incubation at 90°C. However, thermoactivity of these two mutants at 100°C did increase, but at a cost of stability at high temperatures. Although the exact mechanism behind the observed changes in thermostability is still unclear at this stage, it has been suggested that protein thermostability is not maintained by a single mechanism. Instead, it is fostered by a network of numerous factors, including increased van der Waal interactions (Berezovsky et al. 1997), higher core hydrophobicity (Schumann et al. 1993), additional networks of hydrogen bonds (Jaenicke and Bohm 1998), enhanced secondary structure propensity (Querol et al. 1996), ionic interactions (Vetriani et al. 1998), increased packing density (Hurley et al. 1992), and decreased length of surface loops (Thompson and Eisenberg 1999). In Table 4, a comparison of the hydrolysis of OP nerve agents, DFP, *p*-nitrophenyl soman, and prolidase specific substrate, Gly-Pro, between the prolidases from *Alteromonas* sp. strain JD6.5 and *P. furiosus* was shown (Vyas et al. 2010). This is the first study demonstrating that *P. furiosus* prolidases are able to hydrolyze OP nerve agents,

Table 4 Specific activity of OP nerve agents and Gly-Pro with *Alteromonas* sp. strain JD6. 5 OPAA/prolidase and *Pyrococcus furiosus* prolidases

Prolidases	Specific activity (U/mg)		
	DFP	Gly-Pro	<i>p</i> -Nitrophenyl soman
<i>Alteromonas</i> OPAA/prolidase ^a	439 ^a	13.5 ^a	4.8 ^a
WT- <i>Pfprol</i>	0.73	347	0.50
G39E <i>Pfprol</i>	1.63	130	0.86
R19G/K71E/S229T <i>Pfprol</i>	0.89	163	1.02
R19G/G39E/K71E/S229T <i>Pfprol</i>	5.45	36	1.70

Pyrococcus prolidases were assayed at 50°C for DFP, 100°C for Gly-Pro, and 70°C for *p*-nitrophenyl soman. All assays contained 50 mM MOPS 200 mM NaCl pH 7.0, 0.2 mM CoCl₂, 4 mM Gly-Pro and 3 mM for DFP and soman. Units for OP nerve agents are defined as micromoles of product formed per minute per milligram, and for Gly-Pro, units represent micromoles of proline released per minute per milligram

^a Results are taken from Vyas et al. (2010)

DFP and *p*-nitrophenyl soman, with the mutants exhibiting higher activity at lower temperature relative to WT-*Pf*prol. The wild-type and mutant *P. furiosus* prolidases all remain reactive at 12 h and even up to 48 h when incubated at 70°C, which is promising given the DTRA guidelines. The development of an enzyme-based cocktail for response decontamination of chemical warfare agents is being considered by the US Department of Defense and requires that enzyme catalysts retain reactivity in solutions over a range of temperatures and have a pot life of at least 12 h. Other desirable qualities include enzymes that are active and stable over broad ranges of pH and remain functional in the presence of salts and other surfactants. It would be beneficial to include OPAA/prolidase in a cocktail, along with other prolidases, such as the mutant *P. furiosus* prolidases that are stable and active over a broad temperature and pH range.

The ability of OPAA to hydrolyze OP nerve agents in addition to Xaa-Pro dipeptides has been observed for many years, while the reaction mechanism has taken longer to elucidate (Cheng et al. 1998, 1999). OPAA/prolidase has the same pita-bread fold in the C-terminal region as do prolidases and also contains a binuclear Mn(II) active site with metal ligand residues Asp-244, Asp-255, His-336, Glu-381, and Glu-420 with bound glycolate, a surrogate of glycine (Vyas et al. 2010). These residues are homologous in most pita-bread enzyme C-terminal catalytic domains, including enzymes methionine aminopeptidase and aminopeptidase P (AMPP) (Lowther and Matthews 2002). *P. furiosus* prolidase is almost identical to OPAA, although it contains a Co(II) dinuclear active site consisting of metal ligand residues Asp-209, Asp-220, His-284, Glu-313, and Glu-327, with a bridging water molecule at W176 (Maher et al. 2004). Detailed comparisons of AMPP and OPAA indicated that the substrate specificity is similar due to the hydrophobic proline binding pocket provided by residues His-350 and Arg-404 in AMPP that interact with the prolyl ring of substrate P₁' Pro; these interactions and residues are conserved in OPAA/prolidase at His-332 and Arg-418 (Graham et al. 2006). The reaction mechanism of these two enzymes is similar, where in *Pf*prol, the bridging hydroxide nucleophile, attacks the carbonyl oxygen of the scissile peptide bond of the dipeptide substrate, and it also can attack the phosphorus center of OP nerve agents, like DFP, which explains the crossover in substrate specificity (Lowther and Matthews 2002; Vyas et al. 2010). However, there could be subtle differences introduced when the four subunits form a tetramer of AMPP, whereas only one subunit forms the monomer of OPAA/prolidase (Fig. 1c).

This study provides experimental evidence that supports the idea that through the use of directed evolution methods, thermophilic enzymes can be engineered to yield cold-adapted variants. Our efforts focused on the improvement

of *P. furiosus* prolidase activity at low temperatures (10°C to 70°C) and resulted in identification of amino acid residues critical in supporting low-temperature activity of the enzyme.

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References

- Adams MW, Perler FB, Kelly RM (1995) Extremozymes: expanding the limits of biocatalysis. *Biotechnology* (N Y) 13:662–668
- Berezovsky IN, Tumanyan VG, Esipova NG (1997) Representation of amino acid sequences in terms of interaction energy in protein globules. *FEBS Lett* 418:43–46
- Booth M, Jennings PV, Ni Fhaolain I, O'Cuinn G (1990) Endopeptidase activities of *Streptococcus cremoris*. *Biochem Soc Trans* 18:339–340
- Browne P, O'Cuinn G (1983) The purification and characterization of a proline dipeptidase from guinea pig brain. *J Biol Chem* 258:6147–6154
- Cheng TC, Rastogi VK, DeFrank JJ, Sawiris GP (1998) G-type nerve agent decontamination by *Alteromonas* prolidase. *Ann N Y Acad Sci* 864:253–258
- Cheng TC, DeFrank JJ, Rastogi VK (1999) *Alteromonas* prolidase for organophosphorus G-agent decontamination. *Chem Biol Interact* 119–120:455–462
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645
- Defense Threat Reduction Agency (2008) Joint Science and Technology Office for Chemical and Biological Defense FY 10/11—new initiatives. Defense Threat Reduction Agency, Fort Belvoir, pp 1–53
- DeFrank JJ, Cheng TC (1991) Purification and properties of an organophosphorus acid anhydrase from a halophilic bacterial isolate. *J Bacteriol* 173:1938–1943
- Du X, Tove S, Kast-Hutcheson K, Grunden AM (2005) Characterization of the dinuclear metal center of *Pyrococcus furiosus* prolidase by analysis of targeted mutants. *FEBS Lett* 579:6140–6146
- Endo F, Hata A, Indo Y, Motohara K, Matsuda I (1987) Immunochemical analysis of prolidase deficiency and molecular cloning of cDNA for prolidase of human liver. *J Inher Metab Dis* 10:305–307
- Fernandez-Espla MD, Martin-Hernandez MC, Fox PF (1997) Purification and characterization of a prolidase from *Lactobacillus casei* subsp. *casei* IFPL 731. *Appl Environ Microbiol* 63:314–316
- Fujii M, Nagaoka Y, Imamura S, Shimizu T (1996) Purification and characterization of a prolidase from *Aureobacterium esteraromaticum*. *Biosci Biotechnol Biochem* 60:1118–1122
- Fukasawa KM, Fukasawa K, Higaki K, Shiina N, Ohno M, Ito S, Otagoto J, Ota N (2001) Cloning and functional expression of rat kidney dipeptidyl peptidase II. *Biochem J* 353:283–290
- Ghosh M, Grunden AM, Dunn DM, Weiss R, Adams MW (1998) Characterization of native and recombinant forms of an unusual cobalt-dependent proline dipeptidase (prolidase) from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* 180:4781–4789

- Graham SC, Lilley PE, Lee M, Schaeffer PM, Kralicek AV, Dixon NE, Guss JM (2006) Kinetic and crystallographic analysis of mutant *Escherichia coli* aminopeptidase P: insights into substrate recognition and the mechanism of catalysis. *Biochemistry* 45:964–975
- Grunden AM, Ray RM, Rosentel JK, Healy FG, Shanmugam KT (1996) Repression of the *Escherichia coli* modABCD (molybdate transport) operon by ModE. *J Bacteriol* 178:735–744
- Hill CM, Li WS, Cheng TC, DeFrank JJ, Raushel FM (2001) Stereochemical specificity of organophosphorus acid anhydrolase toward *p*-nitrophenyl analogs of soman and sarin. *Bioorg Chem* 29:27–35
- Hurley JH, Baase WA, Matthews BW (1992) Design and structural analysis of alternative hydrophobic core packing arrangements in bacteriophage T4 lysozyme. *J Mol Biol* 224:1143–1159
- Jaenicke R, Bohm G (1998) The stability of proteins in extreme environments. *Curr Opin Struct Biol* 8:738–748
- Jalving R, Bron P, Kester HC, Visser J, Schaap PJ (2002) Cloning of a prolidase gene from *Aspergillus nidulans* and characterisation of its product. *Mol Genet Genomics* 267:218–222
- Lebbink JH, Kaper T, Bron P, van der Oost J, de Vos WM (2000) Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* beta-glucosidase CelB by directed evolution. *Biochemistry* 39:3656–3665
- Lowther WT, Matthews BW (2002) Metalloaminopeptidases: common functional themes in disparate structural surroundings. *Chem Rev* 102:4581–4608
- Maher MJ, Ghosh M, Grunden AM, Menon AL, Adams MW, Freeman HC, Guss JM (2004) Structure of the prolidase from *Pyrococcus furiosus*. *Biochemistry* 43:2771–2783
- Maloy SR, Nunn WD (1981) Selection for loss of tetracycline resistance by *Escherichia coli*. *J Bacteriol* 145:1110–1111
- Morel F, Frot-Coutaz J, Aubel D, Portalier R, Atlan D (1999) Characterization of a prolidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with an unusual regulation of biosynthesis. *Microbiology* 145(Pt 2):437–446
- Myara I, Cosson C, Moatti N, Lemonnier A (1994) Human kidney prolidase—purification, preincubation properties and immunological reactivity. *Int J Biochem* 26:207–214
- Querol E, Perez-Pons JA, Mozo-Villarias A (1996) Analysis of protein conformational characteristics related to thermostability. *Protein Eng* 9:265–271
- Schumann J, Bohm G, Schumacher G, Rudolph R, Jaenicke R (1993) Stabilization of creatinase from *Pseudomonas putida* by random mutagenesis. *Protein Sci* 2:1612–1620
- Sjostrom H, Noren O, Josefsson L (1973) Purification and specificity of pig intestinal prolidase. *Biochim Biophys Acta* 327:457–470
- Studier FW (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* 41:207–234
- Suga K, Kabashima T, Ito K, Tsuru D, Okamura H, Kataoka J, Yoshimoto T (1995) Prolidase from *Xanthomonas maltophilia*: purification and characterization of the enzyme. *Biosci Biotechnol Biochem* 59:2087–2090
- Suzuki T, Yasugi M, Arisaka F, Yamagishi A, Oshima T (2001) Adaptation of a thermophilic enzyme, 3-isopropylmalate dehydrogenase, to low temperatures. *Protein Engineering* 14:85–91
- Theriot CM, Tove SR, Grunden AM (2009) Characterization of two proline dipeptidases (prolidases) from the hyperthermophilic archaeon *Pyrococcus horikoshii*. *Appl Microbiol Biotechnol* 86:177–188
- Thompson MJ, Eisenberg D (1999) Transproteomic evidence of a loop-deletion mechanism for enhancing protein thermostability. *J Mol Biol* 290:595–604
- Vetriani C, Maeder DL, Tolliday N, Yip KS, Stillman TJ, Britton KL, Rice DW, Klump HH, Robb FT (1998) Protein thermostability above 100 degreesC: a key role for ionic interactions. *Proc Natl Acad Sci USA* 95:12300–12305
- Vyas NK, Nickitenko A, Rastogi VK, Shah SS, Quiocho FA (2010) Structural insights into the dual activities of the nerve agent degrading organophosphate anhydrolase/prolidase. *Biochemistry* 49:547–559
- Wetherell J, Price M, Mumford H (2006) A novel approach for medical countermeasures to nerve agent poisoning in the guinea-pig. *Neurotoxicology* 27:485–491
- Zecchinon L, Claverie P, Collins T, D'Amico S, Delille D, Feller G, Georgette D, Gratia E, Hoyoux A, Meuwis MA, Sonan G, Gerday C (2001) Did psychrophilic enzymes really win the challenge? *Extremophiles* 5:313–321