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Characterization of two proline dipeptidases (prolidases) from the hyperthermophilic archaeon *Pyrococcus horikoshii*

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Abstract Prolidases hydrolyze the unique bond between X-Pro dipeptides and can also cleave the P–F and P–O bonds found in organophosphorus compounds, including the nerve agents, soman and sarin. The advantages of using hyperthermophilic enzymes in biodetoxification strategies are based on their enzyme stability and efficiency. Therefore, it is advantageous to examine new thermostable prolidases for potential use in biotechnological applications. Two thermostable prolidase homologs, PH1149 and PH0974, were identified in the genome of *Pyrococcus horikoshii* based on their sequences having conserved metal binding and catalytic amino acid residues that are present in other known prolidases, such as the previously characterized *Pyrococcus furiosus* prolidase. These *P. horikoshii* prolidases were expressed recombinantly in the *Escherichia coli* strain BL21 (λDE3), and both were shown to function as proline dipeptidases. Biochemical characterization of these prolidases shows they have higher catalytic activities over a broader pH range, higher affinity for metal and are more stable compared to *P. furiosus* prolidase. This study has important implications for the potential use of these enzymes in biotechnological applications and provides further information on the functional traits of hyperthermophilic proteins, specifically metalloenzymes.

Keywords Prolidase · *Pyrococcus horikoshii* · Hyperthermophile · Metalloenzyme · Cobalt enzyme

Introduction

Pyrococcus horikoshii is a hyperthermophilic, anaerobic organism that was isolated from a deep (1,400-m depth) hydrothermal vent in the Okinawa Trough in the northeastern Pacific Ocean (Gonzalez et al. 1998). Closely related *Pyrococcus furiosus* was isolated from a shallow marine solfatara at Vulcano Island off the coast of Italy (Fiala and Stetter 1986). *P. furiosus* is one of the most studied hyperthermophiles to date (Adams 1993). Both *Pyrococcus* species grow optimally at extreme temperatures of 98–100 °C and at pH 7.0 (Gonzalez et al. 1998). They are both obligately heterotrophic anaerobes that are able to use selected peptides and proteins to produce organic acids, CO_2, and H_2 (Fiala and Stetter 1986; Gonzalez et al. 1998). *P. furiosus* grows on maltose, starch, and pyruvate, whereas *P. horikoshii* cannot and is only able to utilize proteinaceous media substrates such as yeast extract, tryptone, or a 21-amino-acid mixture supplemented with vitamins (Gonzalez et al. 1998; Schut et al. 2003). *P. horikoshii*, unlike other closely related species such as *P. furiosus* and *Pyrococcus abyssi*, requires tryptophan and histidine for growth and cannot grow on a nonpeptide carbon source (Gonzalez et al. 1998; Lecompte et al. 2001). The growth profile for these three organisms is similar (Gonzalez et al. 1998), with the main differences being the substrates they are able to use for energy, which could lead to subtle differences in the regulation of metabolism.

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The genomes of *P. furiosus* and *P. horikoshii* have been compared. The genomes consist of 1.908 and 1.738 mbp for *P. furiosus* and *P. horikoshii*, respectively (Maeder et al. 1999). The missing 170-kbp gap between the two organisms contains sequences that code for amino acid biosynthetic pathways, specifically *trp*, *his*, *aro*, *leu-ile-val*, *arg*, *pro*, *cys*, *thr*, and *mal* operons (Maeder et al. 1999). However, *P. horikoshii* has many genes for chemotaxis that *P. furiosus* lacks (Lecompte et al. 2001; Maeder et al. 1999). The reliance of *P. horikoshii* on proteinaceous substrates for energy production and metabolism suggests that *P. horikoshii* may require more proteases that function to provide the organism with amino acids that it is unable to make on its own. This could explain why *P. horikoshii* possesses more than one functional proline dipeptidase.

Enzymes that are able to catalyze the hydrolysis of proteins into smaller peptide fractions and amino acids are defined as proteases. There are few proteases that are able to cleave a peptide bond adjacent to a proline residue. This is due to the conformational constraint that the cyclic structure of proline puts on a peptide bond (Cunningham and O'Connor 1997). Prolidase is one of these proteases that is able to hydrolyze dipeptides with proline in the C-terminus, X-Pro, and a nonpolar amino acid in the N-terminus (Lowther and Matthews 2002). Prolidases are ubiquitous in nature and can be found in archaea, bacteria, and mammals (Endo et al. 1989; Fernandez-Espla et al. 1997; Ghosh et al. 1998; Suga et al. 1995). It is unclear what role prolidase plays in archaea and bacteria, but it has been suggested to aid in protein degradation and could be responsible for the recycling of proline. Due to its reaction mechanism, it could also play a role in regulating biological processes (Cunningham and O'Connor 1997). In humans, however, prolidase has been shown to be involved in the final stage of the degradation of endogenous and dietary protein and is important in the breakdown of collagen. PD, or prolidase deficiency in humans, is a recessive disorder marked by mutations and or deletions in the human prolidase gene (Ledoux et al. 1996). PD is characterized by skin ulcerations, mental retardation, and recurrent infections of the respiratory tract (Endo et al. 1989; Forlino et al. 2002).

The specificity of prolidase reactions and the substrates that it is able to cleave may be dependent on the metal center it possesses. Prolidase belongs to a small class of metal-loenzymes known as the "pita bread enzymes" because they contain the same pita bread fold encompassing a similar metal center and substrate-binding pocket (Lowther and Matthews 2002). Other enzymes within this class include methionine aminopeptidase, aminopeptidase P, and creatinase, each having slightly different substrate specificity, but the same conserved metal binding pocket suggesting they might have a conserved catalytic mechanism (Lowther and Matthews 2002). Most enzymes in this class require one or two divalent ions such as Co^{2+}, Mn^{2+}, or Zn^{2+} to be present in their active sites to catalyze a reaction, with one of the metal atoms being more tightly bound than the other (Lowther and Matthews 2002). The first prolidase structurally and biochemically characterized was from the hyperthermophilic archaeon *P. furiosus* (Ghosh et al. 1998; Grunden et al. 2001; Maher et al. 2004). *P. furiosus* prolidase showed maximum activity at 100 °C and pH 7.0 (Ghosh et al. 1998; Grunden et al. 2001) and a narrow substrate specificity, only hydrolyzing dipeptides with a proline in the C-terminus and nonpolar amino acid (Leu, Met, Val, Phe, or Ala) in the N-terminus (Ghosh et al. 1998). This dipeptidase is maximally active with the addition of divalent cations Co^{2+} and Mn^{2+} but cannot be substituted with other divalent cations (Mg^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}; Ghosh et al. 1998). It requires two cobalt atoms occupying the metal binding sites, one tight binding at residues E313 and H284 and one loose binding at D209, with a K_d of 0.24 mM (Du et al. 2005). *P. furiosus* prolidase has also been shown to be maximally active with Fe^{2+} (1,434 U/mg) and somewhat less active with Co2+ (573.6 U/mg) under anaerobic conditions, suggesting that the metal preference in vivo may be for Fe^{2+} rather than Co^{2+} (Du et al. 2005).

Recombinant prolidase has use in several biotechnological applications (Theriot et al. 2009). It is used by the food and dairy industry in the fermentation process, specifically in cheese taste and texture development (Bockelmann 1995). Recombinant prolidase is also being examined for enzyme replacement therapy for patients that have PD. However, it has also been shown to degrade organophosphorus (OP) nerve agents. OP nerve agents act by inhibiting acetylcholinesterase (AChE), which leads to a buildup of acetylcholine in the body and can lead to hypersecretion, convulsions, respiratory problems, coma, and finally, death. OPAAs or organophosphorus acid anhydrolases from *Alteromonas* have shown the ability to hydrolyze OPs because they are able to cleave the P–F, P–O, P–CN, and P–S bonds of the nerve agents, sarin and soman (Cheng et al. 1998). OPAA can also hydrolyze specific proline dipeptides characterized by [Xaa-Pro], specifically Leu-Pro and Ala-Pro, therefore it is now considered a prolidase. OPAA's target substrates (soman and sarin) mimic that of the prolidase specific substrates [Xaa-Pro] in shape, size, and surface charges (Cheng and DeFrank 2000). Prolidase could be a potential biodecontaminant for detoxification of OP nerve agents in the field. An activity of 30 U mg^{-1} was seen when purified *P. furiosus* prolidase was tested at 55 °C against G-series OP nerve agent diisopropylfluorophosphate (DFP; Theriot et al. 2009). Currently, *Alteromonas* OPAA-1 and OPAA-2 prolidases are used in a foam formulation DS2 for biodecontamination,

although metal is still required for enzyme activity (Cheng et al. 1999). It is necessary to study other candidate prolidases in the hopes of identifying an enzyme that will be more efficient in detoxifying OP nerve agents under harsh conditions that exist with field applications. To further evaluate new enzymes that could potentially be used for decontamination of OP nerve agents, two putative prolidases from *P. horikoshii* have been expressed and biochemically characterized.

Materials and methods

Identification and cloning of the *P. horikoshii* prolidase-encoding genes

The *Pf*prol accession number (AAL81467) was entered into the Basic Local Alignment Search Tool (BLAST) or blastp to look for similar structures based on *Pf*prol. The results included homologous protein structures *Ph*prol (BAA30249) and *Ph1*prol (BAA30071).

P. horikoshii genomic DNA was obtained from ATCC, strain 700860D-5. *P. horikoshii* prolidase (PH1149), prolidase homolog 1 (PH0974), and prolidase homolog 2 (PH1902) genes were amplified by PCR for subsequent cloning of these genes into the T7-polymerase-driven expression vector pET-21b (Novagen). For the PCR amplification of *P. horikoshii* prolidase, prolidase homolog 1, and prolidase homolog 2 genes, two primers were designed for each: *Ph*prol primer 1 (5′-AAGATCAAGGAGGT**CAT ATG**GACATAA-3′; forward, containing an *Nde*I restriction site shown in bold) and primer 2 (5′-CCTACTA**AAGCTT** GCTAGATGAGTTCTC-3′; reverse, containing an *Hind*III restriction site shown in bold); *Ph1*prol primer 1 (5′ AAC**CATATG**AGGCTTGAAAAGTTCATTCAC-3′; forward, containing an *Nde*I restriction site shown in bold), and primer 2 (5′-TGA**GTCGAC**AGTAGTAGAATAA TAACA-3′; reverse, containing a *Sal*I restriction site shown in bold); *Ph2*prol primer 1 (5′-GAACT**CATATG** GTTATGAGAGGGAACA-3′; forward, containing an *Nde*I restriction site shown in bold) and primer 2 (5′-CACTG **GTCGAC**ATAGACATTCTAATAA-3′; reverse, containing a *Sal*I restriction site shown in bold). All primers were designed using MacVector (Accelrys, San Diego, CA) computer software.

PCR amplification was performed using native *P. furiosus* DNA polymerase (0.2 μl) in a 50 μl reaction solution containing 5.0 μl 10× Taq buffer, 0.4 μl dNTP (25 mM), 0.5 μl forward primer (40 μM), 0.5 μl reverse primer (40 μM), 0.5 μl Taq polymerase, and 1.0 μl *P. horikoshii* genomic DNA (100 ng/μl). The following PCR protocol was run on the thermocycler (Biorad, Hercules, CA): Two initial cycles for 4 min at 94 °C for denaturation, 1 min at 55 °C for annealing, 1 min at 72 °C for extension; followed by 39 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; and one final cycle at 72 °C for 7 min. The prolidase PCR product sizes were 1.08 kb, 1.07 kb, and 1.09 kb for *Ph*prol, *Ph1*prol, and *Ph2*prol, respectively. PCR products were electrophoresed through a 1% agarose gel for visual inspection.

The amplified prolidase genes were subsequently cloned into the *EcoR*V site of plasmid pCR-Script (Stratagene) to yield plasmids *ph*prol-script, *ph1*prol-script, and *ph2*prol-script. Plasmids were transformed into *Escherichia coli* strain XL1-Blue (Novagen), and the transformed cells were plated on Luria–Bertani (LB) plates supplemented with ampicillin (100 μg/ml) and X-gal (40 μg/ml) and incubated at 37 °C overnight. Blue-white screening was used to select colonies for plasmid isolation.

Plasmids with insert were isolated from white colonies and digested with N*de*I and *Hind*III for the *ph*prol gene and *Nde*I and S*al*I to excise prolidase homolog 1 and 2 genes, respectively. The excised prolidase and homolog genes were subsequently cloned into the N*de*I and *Hind*III or N*de*I and S*al*I (NEB) sites in expression vector pET-21b (Novagen), resulting in plasmids pET-*Ph*prol, pET-*Ph1*prol, and pET-*Ph2*prol. All plasmids were sent to MWG Biotech (High Point, NC) for sequencing to ensure that no sequence changes occurred in the cloning process.

Overexpression of *P. horikoshii* prolidases

P. horikoshii prolidase expression plasmids and the rare arginine, leucine, and isoleucine tRNA encoding plasmid pRIL (Stratagene) were transformed into *E. coli* BL21(λDE3) cells (Novagen), which has isopropyl-β-D-thiogalactopyranoside inducible expression of T7-RNA polymerase encoded on the chromosome. Transformants were selected on LB–Ampicillin–Chloramphenicol plates after incubation at 37 °C overnight.

Large-scale protein expression was done for *Ph*prol and *Ph1*prol by inoculating 1-L cultures of autoinduction media (Studier 2005) supplemented with 100 μg/ml of ampicillin and 34 μg/ml chloramphenicol for plasmid maintenance. Cells were grown with shaking (200 rpm) at 37 °C for 14 h. Cells were harvested by centrifugation and stored at −80 °C until broken. Recombinant protein expression was evaluated throughout this process using SDS-PAGE analysis.

Purification of recombinant *P. horikoshii* prolidases

Cell pellets containing the *Ph*prol and *Ph1*prol protein were suspended in 50 mM Tris–HCl, pH 8.0 containing 1 mM benzamidine–HCl, and 1 mM DTT. The cell suspension was passed through a French pressure cell (20,000 lb/in^2)

two times. The lysed suspension was centrifuged at 38,720 g for 30 min to remove cell debris. The supernatant was made anaerobic and was heated at 80 °C for 30 min. *E. coli* cell debris and denatured protein were removed by centrifugation of the heated supernatant at 38,720 g for 30 min. The clarified extract was applied to a 20-ml phenyl sepharose column. Before adding the extract to the column, ammonium sulfate, $[NH_4]_2SO_4$, was added to the heat-treated extract gradually to a final concentration of 1.5 M. For phenyl sepharose chromatography, the binding buffer used was 50 mM Tris–HCl, 1.5 M $[NH_4]_2SO_4$, pH 8.0, and the elution buffer was 50 mM Tris–HCl, pH 8.0. The fractions with active enzyme were applied to a 5 ml Q-column, which used a binding buffer of 50 mM Tris–HCl, pH 8.0 and an elution buffer of 50 mM Tris–HCl, 1 MNaCl, pH 8.0. All fractions were visualized on 12.5% SDS-polyacrylamide gels, and enzyme assays were performed as well to ensure activity after each purification step.

Prolidase enzyme assay

The enzyme reaction mixture and assay is based on a previously described method from Ghosh et al. 1998 and Du et al. 2005. The (500 µl) reaction mixture contains 50 mM MOPS (3-[N-morpholino]propanesulfonic acid) buffer (pH 7.0), 200 mM NaCl, 5% (*v*/*v*) glycerol, 0.1 mg/ml BSA protein and 1.2 mM $CoCl_2$ (metal), and finally the enzyme. The reaction mixture was heated for 5 min at 100 °C in order for the metal and enzyme to interact. The reaction was initiated with the addition of the substrate, Met-Pro (final concentration of 4 mM). The reaction was heated for an additional 10 min at 100 °C. To stop the reaction, glacial acidic acid (500 µl) was added, and then (3% [wt. vol]) ninhydrin reagent (500 µl) was added. The mixture was heated again for 10 min at 100 °C and then cooled to 23 °C. The absorbance was determined at 515 nm with an extinction coefficient of 4,570 M^{-1} cm^{-1} for the ninhydrin-proline complex. One unit of prolidase activity is defined as the amount of enzyme that liberates one micromole of proline per minute (Ghosh et al. 1998). For assays conducted at different pH values, the following buffers were used at final concentrations of 100 mM: pH 5.0, Sodium Acetate; pH 6.0–8.0, MOPS; pH 9.0, CHES; pH 10.0, CAPS. For assays that evaluate metal preference, the following metals were substituted for Co^{2+}: Mn^{2+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, and Cu^{2+} at final concentrations of 1.2 mM. Also, when evaluating substrate specificity the following dipeptides: Leu-Pro, Gly-Pro, Ala-Pro, Arg-Pro, Phe-Pro, and Pro-Ala, were substituted for Met-Pro at a final concentration of 4 mM. Enzyme kinetic assays were done with increasing concentrations of Met-Pro and a metal concentration of Co^{2+} of 0.2 mM.

Anaerobic enzyme assay to determine metal preference

The active site metals were stripped from the purified prolidases by dialyzing 1 ml of 0.1 mg/ml of purified protein in 1 L of buffer (50 mM MOPS pH 7.0, 0.5 mM EDTA) for 1 h, followed by two more washes in 1 L of 50 mM MOPS pH 7.0 (repeated twice for 1 h each) to remove any EDTA. The metal centers were reconstituted by incubating the apo-proteins with 7.5 µM (this corresponds to threefold molar equivalents of metal/prolidase monomer) of the following metals, $CoCl_2$, $FeSO_4$, $ZnCl_2$, under anaerobic conditions for 15 min at 80 °C. A final concentration of 1 mM of DT or sodium dithionite was added to the $FeSO_4$ in order to keep the iron reduced to Fe (II). The reconstituted enzyme preparations were assayed following the same protocol described above. All assays were done under anaerobiosis in a Coy Anaerobic Chamber.

Metal content analysis of purified *P. horikoshii* prolidases

For preparation of protein samples for metal content determinations, 5 ml of 1 mg/ml purified prolidase protein was dialyzed in 2 L 50 mM MOPS, pH 7.0 overnight at 4 °C. The dialyzed protein sample and a dialysis buffer sample were collected. Metal content of the prolidases was determined by ICP emission spectrometry at the North Carolina State University Analytical Service Laboratory.

Metal competition experiments

The active site metals were stripped from the purified prolidases by dialyzing 1 ml of 1 mg/ml of protein in 1 L of buffer (50 mM MOPS pH 7.0, 5 mM EDTA) for 1 h, followed by two more washes in 1 L of 50 mM MOPS pH 7.0 (repeated twice for 1 h each) to remove any residual EDTA. The metal centers were reconstituted by incubating the apo-proteins with 125 µM (this corresponds to fivefold molar equivalents of metal/prolidase monomer) of the following metals, $CoCl_2$, $MnCl_2$, and $ZnCl_2$, and 62.5 µM (this corresponds to 2.5-fold molar equivalents of metal/prolidase monomer) of the following metal mixtures: $CoCl_2$ and $MnCl_2$ and $CoCl_2$ and $ZnCl_2$ under anaerobic conditions for 15 min at 80 °C. Metal reconstituted enzyme samples were dialyzed in 50 mM MOPS pH 7.0 at 4 °C overnight. Enzyme assays were done following metal removal and after overnight dialysis.

Thermostability assay

Each enzyme (0.2 mg/ml in 100 mM MOPS, pH 7.0) was incubated anaerobically in a sealed vial in a 100 °C water

bath for 8 h. Samples were taken every hour, and an enzyme assay was done to monitor activity. To calculate the thermal half-life $t_{50\%}$, enzymes were prepared as described above but were incubated in a 90 °C oven for 48 h. Samples were taken at specific time points to monitor activity. Enzyme assays contained 0.2 mM $CoC1_2$ and 4 mM Met-Pro.

Amino acid sequence accession numbers

The prolidase amino acid sequences in the Clustal alignment (Fig. 1) can be accessed in the GenBank database with accession numbers: *P. furiosus* prolidase (AAL81467), *P. horikoshii* prolidase (BAA30249), *P. horikoshii* prolidase homolog 1 (BAA30071), *P. horikoshii* prolidase homolog 2 (NP 143731), *Alteromonas* OPAA-2 (AAB05590), prolidases from *Lactobacillus delbrueckii* (CAB07978), human (AAA60064), and *E. coli* (P15034).

Results

Identification of *P. horikoshii* prolidase homolog genes

P. horikoshii prolidase or *Ph*prol (PH1149), *P. horikoshii* prolidase homolog 1 or *Ph1*prol (PH0974) and *P. horikoshii* prolidase homolog 2 or *Ph2*prol (PH1902) each show 88%, 55%, and 27% similarity to previously characterized *P. furiosus* prolidase, respectively (Fig. 1). *Ph*prol and *Ph1*prol both contain all five conserved metal center-liganding amino acid residues that are required for catalysis (Asp-209, Asp-220, His-192, Glu-313, Glu-327; *Pf*prol numbering; Maher et al. 2004). *Ph2*prol only contains two out of the five conserved residues and has different spacing between the residues signifying that it may be a different class of enzyme. National Center for Biotechnology Information (NCBI) lists the following annotations for each protein as follows: PH1149, X-Pro dipeptidase; PH0974, dipeptidase, and PH1902, hypothetical protein.

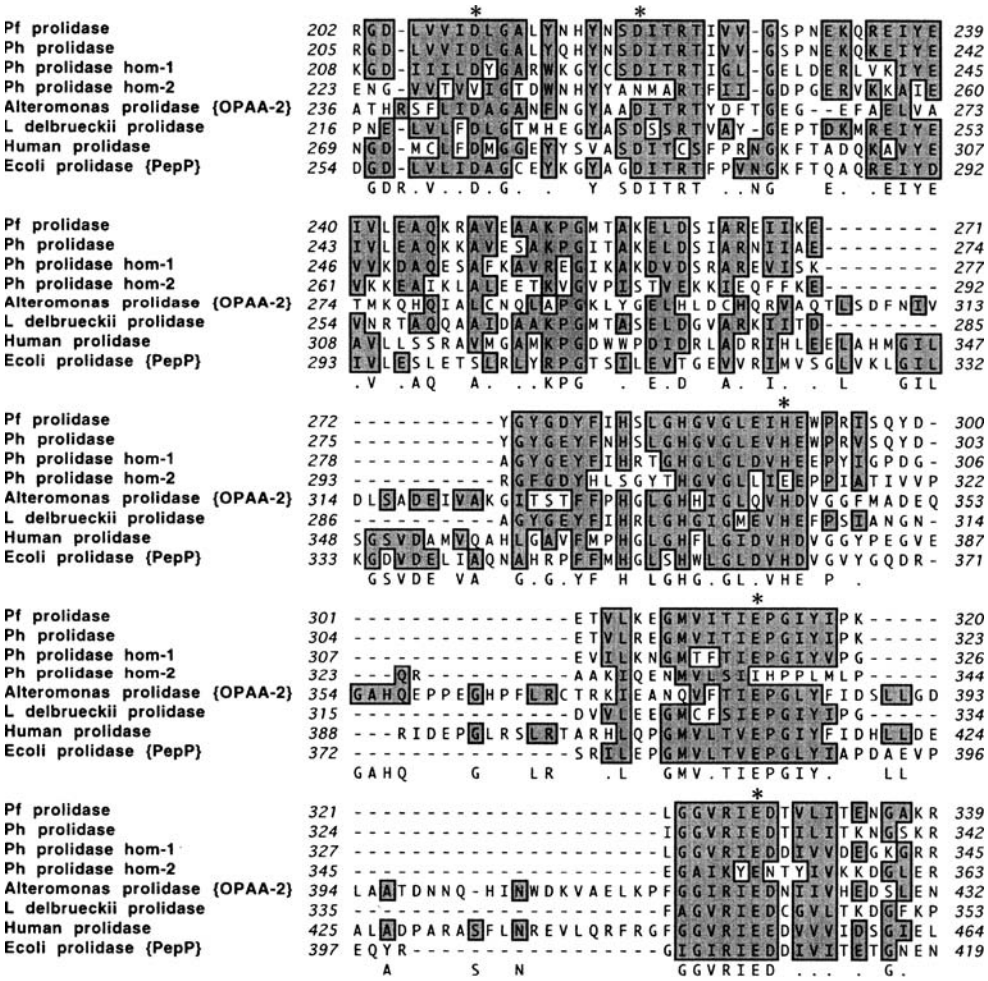

Fig. 1 Clustal alignment of *P. horikoshii* prolidase (PH1149) and prolidase homolog 1 (PH0974) and 2 (PH1902) along with previously characterized *P. furiosus* prolidase (PF1343) and other characterized prolidases from *Alteromonas* OPAA-2 (AAB05590), *Lactobacillus delbrueckii* (CAB07978), human (AAA60064), and *E. coli* (P15034). *Gray shading* designates identical and similar residues. *Asterisks* indicate the five-conserved residues in the prolidases identifying the dinuclear cobalt metal-binding sites

Expression and purification of recombinant prolidases from *P. horikoshii*

Prolidases from *P. horikoshii* were expressed in BL21 (λDE3) *E. coli* cells using autoinduction media (Studier 2005). Maximum recombinant protein expression levels were obtained after incubation of 14 h with autoinduction cultures. Assays were done using crude cell extract and heat-treated cell extract (heated at 80 °C for 20 min) to determine whether the recombinant prolidases had activity.

Although there was an overexpressed protein (41.78 kDa) that could be seen by SDS-gel analysis for the PH1902 expressed cell extract, there was no enzymatic activity observed for *Ph2*prol (PH1902) using the nonheat treated or heat-treated crude extracts and the entire battery of metals and dipeptide substrates. Therefore, the recombinantly expressed PH1902 protein was determined not to be a prolidase and was not subjected to further purification and characterization.

*Ph*prol (PH1149) and *Ph1*prol (PH0974) showed high activity in crude and heat-treated cell extract, and multicolumn purification was performed to purify both enzymes. The overexpressed prolidases were identified using SDS-PAGE analysis, and protein bands of 39.27 kDa (*Ph*prol) and 40.04 kDa (*Ph1*prol) were followed throughout the purification process, as was prolidase activity (Supplemental Fig. 1a and 1b).

To evaluate the metal content of each enzyme after purification, ICP emission spectrometry was used. The metal present in the highest amounts for purified *Ph*prol and *Ph1*prol was zinc (0.268-g atoms of Zn/mol of subunit and 1.136-g atoms of Zn/mol of subunit, respectively; Supplemental Table 1). Cobalt was detected at levels less than 0.001-g atoms of Co/mol of subunit for both enzymes.

Catalytic properties of recombinant prolidases *Ph*prol (PH1149) and *Ph1*prol (PH0974)

The temperature profile showed maximal activity of 1,938 U/mg for *Ph*prol and 2,355 U/mg for *Ph1*prol at 100 °C (Supplemental Fig. 2). The optimum pH for both enzymes was at pH 7.0, although the second highest activity was recorded at pH 6.0 closely followed by pH 5.0 (Fig. 2). Previously characterized *Pf*prol was shown to have the highest activity with the substrate Met-Pro and the next highest activity with Leu-Pro (Ghosh et al. 1998). *Ph*prol and *Ph1*prol showed the same trends as *Pf*prol (1,350 U/mg) with these dipeptides, having 100% relative activity with Met-Pro, correlating to specific activities of 1,824 U/mg and 2,751 U/mg, respectively (Table 1). All substrate specificity assays were done using 1.2 mM $CoCl_2$ and 4 mM of each substrate. There were slight differences in activities for the following dipeptide substrates: Gly-Pro, Ala-Pro, Arg-Pro, and Phe-Pro. Both *Ph*prol (10% relative activity) and *Ph1*prol (8% relative activity) showed slightly higher activity with Gly-Pro as the substrate compared to *Pf*prol (1% relative activity; Ghosh et al. 1998). While *Ph1*prol appeared to have more than twice the relative activity with Ala-Pro, Arg-Pro, and Phe-Pro compared to *Ph*prol, all the prolidases showed very little to no activity with the substrate Pro-Ala.

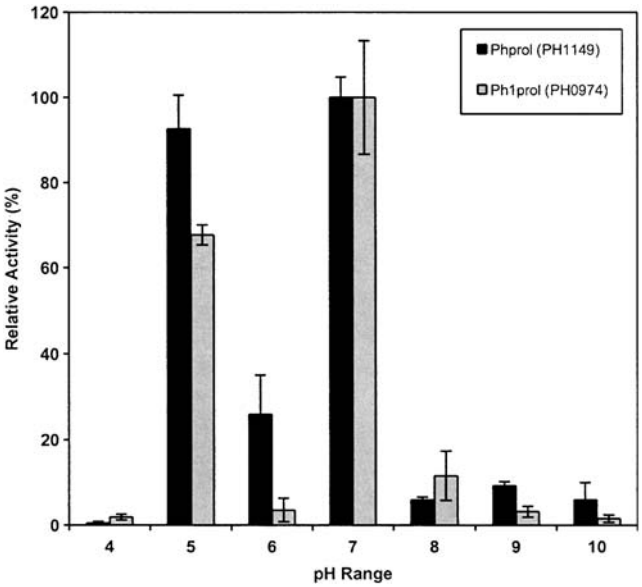

Fig. 2 Activity of *P. horikoshii* prolidases over a pH range of 4.0–10.0. Prolidase assays contained 14.8 ng for *Ph*prol and 6.2 ng *Ph1*prol, Met-Pro (4 mM), and $CoCl_2$ (1.2 mM). The following buffers were used for each pH at a final concentration of 100 mM: pH 5.0, Sodium Acetate; pH 6.0–8.0, MOPS; pH 9.0, CHES; pH 10.0, CAPS. One hundred percent specific activity corresponds to 2,321 U/mg for *Ph*prol and 3,357 U/mg for *Ph1*prol

*Ph*prol and *Ph1*prol are both very thermostable, showing no significant change in activity after 8 h at 100 °C. The $t_{50\%}$ value at 90 °C for both *Ph*prol and *Ph1*prol was 21.5 and 21 h, respectively.

Aerobic activity assay conditions

P. furiosus and *P. horikoshii* both require an anaerobic environment to grow optimally. Therefore, prolidases from these anaerobic organisms should be assayed anaerobically to evaluate their enzymatic properties under physiologically relevant conditions. However, for prolidases to be useful in biotechnological applications, they will need to be active under aerobic conditions. As such, it is important in this case to screen the *P. horikoshii* prolidases under both aerobic and anaerobic conditions.

For the aerobic assays, both prolidases were prepared with different divalent metal cations (1.2 mM), and the highest activity was seen with Co^{2+} followed by Mn^{2+}, while Cu^{2+},

Table 1 Substrate specificity of recombinant *P. furiosus* and *P. horikoshii* prolidases with different dipeptides

Substrate	Relative activity (%)		
	*Pf*prol[a]	*Ph*prol	*Ph1*prol
Met-Pro	100	100	100
Leu-Pro	79	71	74
Gly-Pro	1	10	8
Ala-Pro	17	8	40
Arg-Pro	10	10	27
Phe-Pro	24	15	31
Pro-Ala	0	2	4

Prolidase assays contained prolidase (14.8 ng of *Ph*prol and 6.2 ng *Ph1*prol), $CoCl_2$ (1.2 mM), and 4 mM of each substrate. One hundred percent activity can be seen with Met-Pro and correlates to an activity of 1,350 U/mg for *Pf*prol, 1,824 U/mg for *Ph*prol, and 2,751 U/mg for *Ph1*prol

[a] Results are taken from previous paper: Ghosh et al. 1998

Fe^{2+}, Zn^{2+}, and Ni^{2+} could not restore activity (Fig. 3). When assayed with different concentrations of Co^{2+} and Mn^{2+}, the enzymes performed optimally at 0.2 mM Co^{2+} for *Ph*prol with an activity of 2,075 U/mg and for *Ph1*prol with 0.15 mM Co^{2+} and an activity of 4,901 U/mg (Fig. 4). With Mn^{2+}, the optimum activity was almost 724 U/mg for *Ph*prol, and for *Ph1*prol, the activity reached 2,139 U/mg at a final concentration of 1.6 mM.

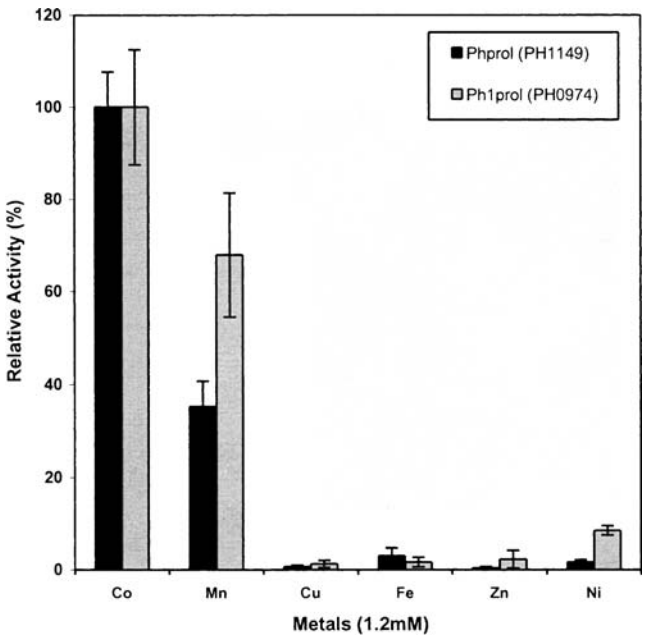

Fig. 3 Activity of *P. horikoshii* prolidases with different divalent cations: Co^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and Ni^{2+}. Prolidase assays contained 14.8 ng for *Ph*prol and 6.2 ng *Ph1*prol, Met-Pro (4 mM), and metals (1.2 mM). One hundred percent specific activity correlates to 2,321 U/mg for *Ph*prol and 2,751 U/mg for *Ph1*prol

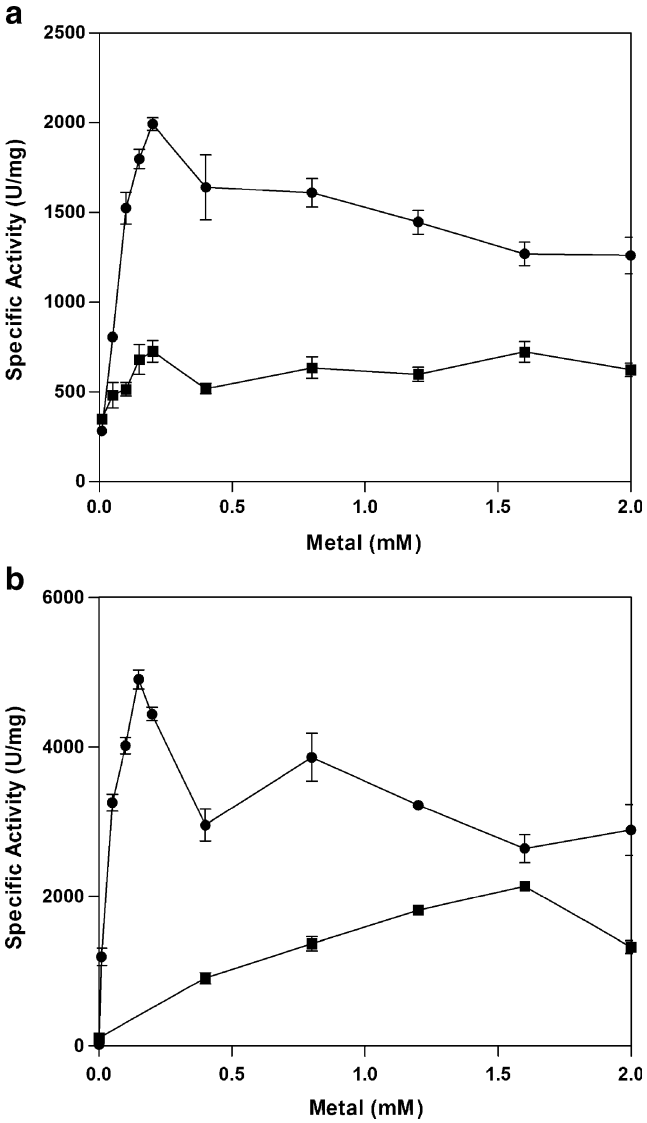

Fig. 4 The effects of metal ion concentrations of Co^{2+} *(circles)* and Mn^{2+} *(squares)* on activities of purified *Ph*prol (**a**) or *Ph1*prol (**b**). The reactions contained Met-Pro (4 mM) and 7.4 ng of enzyme when assayed with Co^{2+} and 14.8 ng of enzyme with Mn^{2+}

The kinetic parameters of *Ph*prol and *Ph1*prol were determined using lower metal concentrations (0.2 mM) than were used for *Pf*prol (1.2 mM; Ghosh et al. 1998). The kinetic analysis was done with Met-Pro and 0.2 mM of $CoCl_2$ because their inclusion in the assay reactions provided the highest activity. The affinity or K_m of *Ph*prol for the substrate Met-Pro, reported here as 3.4 mM is in line with *Pf*prol, both the native (2.8 mM) and recombinant (3.3 mM) versions, whereas *Ph1*prol has a K_m of around 1.9 mM. The V_{max} of both enzymes was very high (3,997 and 5,714 μmol min^{-1} mg^{-1} for *Ph*prol and *Ph1*prol, respectively), which were over threefold higher than that of recombinant *Pf*prol (Table 2). The k_{cat}/K_m for *Ph*prol and

*Ph1*prol were high as well (2,617 and 3,812 s^{-1} for *Ph*prol and *Ph1*prol, respectively).

Anaerobic activity assay conditions

Although aerobic metal analysis demonstrated that Co^{2+} supported the highest activity, when the assays were conducted under anaerobic conditions, very different results were seen. The *Ph*prol and *Ph1*prol purified enzymes were stripped of metals using EDTA, followed by metal reconstitution under anaerobiosis with Fe^{2+}, Co^{2+}, and Zn^{2+} at 80 °C for 20 min. The highest activity for the anaerobic assays was observed with Fe^{2+}, not Co^{2+}. With Fe^{2+}, *Ph*prol showed the highest activity of 2,371 U/mg while the activity for *Ph1*prol was 1,357 U/mg. The specific activity with Co^{2+} remained around 30% of the activity seen with Fe^{2+} (Supplemental Fig. 3 and Supplemental Table 2). Zn^{2+} was shown to only provide 1% of the relative activity in *Ph1*prol and did not support any activity in *Ph*prol.

Metal competition experiment

Other than *Pf*prol, which has a solved Co–Co binuclear metal center, prolidases and metalloenzymes from different organisms and under varying conditions have been found to incorporate alternate divalent metal ions in their active sites (Besio et al. 2009; Lupi et al. 2006; Maher et al. 2004). Since, as purified, the *P. horikoshii* prolidases were shown to have been bound with zinc rather than with metals that support catalysis such as Co^{2+}, Mn^{2+}, and Fe^{2+}; it became necessary to evaluate how different metals and or metal combinations affect catalysis of these enzymes. *Ph*prol and *Ph1*prol were dialyzed in 50 mM MOPS buffer containing EDTA to strip the enzyme of metal. Metal reconstitution was done under the following metal conditions, no metal addition (apo-protein) and the addition of the following metal or metal combinations, Co^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}/Mn^{2+}, Co^{2+}/Zn^{2+}. The activity of the WT-*Ph1*prol and WT-*Ph*prol, pre-EDTA treatment, showed the normal activity seen with the addition of Co^{2+} (0.2 mM), Mn^{2+}, or Zn^{2+} (1.2 mM) to an enzyme assay conducted at 100 °C (Tables 3 and 4). Prolidase activity with the addition of cobalt in the assay yielded the highest activity at 4,778 U/mg and 2,174 U/mg for WT-*Ph1*prol and WT-*Ph*prol, respectively. Apo-protein represents protein that had EDTA treatment but contained no metal in the metal reconstitution process. Without the addition of metals to the enzyme assay, the apo-protein had very little to nondetectable activity (data not shown). When Co^{2+} ions were added to the assay with apo-protein, only 55% of the enzyme activity was restored for *Ph1*prol, and the activity was almost twofold higher for *Ph*prol. When adding Mn^{2+} to the assay, both *Ph1*prol and *Ph*prol apo-protein activity were almost fully restored. The highest activities for *Ph1*prol were observed when Co^{2+}, Mn^{2+}, Co^{2+}/Mn^{2+}, and Co^{2+}/Zn^{2+} were used in the reconstitution of the metal center and Co^{2+} was added to the assay mix (Table 3). The same trend was seen with *Ph*prol, except the difference was almost a twofold increase in activity when reconstituting with Co^{2+}, Mn^{2+}, Co^{2+}/Mn^{2+}, and Co^{2+}/Zn^{2+}, when the assay contained Co^{2+} (Table 4). When adding Mn^{2+} to the assay conditions with *Ph*prol and *Ph1*prol reconstituted with different metals, the activity was restored but not to the high levels detected when Co^{2+} was added. The addition of Zn^{2+} to assay conditions resulted in minimal activity (less than 1% relative activity) for *Ph1*prol and little to no detected activity for *Ph*prol (Tables 3 and 4).

Discussion

Biochemical characterization of both *Ph*prol (PH1149) and *Ph1*prol (PH0974) supports their classification as prolidases or X-Pro dipeptidases. Although, *Ph2*prol possesses similar motifs to Xaa-Pro aminopeptidases when using pBLAST for protein comparison, no activity was seen when assays were done with *Ph2*prol crude cell extract when prolidase specific substrates and metals were supplied. Closer inspection of the *P. furiosus* genome shows that it also contains two annotated prolidase genes, one being PF1343,

Table 2 Kinetic parameters of prolidases from *P. furiosus* and *P. horikoshii*

Prolidase	Substrate	K_m (mM)	V_{max} (μmol/min/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
N-*Pf*prol[a]	Met-Pro	2.8	645	271	97
R-*Pf*prol[a]	Met-Pro	3.3	1,250	525	159
*Ph*prol	Met-Pro	3.4	3,997	2,617	768
*Ph1*prol	Met-Pro	1.9	5,714	3,812	2,006

All assays were done at 100 °C in 50 mM MOPS pH 7.0 with $CoCl_2$ (0.2 mM). N-*Pf*prol and R-*Pf*prol represent the native and recombinant *P. furiosus* prolidase

[a] Results are taken from Ghosh et al. 1998

Table 3 Effect of metal ions on *Ph1*prol activity

Metal ions added in the assay	Specific activity (U/mg)						
	Metal reconstitution conditions						
	WT-*Ph1*prol	Apo-*Ph1*prol	Co-*Ph1*prol	Mn-*Ph1*prol	Zn-*Ph1*prol	Co/Mn-*Ph1*prol	Co/Zn-*Ph1*prol
Co^{2+}	4,778	2,605	4,688	5,352	2,652	4,903	3,407
	±646	±1277	±37	±230	±357	±547	±345
Mn^{2+}	1741	1,737	2,035	2,320	1,153	2,527	1,668
	±196	±62	±130	±35	±214	±81	±485
Zn^{2+}	63	25	33	28	24	33	39
	±52	±1	±0.1	±1	±5	±1	±5

All assays were done at 100 °C in 50 mM MOPS pH 7.0 with the addition of $CoCl_2$ (0.2 mM), $MnCl_2$, or $ZnCl_2$ (1.2 mM). Prolidase was stripped of metal by dialysis in 50 mM MOPS buffer (pH 7.0) containing 5 mM EDTA. Metal reconstitution was done using 2.5–5 molar equivalents of metal per subunit with the following metal or metal combinations: Co^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}/Mn^{2+}, and Co^{2+}/Zn^{2+}. Apo-*Ph1*prol was subjected to the reconstitution incubation at 80 °C as were the metal reconstituted enzymes; however, with no metal added

which is the prolidase designated *Pf*prol, and PF0747, which we have classified as *Pf2*prol. *Pf2*prol is similar to *Ph2*prol in that it only contains two out of five conserved metal-binding residues and shows low percent similarity to *Pf*prol. *Pf2*prol and *Ph2*prol cannot be confirmed as prolidases, and their function is still unknown at this time.

The catalytic properties of *P. horikoshii* prolidases are fairly similar to those observed for *P. furiosus* prolidase; however, there are key differences that could make them more ideal candidates for use in biotechnological applications. The temperature optima results for *Ph*prol and *Ph1*prol were very similar to *Pf*prol in that the highest activity was seen at 100 °C. This is not surprising since optimal growth of *Pyrococci* is at 98–100 °C (Gonzalez et al. 1998). This would suggest that proteins isolated from these organisms should be very stable at high temperatures. Previously, when testing the thermal half-life of *Pf*prol under similar pH (7.0) and temperature conditions, Ghosh et al. found the $t_{50\%}$ of *Pf*prol (0.3 mg/ml) was 3 h. *P. horikoshii* prolidases (0.2 mg/ml) are even more thermostable with $t_{50\%}$ values of over 20 h at 90 °C, which is significantly higher than recombinant *Pf*prol and other characterized prolidases. Even after 48 h at 90 °C, *Ph*prol and *Ph1*prol were still active with a relative activity of 28% and 20%, respectively.

The *P. horikoshii* prolidases are more active over a broader pH range than *Pf*prol, showing significantly higher activity at the lower pH range, specifically pH 5.0–7.0, and they continue to be active at pH 8.0 to a greater extent than is *Pf*prol. The enhanced stability of *P. horikoshii* prolidases

Table 4 Effect of metal ions on *Ph*prol activity

Metals ions added in the assay	Specific activity (U/mg)						
	Metal reconstitution conditions						
	WT-*Ph*prol	Apo-*Ph*prol	Co-*Ph*prol	Mn-*Ph*prol	Zn-*Ph*prol	Co/Mn-*Ph*prol	Co/Zn-*Ph1*prol
Co^{2+}	2,174	4,112	3,615	3,756	2,080	4,064	4,421
	±384	±119	±192	±168	±310	±37	±197
Mn^{2+}	818	513	725	901	705	670	831
	±129	±140	±93	±75	±89	±23	±31
Zn^{2+}	9.6 ±6	ND	ND	ND	ND	ND	ND

ND not detected

All assays were done at 100 °C in 50 mM MOPS pH 7.0 with the addition of $CoCl_2$ (0.2 mM), $MnCl_2$, or $ZnCl_2$ (1.2 mM). Prolidase was stripped of metal by dialysis in 50 mM MOPS buffer (pH 7.0) containing 5 mM EDTA. Metal reconstitution was done using 2.5–5 molar equivalents of metal per subunit with the following metal or metal combinations: Co^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}/Mn^{2+}, and Co^{2+}/Zn^{2+}. Apo-*Ph*prol was subjected to the reconstitution incubation at 80 °C as were the metal reconstituted enzymes; however, with no metal added

and catalysis over a larger pH range are qualities that could help these enzymes withstand other potential denaturants in OP nerve agent decontamination formulations. The kinetic parameters for the recombinant *P. horikoshii* prolidases are more promising for biotechnological applications compared to those determined for *Pf*prol. The affinity for the substrate Met-Pro is highest in *Ph1*prol with a K_m of 1.9 mM, and the reaction turnover rates are significantly higher than for *Pf*prol. The substrate profile is similar to other prolidases in that they show high specificity to dipeptides with proline in the C-terminus and a nonpolar amino acid in the N-terminal region and rarely are able to cleave dipeptides with proline in the N-terminus, Pro-X. The differences in relative activities between *Ph*prol and *Ph1*prol could suggest differences in their roles in vivo. *P. horikoshii*, unlike *P. furiosus*, lacks many genes and operons that are responsible for de novo synthesis of amino acids; therefore, it is important that this organism obtain them from their environment. *P. horikoshii* may have two functional prolidases to aid in their amino acid metabolism requirements.

When examining the metal content of purified prolidases from *P. horikoshii*, it is not surprising that the incorporation of zinc, not cobalt was found in significant amounts. Previously in Ghosh, et al., both the native and recombinant *Pf*prol contained one Co atom per molecule. When further chemical analysis was done, Zn was found in variable to significant amounts, but was attributed to nonspecific binding due to its inability to restore enzyme catalysis. In addition, during the crystallization process used to solve the structure of *Pf*prol, Zn replaced Co, the metal needed for catalysis, in the active site of the crystallized prolidase enzyme (Ghosh et al. 1998; Maher et al. 2004). The Maher study (Maher et al. 2004) warned when purifying or crystallizing a metalloenzyme such as prolidase, other metals could be introduced based on the crystallization medium used, and this metal may not necessarily be physiologically or catalytically relevant to the enzyme.

The highest activity of *Ph*prol and *Ph1*prol when assayed aerobically was observed when lower concentrations of Co^{2+} were introduced into the assay reaction than was used for *Pf*prol activity assays (0.2 mM Co^{2+} for *Ph*prol and *Ph1*prol versus 1.2 mM for *Pf*prol). For both *Ph*prol and *Ph1*prol, the association constant for Co^{2+} appears to be much lower, between 10–50 μM, while for *Pf*prol, it was previously projected to be 0.5 mM (500 μM) for recombinant *P. furiosus* prolidase (Ghosh et al. 1998). For Mn^{2+}, the association constant was determined to be around 0.6 mM for *Ph1*prol, which is also consistent with *Pf*prol, which was shown to be 0.66 mM (Ghosh et al. 1998). The addition of metal for enzyme catalysis is one of the limitations in using this enzyme in applications because adding metal to the environment can be harmful. The *P. horikoshii* prolidases appear to be more active and more specific for their substrate and require less metal for full catalysis than *Pf*prol, making them good candidates in the future for biotechnological applications.

Metal competition experiments with *Ph*prol and *Ph1*prol also show variations in the preferred metal or mixed metals needed for optimum activity. Interestingly, the highest activities were seen with the enzyme reconstituted with Co^{2+}, Mn^{2+}, or a combination of Co^{2+}/Mn^{2+}, with the addition of Co^{2+} to the assay conditions. This may suggest optimum activities could be achieved by a mixed metal center using Co and Mn ions in the active sites, rather than solely Co ions. Recently, in Besio et al. (2009), for the first time, structural ICP-MS and XAS data were presented of the recombinant human prolidase, showing that an active prolidase can use a heterogeneous dimeric metal site, employing both Zn and Mn ions (Besio et al. 2009). Recombinant human prolidase had previously been characterized and was shown to contain a fully loaded Mn active site for maximum catalysis. However, the Besio et al. study indicated that human prolidase is also enzymatically active when one of two active sites is occupied by two Zn ions and the other with one Mn and one Zn ion (Besio et al. 2009). It has been suggested that zinc could play a structural role, while other divalent cations play a role in catalysis. The incorporation of zinc, whether nonspecific or structural, may be present due to the host system used in the expression process. By using the *E. coli* host system to express recombinant proteins, zinc is more prevalent in vivo than any other divalent cations, which support prolidase activity (Maher et al. 2004; Willingham et al. 2001).

Although, cobalt is the preferred metal for catalysis aerobically, Fe^{2+} shows the highest activity anaerobically with *Ph*prol and *Ph1*prol. In Du et al., it was reported that *Pf*prol also had the highest activity with Fe^{2+} followed by Co^{2+} under anaerobic conditions (Du et al. 2005). These findings are not particularly surprising considering the questions arising in the reported literature about the metal center in the closely related enzyme methionine aminopeptidase (Chai et al. 2008; Copik et al. 2005; Cosper et al. 2001; D'Souza et al. 2000, 2002; D'Souza and Holz 1999; Walker and Bradshaw 1998).

Methionine aminopeptidases share very similar structures with prolidases, as they both contain the same unique pita-bread fold and the same five metal ligands in the C-terminal region of the enzyme (Lowther and Matthews 2002). Other enzymes that are members of this family include aminopeptidase P and creatinase, and they all require metal for catalysis (Lowther and Matthews 2002). Purified MetAP-I apoenzyme from *E. coli* can be activated by divalent cations such as Co^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}, with cobalt supporting the highest activity, but it is

considered unlikely that Co^{2+} is the native cofactor in vivo based on whole cell metal analysis and MetAP inhibitor studies (Chai et al. 2008; D'Souza and Holz 1999; Ye et al. 2006). In 1998, Walker and Bradshaw first demonstrated that Zn^{2+} was the native metal required in yeast MetAP-I, not Co^{2+} (Walker and Bradshaw 1998). Under physiologically relevant conditions and with inhibitors, *E. coli* MetAP-I (*Ec*MetAP-I) was found to use Fe^{2+} or Mn^{2+} for maximal activity, while Fe^{2+} was also found to maximally activate *P. furiosus* MetAP-II or *Pf*MetAP-II at its physiologically relevant temperature of 80 °C (D'Souza et al. 2000, 2002; D'Souza and Holz 1999; Meng et al. 2002). These two enzymes have also been suggested to function in vivo as mononuclear enzymes (Chai et al. 2008; Copik et al. 2005). More recently, a study by Chai et al. reports that Fe^{2+} is the native metal for MetAPs in both *E. coli* and two other *Bacillus* strains (Chai et al. 2008). Studies of prolidases and other related metallopeptidases continue to provide much needed information on the relationship and interactions between proteins and metals.

Many different prolidases are being studied for use in biotechnological applications including nerve agent detoxification, the development of cheese ripening and flavor in fermented foods, and most recently in enzyme therapy and drug delivery studies for patients with PD, and even cancer (Theriot et al. 2009). Currently, prolidases are being characterized from lactic acid bacteria evaluating their role in the food fermentation process (Yang and Tanaka 2008). Enzyme therapy and drug delivery studies are underway using both recombinant human and prokaryotic prolidases (Lupi et al. 2006; Mittal et al. 2005, 2007). By studying new prolidases from different organisms, we are able to explore the structure-function relationship of these enzymes and their interactions with metal.

Current biodecontamination formulations that incorporate *Alteromonas* prolidases have limited utility when used under harsh field conditions. The main limitations are in the long-term stability of the enzyme in a formulation mixture that includes other solvents and denaturing solutions and the need to add excess metal to reach full activity. An enzyme is needed with higher activity at a lower pH, over a broader temperature range, long-term stability under harsh conditions, and low metal requirement (Cheng and DeFrank 2000). Both prolidases characterized from *P. horikoshii* show promising enzymatic properties that make these enzymes potential candidates for future optimization studies for OP degradation. Compared to *Pf*prol, *Ph*prol and *Ph1*prol show higher activity at a lower pH range, long-term stability, higher affinity for the substrate, and significantly lower metal requirement for catalysis. Future studies will focus on broadening the enzyme catalysis temperature range to include those suitable for application conditions. The differences in the structures of *Pf*prol compared to *Ph*prol and *Ph1*prol could also provide insight into the properties needed for generating an enzyme with a higher affinity for metal. By utilizing genetic engineering strategies, we may be able to make a more catalytically active enzyme for detoxification of OP nerve agents.

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