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Characterization of Native and Recombinant Forms of an Unusual Cobalt-Dependent Proline Dipeptidase (Prolidase) from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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Proline dipeptidase (prolidase) was purified from cell extracts of the proteolytic, hyperthermophilic archaeon *Pyrococcus furiosus* by multistep chromatography. The enzyme is a homodimer (39.4 kDa per subunit) and as purified contains one cobalt atom per subunit. Its catalytic activity also required the addition of Co^{2+} ions (K_d, 0.24 mM), indicating that the enzyme has a second metal ion binding site. Co^{2+} could be replaced by Mn^{2+} (resulting in a 25% decrease in activity) but not by Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, or Ni^{2+}. The prolidase exhibited a narrow substrate specificity and hydrolyzed only dipeptides with proline at the C terminus and a nonpolar amino acid (Met, Leu, Val, Phe, or Ala) at the N terminus. Optimal prolidase activity with Met-Pro as the substrate occurred at a pH of 7.0 and a temperature of 100°C. The N-terminal amino acid sequence of the purified prolidase was used to identify in the *P. furiosus* genome database a putative prolidase-encoding gene with a product corresponding to 349 amino acids. This gene was expressed in *Escherichia coli* and the recombinant protein was purified. Its properties, including molecular mass, metal ion dependence, pH and temperature optima, substrate specificity, and thermostability, were indistinguishable from those of the native prolidase from *P. furiosus*. Furthermore, the K_m values for the substrate Met-Pro were comparable for the native and recombinant forms, although the recombinant enzyme exhibited a twofold greater V_{max} value than the native protein. The amino acid sequence of *P. furiosus* prolidase has significant similarity with those of prolidases from mesophilic organisms, but the enzyme differs from them in its substrate specificity, thermostability, metal dependency, and response to inhibitors. The *P. furiosus* enzyme appears to be the second Co-containing member (after methionine aminopeptidase) of the binuclear N-terminal exopeptidase family.

Pyrococcus furiosus is a fermentative archaeon which grows optimally at temperatures near 100°C (26). Like many heterotrophic hyperthermophiles, it utilizes proteins and peptides as growth substrates and produces organic acids, CO_2, and H_2. Several enzymes involved in the catabolism of amino acids have been purified from *P. furiosus* (2), including aminotransferases (3), glutamate dehydrogenase (34), 2-keto acid oxidoreductases (31, 37, 38), and acetyl coenzyme A synthetases (39). In addition, this organism produces perhaps a dozen or more proteolytic-type enzymes, which are assumed to generate small peptides from the protein-based growth substrates (6, 8, 21, 23, 45). So far, three proteases have been characterized from *P. furiosus*. These are a membrane-associated serine protease (48), an intracellular protease with trypsin- and chymotrypsin-like activities (28, 29), and an intracellular endopeptidase that cleaves at prolyl residues (30). In addition, two proteases have been purified from other members of the family *Thermococcales*, including a thiol protease from an unclassified species of *Pyrococcus* (40) and a serine protease from *Thermococcus stetteri* (33). To date, however, there have been no reports on the properties of amino acid-yielding peptidases from *P. furiosus* or related species. In order to further understand the pathways of peptide metabolism in these organisms, we examined *P. furiosus* for dipeptidase activities. Cell extracts contained very high concentrations of prolidase, a proline-specific dipeptidase, and the characterization of this enzyme is reported herein.

Since prolyl residues confer a conformational constraint on a peptide chain due to the cyclic nature of its pyrrolidine side group, only a few proteases are known that are able to hydrolyze bonds adjacent to proline (22, 49). These enzymes include (i) proline-specific endopeptidase, which hydrolyzes peptides on the carboxyl side of prolyl residues located internally within a polypeptide (-X--Pro-/-X-); (ii) prolyl aminopeptidase, which cleaves the bond between any N-terminal amino acid and a penultimate prolyl residue (NH_2-X-/-Pro--X-) in peptides of various lengths; (iii) proline iminopeptidase, which catalyzes cleavage of unsubstituted N-terminal prolyl residues from dipeptides, tripeptides, and polypeptides (Pro-/-X-); (iv) proline specific C-terminal exopeptidase (-X--Pro-/-X-COOH), which releases an amino acid from the C terminus of a peptide with a penultimate proline residue; and (v) prolidase, which only cleaves dipeptides with proline at the C terminus (NH_2-X-/-Pro-COOH). These proline-specific enzymes are thought to participate, in concert with other endo- and exopeptidases, in the terminal degradation of intracellular proteins and may also function in the recycling of proline.

Prolidase (iminodipeptidase, EC 3.4.3.7) is widespread in nature and has been isolated from different mammalian tissues (12, 24, 44) as well as from bacteria such as species of *Lactobacillus* (10, 25) and *Xanthomonas* (46). While the physiological role of prolidase in bacteria is unclear, a deficiency of this enzyme in humans results in abnormalities of the skin and

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other collagenous tissues (43). Prolidase also has biotechnological applications. For example, it has a potential use in the dairy industry as a cheese-ripening agent (9) since proline release from proline-containing peptides in cheese reduces bitterness. In addition, it was recently reported (18) that prolidase and an enzyme termed organophosphorus acid anhydrolase (OPAA, EC 3.1.8.1) appear to be one and the same. OPAA hydrolyzes highly toxic, organophosphorus, acetylcholinesterase inhibitors, which include various chemical warfare agents and pesticides. Such enzymes have been characterized from various species of *Pseudomonas*, *Flavobacterium*, and *Alteromonas* and also from eucaryotes (5, 17, 36). The sequence of the OPAA from *Alteromonas* sp. strain JD6.5 shows similarity to that of human prolidase and, like the latter enzyme, OPAA catalyzes the Mn^{2+}-dependent hydrolysis of X-Pro dipeptides but not of Y-Pro-X tripeptides (18). It seems reasonable to conclude, therefore, that the natural function of OPAA involves peptide metabolism rather than detoxification. Conversely, previously characterized prolidases may be found to be biotechnologically relevant in detoxification strategies.

Prolidase has yet to be characterized from an archaeon or a hyperthermophile. It was therefore of some interest to determine the properties of this enzyme from *P. furiosus*. In addition, the gene encoding *P. furiosus* prolidase was cloned and expressed in *Escherichia coli*, and this allowed a biochemical comparison to be made between the native form (from *P. furiosus*) and the recombinant form.

MATERIALS AND METHODS

Growth of microorganisms. *P. furiosus* (DSM 3638) was grown at 95°C in a 500-liter fermentor with maltose as the carbon source as described previously (13). *E. coli* BL21(λDE3) (F^- *ompT* [*lon*] *hsdS*) was grown in a 100-liter fermentor at 37°C in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) as needed.

Enzyme assay. The prolidase activity assay used was based on the amount of proline liberated from the hydrolysis of dipeptides that contain proline at the C terminus. The proline concentration was determined by a modification of the colorimetric ninhydrin method of Yaron and Mlynar (51). The ninhydrin reagent was prepared by the addition of ninhydrin (3.0% [wt/vol]) to a mixture of 60% (vol/vol) glacial acetic acid and 40% (vol/vol) phosphoric acid followed by a 30-min incubation at 70°C (51). The assay mixture (500 μl) for prolidase contained 50 mM MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer (pH 7.0), 4 mM Met-Pro (substrate), and 1.2 mM $CoCl_2$ and was incubated at 100°C for 5 min. The reaction was initiated by addition of the enzyme or extract. The mixture was incubated at 100°C for a further 10 min, and the reaction was stopped by the addition of glacial acetic acid (500 μl) followed by the ninhydrin reagent (500 μl). After heating at 100°C for 10 min, the solution was cooled to 23°C and the absorption at 515 nm was determined with an extinction coefficient of 4,570 $M^{-1} \cdot cm^{-1}$ 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 under these assay conditions.

Purification of *P. furiosus* prolidase. Prolidase was purified from *P. furiosus* under anaerobic conditions at 23°C. Frozen cells (500 g [wet weight]) were thawed in 1,800 ml of 50 mM Tris-HCl buffer (pH 8.0) containing lysozyme (1 mg/ml) and DNase (10 μg/ml) and were lysed by incubation at 37°C for 2 h followed by sonication (Branson 8200 sonicator) for 1 h. A cell extract was obtained by ultracentrifugation at 50,000 × *g* for 2 h. The supernatant (1,800 ml) was loaded onto a column (10 by 14 cm) of DEAE Fast Flow (Pharmacia, Piscataway, N.J.) equilibrated with 50 mM Tris (pH 8.0) containing 10% (vol/vol) glycerol. The column was eluted at a flow rate of 10 ml/min with a 10-liter linear gradient of 0 to 1.0 M NaCl in the same Tris-glycerol buffer. Prolidase activity was detected as 0.25 to 0.40 M NaCl was applied to the column. The active fractions were combined (1,500 ml), and solid ammonium sulfate was added to a final concentration of 1.5 M. This solution was applied to a column (3.5 by 10 cm) of phenyl Sepharose (Pharmacia) equilibrated with Tris-glycerol buffer containing 1.5 M ammonium sulfate. The column was eluted with a gradient (1 liter) from 1.5 to 0 M ammonium sulfate in the Tris-glycerol buffer at a flow rate of 7 ml/min. Prolidase eluted as 0.45 to 0.78 M ammonium sulfate was applied to the column. The prolidase-containing fractions (250 ml) were concentrated to a volume of 7 ml by ultrafiltration (PM-30 membrane filter; Amicon, Beverly, Mass.) and applied to a column (3.5 by 60 cm) of Superdex-200 (Pharmacia) equilibrated with 50 mM Tris (pH 8.0) containing 0.5 M NaCl at a flow rate of 0.5 ml/min. The active fractions from the Superdex 200 column were applied to a column of HiTrap-Q (1.6 by 2.5 cm; Pharmacia) equilibrated with 50 mM Tris (pH 8.0), and the enzyme was eluted with a gradient (100 ml) from 0 to 0.5 M NaCl in the same buffer at a flow rate of 4 ml/min. Fractions containing prolidase activity (10 ml) eluted as 0.29 to 0.40 M NaCl was applied and were stored at −80°C until being required.

Cloning and expression of the prolidase-encoding gene. Recombinant *P. furiosus* prolidase was obtained by PCR amplification of the *P. furiosus* prolidase gene and subsequent cloning of this gene into the T7-polymerase-driven expression vector pET-21b (Novagen, Milwaukee, Wis.). For the PCR amplification of the prolidase gene, two primers were designed. Primer 1 (ATAGGATCCGGT GAGGAGGTTGTATGAAAGAAAGACTTGAA; Stratagene, La Jolla, Calif.) contained an engineered *Bam*HI site and spans from −21 to +6 on the coding strand. Primer 2 (ATAGGATCCGGTGAGGAGGTTGTATGAAAGAAAG AC; Stratagene) had an engineered *Not*I site and corresponds to sequence ranging from +1511 to +1541 on the noncoding strand. PCR amplification was performed with native *P. furiosus* DNA polymerase and a Robocycler 40 (Stratagene) programmed for 39 cycles, each cycle consisting of denaturation at 95°C for 5 min, annealing at 52°C for 2 min, and extension at 72°C for 5 min. The resultant 1.5-kb prolidase gene was first subcloned into the blunt end *Srf*I site in the vector pCR-Script (Stratagene) to yield plasmid pProl. The prolidase insert DNA contained in plasmid pProl was then sequenced to ensure that no mutations were present in the gene. The prolidase gene was then excised from plasmid pProl by restriction digest with the enzymes *Bam*HI and *Not*I (Stratagene) and cloned into the *Bam*HI and *Not*I sites in expression vector pET-21b, resulting in plasmid pET-Prol.

For expression of recombinant prolidase, plasmid pET-Prol was transformed into *E. coli* BL21(λDE3), which has isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression of T7-RNA polymerase. Prolidase was produced in a culture of BL21(λDE3)-pET-Prol grown in a 100-liter fermentor at 37°C. Expression of the plasmid borne prolidase gene was induced with the addition of IPTG (1 mM) when the culture reached an optical density of 1.0. The induced culture was incubated for 4 h prior to the harvesting of the cells.

Purification of recombinant prolidase. Recombinant prolidase was purified in three steps. IPTG-induced BL21(λDE3)-pET-Prol cells (10 g [wet weight]) were suspended in 10 ml of 50 mM Tris-HCl, pH 8.0, containing benzamidine HCl (0.5 mg/ml). The cell suspension was passed through a French pressure cell (20,000 lb/in^2) twice. The lysed extract was centrifuged at 39,000 × *g* for 1 h to remove any cellular debris, and the supernatant was diluted to 300 ml with 50 mM Tris-HCl, pH 8.0. Solid ammonium sulfate was slowly added with stirring to a final concentration of 1.5 M, and the solution was applied to a column (3.5 cm by 10 cm) of phenyl Sepharose (Pharmacia) equilibrated with the same buffer at a flow rate of 7 ml/min. The bound protein was eluted with a gradient (1,000 ml) from 1.5 to 0 M ammonium sulfate in 50 mM Tris-HCl, and the recombinant prolidase was eluted as 0.67 to 1.0 M ammonium sulfate was applied. The prolidase-containing fractions were incubated at 100°C for 2.5 h, and denatured *E. coli* proteins were removed by centrifugation at 27,000 × *g* for 30 min. The supernatant was diluted threefold with 50 mM Tris-HCl, pH 8.0, as it was applied to a column of HiTrap-Q (1.6 by 2.5 cm; Pharmacia) equilibrated with 50 mM Tris-HCl, pH 8.0. A gradient (100 ml) from 0 to 0.5 M NaCl in the same buffer was applied to the column. The prolidase eluted between 0.25 and 0.37 M NaCl and was stored at −80°C until being required.

Other methods. Molecular weights were estimated by gel filtration with a column (1 by 27 cm) of Superdex 200 (Pharmacia LKB) with amylase (200,000), alcohol dehydrogenase (150,000), and bovine serum albumin (66,000) as standard proteins. Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed using 12% polyacrylamide by the method of Laemmli (35). Protein concentrations were determined by the Bradford method (11) with bovine serum albumin as the standard. To determine metal content, exogenous metal ions were removed from the prolidase by either dialyzing (membrane cutoff, 8 kDa) the sample against 100 volumes of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS), pH 8.0, containing 0.5 M NaCl or by gel filtration with a Superdex 200 column. A complete metal analysis (31 elements) was obtained by plasma emission spectroscopy with a Jarrel Ash Plasma Comp 750 instrument at the Chemical Analysis Laboratory of the University of Georgia. The NH_2-terminal sequences of the native and recombinant prolidases were determined by using an Applied Biosystems Model 477 sequencer in the Molecular Genetics Instrumentation Facility (MGIF) of the University of Georgia. Samples were electroblotted onto polyvinylidene difluoride protein-sequencing membranes (Stratagene) from SDS-electrophoresis gels by using a Bio-Rad electroblotting system. Electroblotting was carried out in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11.0, containing methanol (10% [vol/vol]) for 1 h at 50 V. Both strands of the *P. furiosus* prolidase gene present in plasmid pET-Prol were sequenced in their entirety by the MGIF of the University of Georgia. DNA sequence was analyzed using the computer software programs Genetics Computer Group (University of Wisconsin, Madison) and MacVector (International Biotechnologies, Inc., New Haven, Conn.).

Nucleotide sequence accession number. The DNA sequence of the prolidase gene is available from GenBank under accession no. AF060010.

TABLE 1. Purification of prolidase from *P. furiosus*

Step	Activity[a] (U)	Amt of protein (mg)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Cell extract	48,400	21,060	2.3	1	100
DEAE Sepharose	25,600	4,500	5.7	2.5	53
Phenyl Sepharose	10,400	855	12	5.3	21
Superdex 200	2,180	14	153	66.4	4
HiTrap-Q	1,890	3	630	274	4

[a] Activity was measured with Met-Pro (4 mM) as the substrate.

RESULTS

Purification of *P. furiosus* prolidase. Extracts of *P. furiosus* cells grown with maltose as the primary carbon source contained high prolidase activity (approximately 2.3 U/mg at 100°C) with the dipeptide Met-Pro as the substrate. For comparison, this specific activity is more than 100-fold higher than the prolidase activity found in cell extracts of *Lactobacillus casei* grown on casein (25) and 200-fold higher than that in cell extracts of *Xanthomonas maltophilia* grown in nutrient broth (46). The prolidase of *P. furiosus* appeared not to be regulated, as the specific activities of extracts of cells grown with yeast extract (5.0 g/liter) and maltose (1.0 g/liter), with yeast extract (5.0 g/liter), peptone (5.0 g/liter) and maltose (1.0 g/liter), or with yeast extract (5.0 g/liter) and maltose (5.0 g/liter) were similar (2.5 ± 0.3 U/mg). Since maltose-grown cells are routinely used in our laboratory to purify various O_2-sensitive, oxidoreductase-type enzymes (see reference 2 for an example) these cells were also used for prolidase purification. In addition, the procedure was carried out under anaerobic conditions, not because the prolidase was sensitive to O_2, but to allow the purification of enzymes that are from the same batch of *P. furiosus* cells.

Prolidase activity was not detected in the culture supernatant during either log- or stationary-phase growth of *P. furiosus* or in the membrane fraction of a cell extract. The activity was present only in the soluble fraction, indicating that the enzyme is a cytoplasmic protein. The results of a typical purification are summarized in Table 1. The enzyme was purified 274-fold with a yield of 4% and a specific activity of approximately 630 U/mg. It therefore constitutes approximately 0.36% of total cytoplasmic protein. When the purified prolidase was treated with SDS sample buffer at 80°C for 10 min prior to electrophoresis, it migrated as a single band corresponding to a molecular mass of 51 kDa. However, when treated at 100°C for 30 min, the protein band migrated with a molecular mass of 42 kDa (see Fig. 1). Presumably, the former conditions result in a partially denatured protein which is retarded in the electrophoretic gel. The prolidase which was eluted from a gel filtration column corresponded to a molecular mass of 100 ± 10 kDa. This result, together with the electrophoretic data, suggests that the enzyme is a homodimer.

The N-terminal amino acid sequence of the native prolidase was MKERLEKLVKFMDEN. This sequence was used to search the genomic sequence database of *P. furiosus*, which is nearing completion by the use of multiplex sequencing methods (19). A gene was located whose translated N-terminal region matched exactly the sequence obtained from the enzyme. It consisted of 1,047 bp and encoded a protein of 349 residues with a calculated molecular mass of 39.4 kDa (Fig. 2). The latter value is slightly lower than that (42 kDa) obtained from the SDS-gel analysis, suggesting that the protein is not completely denatured under the conditions used. The enzyme also appears to exhibit nonideal behavior when subjected to gel filtration, since the molecular mass estimated by that method (100 kDa) is higher than that expected (78.8 kDa) for a homodimeric protein. A mass of 39.4 kDa for the prolidase subunit was used in all calculations.

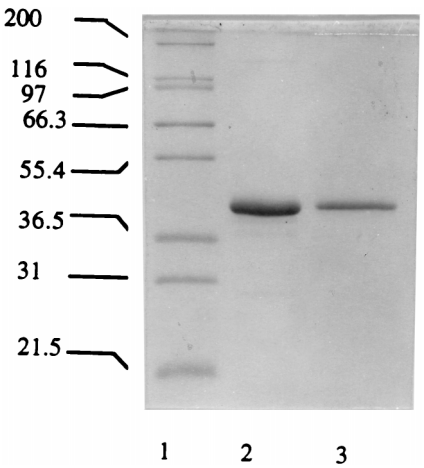

FIG. 1. SDS–12% polyacrylamide gel electrophoresis of purified N- and R-prol. Lane 1, molecular mass markers (kilodaltons): myosin (200), β-galactosidase (116), phosphorylase *b* (97), bovine serum albumin (66.3), glutamic dehydrogenase (55.4), lactate dehydrogenase (36.5), carbonic anhydrase (31); lane 2, R-prol; lane 3, N-prol.

Purification of recombinant *P. furiosus* prolidase. The production of prolidase protein was successfully induced in *E. coli* cells in the presence of IPTG with maximal induction (as determined by prolidase activity) after a 4-h period of induction at 37°C (data not shown). *P. furiosus* prolidase activity could be identified in cell extracts of *E. coli* both by high-temperature (100°C) enzyme assays, which eliminated the host cell prolidase activity, as well as by the appearance of a protein band corresponding to the size of prolidase (42 kDa) after SDS-gel analysis of cell extracts (data not shown). The specific activity of the prolidase in the recombinant *E. coli* cells was approximately 90 U/mg, which is about 40-fold higher than that present in cell extracts of *P. furiosus* (under the same assay conditions when Met-Pro was used as a substrate at 100°C). The results of a typical purification of the recombinant prolidase from a cell extract of *E. coli* BL21(λDE3)-pET-Prol are summarized in Table 2. The enzyme was purified in three steps with a specific activity increase of about 15-fold and a recovery of 54%. It constituted approximately 6.7% of the total cellular protein. However, the specific activity of the purified recombinant form (~ 1,300 U/mg) was about twofold greater than that of the native enzyme from *P. furiosus*. The reasons for this are unclear at present (see below). Nevertheless, the recombinant prolidase was indistinguishable from the native protein from *P. furiosus* when analyzed by SDS-gel electrophoresis, and N-terminal amino acid sequence analysis showed that it contained the same first 15 residues (MKERLEKLVKFM DEN). It should also be noted that the nucleotide sequence of the gene encoding the prolidase was identical to that obtained from the genomic database.

Physical properties of native and recombinant prolidases. Both the native prolidase purified from *P. furiosus* (hereafter referred to as N-prol) and the recombinant prolidase obtained from *E. coli* (hereafter referred to as R-prol) were analyzed for metals. The only ones present in significant amounts (>0.1 g-atom/39.4-kDa subunit) were cobalt and zinc. Both R-prol and N-prol contained 1.0 ± 0.3 (mean ± standard deviation)

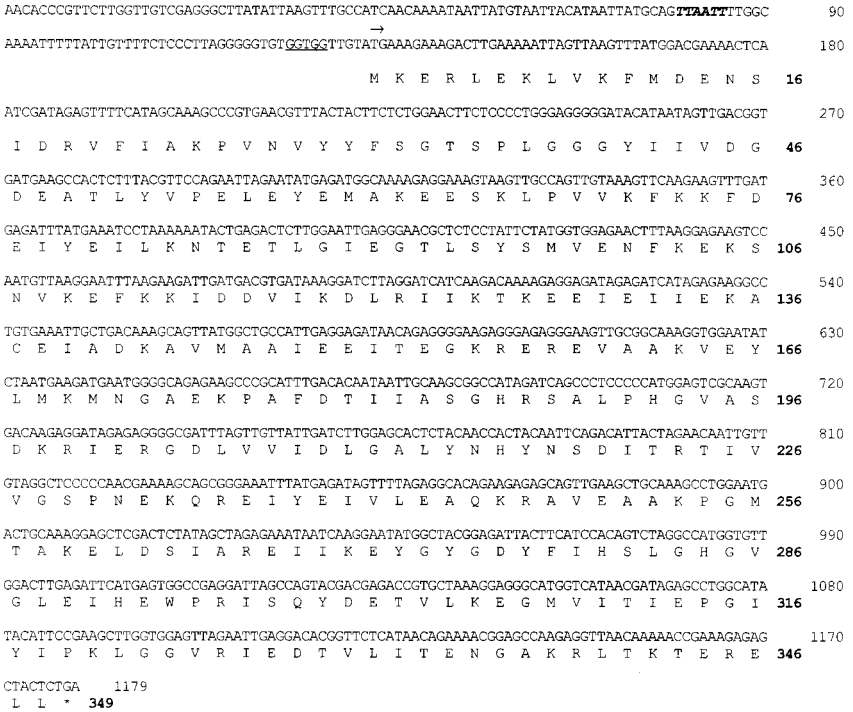

FIG. 2. The 1,047-bp gene encoding *P. furiosus* prolidase and the deduced amino acid sequence (348 amino acids) are shown. A putative TATA box is indicated in boldface. The ribosomal binding site is underlined, and the translation start site is marked by an arrow.

g-atoms of Co/mol of subunit (data from four and eight different prolidase samples, respectively). These data indicate that the difference in the specific activities of N- and R-prol under standard assay conditions was not due to a difference in Co content. R-prol was also purified as described above but with all buffers containing 1 mM $CoCl_2$. The resulting enzyme, after gel filtration (Superdex 200) to remove exogenous Co, also contained 1.0 ($\pm$ 0.2) g-atoms of Co/subunit. However, this and all other prolidase preparations tested were inactive unless Co^{2+} ions were added to the assay medium, suggesting that both the recombinant and native forms of the enzyme require occupancy of a second (or more) Co^{2+} site per subunit for activity and that the two sites have very different affinities (see below). Chemical analysis of R-prol and N-prol also revealed the presence of significant but variable amounts of zinc (1.5 $\pm$ 1.0 and 2.0 $\pm$ 0.5 g-atoms/subunit from 4 and 12 determinations, respectively). However, there was no correlation between the zinc content and the specific activity of an enzyme preparation (whether R- or N-prol), indicating that the zinc is nonspecifically bound. This was confirmed by treating R-prol (containing 2.0 $\pm$ 0.1 g-atoms of Zn/subunit) with EDTA (5 mM in 50 mM EPPS buffer, pH 8.0) for 1 h at 23°C, followed by gel filtration. The resulting enzyme contained only 0.3 $\pm$ 0.1 g-atoms of Zn/subunit, but its specific activity under standard assay conditions was not affected by the chelation treatment.

TABLE 2. Purification of recombinant *P. furiosus* prolidase from *E. coli*

Step	Activity[a] (U)	Amt of protein (mg)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Cell extract	45,400	498	91	1.0	100
Phenyl Sepharose	31,800	105	302	3.3	70
Heat treatment	27,200	24	1,130	12	60
HiTrap-Q	24,400	18	1,360	15	54

[a] Activity was measured with Met-Pro (4 mM) as the substrate.

N-prol was very thermostable, with no loss in activity when a sample (0.3 mg/ml in 100 mM MOPS, pH 7.0) was incubated in a sealed vial for 12 h at 100°C. However, the stability was dependent upon protein concentration, as the same enzyme preparation at a concentration of 0.003 mg/ml lost 50% of its activity after a 4-h incubation at that temperature. R-prol was apparently less thermostable than the native form but also exhibited a concentration-dependent response. The time required for a 50% loss in the activity ($t_{50\%}$) of R-prol at 100°C at a concentration of 0.3 or 0.003 mg/ml was 3 or 1 h, respectively. With R-prol at a concentration of 1.5 mg/ml, the optimal pH for stability was 7.0, with $t_{50\%}$ values at 100°C decreasing from 7.8 to 0.7, 1.3, 6.4 and 4.5 h at pH 2.0 (100 mM glycine-HCl), 4.5 (100 mM sodium acetate), 8.0 (100 mM EPPS), and 10.0 (100 mM CAPS), respectively. Hence, the enzyme was much more stable under alkaline conditions than it was in acidic media. Addition of Co^{2+} ions (1 mM) to either enzyme form during the various heat treatments did not affect the results. These data also demonstrate that both the recombinant and native forms of the prolidase are stable under the routine assay conditions (10 min at 100°C).

Catalytic properties of native and recombinant prolidases. The catalytic activities of N- and R-prol showed virtually identical responses to changes in temperature and pH (Fig. 3). Both showed a pH optimum at 7.0 and a temperature optimum of $\geq$100°C. Indeed, under the assay conditions used, neither form exhibited detectable activity at temperatures of $\leq$40°C. From the temperature-dependent data, the calculated activation energies for N- and R-prol are 11.9 $\pm$ 67.34 and 10.3 $\pm$ 77.86 kcal/mol, respectively. All assay reaction mixtures used

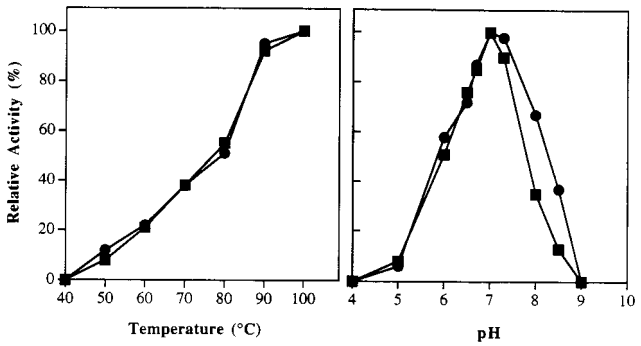

FIG. 3. The effects of pH (A) and temperature (B) on the activities of N-prol (squares) and R-prol (circles). The assay mixtures contained prolidase (0.015 μg), Met-Pro (4 mM), and $CoCl_2$ (1.2 mM). For determination of the effects of pH, the following buffers (each at 50 mM) were used: sodium acetate, pH 5.0; bis-Tris-HCl, pH 6.0; MOPS, pH 7.0; EPPS, pH 8.0; CHES (2-[*N*-cyclohexylamino]-ethanesulfonic acid), pH 9.0; and CAPS, pH 10.0. The assays were carried out at 100°C. For determination of the effects of temperature, the buffer used was 50 mM MOPS, pH 7.0. An N-prol activity level of 100% corresponds to 600 U/mg while 100% R-prol activity corresponds to 1,250 U/mg (with Met-Pro as substrate and measured at 100°C).

for both N- and R-prol included 1.2 mM Co^{2+}, and these ions could not be replaced with other divalent (Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, or Ni^{2+}) or monovalent (Na^+ or K^+) cations (no activity was detected), with the exception of Mn^{2+}. The effects of Co^{2+} and Mn^{2+} concentrations on the activities of N- and R-prol are shown in Fig. 4. The two enzyme forms showed very similar responses, with maximal activities at concentrations of 1.2 mM $CoCl_2$ and 1.6 mM $MnCl_2$, with the latter supporting approximately 75% of the activity of the former. However, both cations caused some inhibition when added above their optimal concentration (Fig. 4). The apparent association constants for Co^{2+} and Mn^{2+} were 0.24 and 0.62 mM for N-prol and 0.5 and 0.66 mM for R-prol, respectively. When N-prol was incubated with 1 mM Co^{2+} ions and then assayed in the absence of the metal, there was no difference in the specific activity (compared to standard conditions where Co^{2+} ions are included in the assay medium). However, when the sample was

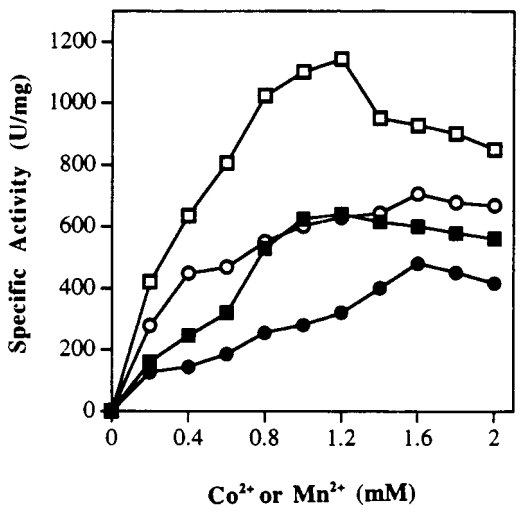

FIG. 4. The effects of Co^{2+} and Mn^{2+} ions on the activities of N- and R-prol. The assay mixtures contained 0.02 μg of N-prol (solid symbols) or R-prol (open symbols), Met-Pro (4 mM), and various concentrations of either $CoCl_2$ (squares) or $MnCl_2$ (circles).

TABLE 3. Substrate specificity of the native and recombinant forms of *P. furiosus* prolidase

Substrate[a]	Relative activity (%)[b]	
	N-prol	R-prol
Met-Pro	100	100
Leu-Pro	75	79
Val-Pro	46	10
Phe-Pro	25	24
Ala-Pro	23	17
Lys-Pro	4	10
Gly-Pro	0	1
Pro-Ala	0	0
Pro-Hydroxypro-Pro	0	0
Lys-Trp-Ala-Pro	1	0
Gly-Arg-Gly-Asp-Thr-Pro	0	0
Pro-Pro-Gly-Phe-Ser-Pro	1	0
Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0	0
N-acetyl Pro	0	0

[a] All substrates were used at a final concentration of 4 mM.
[b] The rate of hydrolysis is expressed as a percentage of the activity compared to that obtained at 100°C with Met-Pro as the substrate. An activity of 100% corresponds to 600 U/mg for N-prol and 1,350 U/mg for R-prol.

preincubated with 1 mM Co^{2+} and then dialyzed (against 3,000 volumes of 50 mM MOPS buffer, pH 7.0), only 5% of the activity remained. Addition of Co^{2+} ions (1.0 mM) to the dialyzed enzyme completely restored enzyme activity (assayed in the absence of Co^{2+} ions), but when EDTA (1 mM) was also added, no activity was detected. It therefore appears that the purified forms of both N- and R-prol contain one tightly bound Co^{2+} ion per subunit, but that one (or more) additional cation(s), which can be either Co^{2+} or Mn^{2+}, is required for activity.

Prolidase was identified by its ability to hydrolyze the dipeptide Met-Pro, and this substrate was used in all routine assays. The activities of N- and R-prol with other peptides are shown in Table 3. Both gave virtually identical results. They only hydrolyzed dipeptides with Pro at the C terminus (not the N terminus), and the nature of the N-terminal residue was critical, with significant activity occurring only with peptides containing nonpolar amino acids. Kinetic analyses were conducted using N-Prol with the five prolyl-containing dipeptides with which it showed significant activity (Table 3). All exhibited normal Michaelis-Menton-type kinetics, and the kinetic constants, shown in Table 4, were calculated from linear double-reciprocal plots. The affinities of the enzyme for Met-Pro and Leu-Pro, the two substrates with which it showed the highest k_{cat}/K_m values, are lower than might have been anticipated, but the k_{cat}/K_m values for all five dipeptides are not too dissimilar, suggesting perhaps that all are of physiological significance.

TABLE 4. Kinetic parameters for substrates of *P. furiosus* prolidase

Prolidase	Substrate[a]	K_m (mM)	V_{max} (μmol/min/mg)	k_{cat}[b] (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
N-prol	Met-Pro	2.8	645	271	97
	Leu-Pro	3.0	645	271	90
	Val-Pro	4.2	175	74	18
	Ala-Pro	8.3	250	105	13
	Phe-Pro	20.0	1,000	420	21
R-prol	Met-Pro	3.3	1,250	525	45

[a] All assays were carried out at 100°C in 50 mM MOPS, pH 7.0, containing 1.2 mM $CoCl_2$.
[b] Based on a minimum molecular mass of 39.4 kDa.

The affinity of R-prol for Met-Pro is comparable to that of the native enzyme (Table 4) although the k_{cat} value is more than twofold higher, consistent with the results obtained under standard assay conditions. The activities of the native and recombinant forms were not significantly affected when they were treated (at 25°C for 30 min, prior to assaying under standard conditions) with any of the following protease (thiol or serine) inhibitors (each at 1 mM final concentration): iodoacetate, ES-64 (L-transepoxysuccinyl-leucylamido (4-guanido) butane), *N*-ethyl maleimide, phenyl methane sulfonyl fluoride, or diisopropylphosphofluoride.

DISCUSSION

P. furiosus contains significant intracellular concentrations of prolidase, an enzyme that appears to hydrolyze only dipeptides that contain Pro at the C terminus and a nonpolar residue at the N terminus. This finding is consistent with the proteolytic nature of the organism, although the rather high K_m values (3 to 20 mM) determined for the enzyme's substrates suggest that such dipeptides must be present at significant intracellular concentrations in vivo. The gene encoding the enzyme was successfully expressed in *E. coli* although, surprisingly, the recombinant form had a higher specific activity than the native prolidase. Why this is the case is unclear since the molecular weight (as judged by SDS-gel electrophoresis), N-terminal amino acid sequence, activation by metal ions (Co^{2+} and Mn^{2+}), temperature and pH dependence, and substrate specificities of the two enzyme forms were indistinguishable. The fact that the recombinant form was slightly less thermostable than the native protein suggests that it may not be completely folded, and perhaps this additional flexibility leads to enhanced catalytic activity.

Kinetic and metal analyses indicated that *P. furiosus* prolidase (both native and recombinant) has at least two binding sites per subunit for Co^{2+} ions. One appears to be an integral part of the protein (and not removed by purification or dialysis) while the other(s) has an association constant of ~0.3 mM and is essential for catalysis. In this regard the *P. furiosus* enzyme resembles certain members of the broad class of binuclear metallohydrolases represented by the N-terminal exopeptidases, the active sites of which also contain two metal ions that typically differ in their exchange kinetics (50). Prototypical members of this family are bovine leucine aminopeptidase (15) and *Aeromonas proteolytica* aminopeptidase (20), each of which contains two Zn^{2+} ions per catalytic unit. The zinc atoms of the *A. proteolyticus* enzyme can be replaced in vitro with cobalt, and apparently like *P. furiosus* prolidase, this amino peptidase can be prepared containing just one metal ion per active site (7). The only naturally occurring, cobalt-dependent members of this enzyme class are the methionine aminopeptidases (4), and these contain two Co^{2+} ions per active site. The crystal structure of the *E. coli* enzyme (42) shows that the two cobalt ions are coordinated by five amino acid residues (Asp97, Asp108, His171, Glu204, and Glu235), and all five are conserved in the sequences of the other methionine aminopeptidases (16, 41, 47). Interestingly, although the amino acid sequence of *P. furiosus* prolidase shows no significant similarity with those of methionine aminopeptidases, all five of the cobalt-coordinating residues are conserved in the *P. furiosus* enzyme (Asp209, Asp220, His280, Glu313, and Glu327) (Fig. 5). Clearly, spectroscopic and structural analyses of the *P. furiosus* enzyme will be required to determine if it does, in fact, contain a binuclear cobalt site and if the site is analogous to that of the methionine aminopeptidase. Such studies are in progress. In this regard, the ability of *P. furiosus* prolidase to be activated by Mn^{2+} ions suggests that an active enzyme form containing a Co-Mn binuclear center should be possible, and this should facilitate the interpretation of spectroscopic data. On the other hand, both R- and N-prol also contained significant amounts of zinc. This appears to be nonspecifically bound, however, as Zn^{2+} ions did not support enzyme activity (in place of Co^{2+} or Mn^{2+} ions) and typical zinc-binding motifs, e.g., HEXXH (32), were not present in the sequence.

Database searches indicated that the amino acid sequence of *P. furiosus* prolidase showed significant similarity to the sequences of all known prolidases and to a putative prolidase in the genome sequence of the archaeon *Methanococcus jannaschii* (14). The *P. furiosus* enzyme showed overall similarities of 69, 61, 58, 56, and 53% with respect to the prolidases from *M. jannaschii*, *Lactobacillus delbrueckii*, *Haemophilus influenzae*, and *E. coli* and the human prolidase, respectively. In all of these enzymes, there are three extended regions of identity in the C terminus, YFXHXLGHXVGLEVHE (*P. furiosus* prolidase residues 277 to 292), GMVXTIEPGIY (residues 307 to 317), and GGVRIED (residues 322 to 328). These regions contain three of the five putative Co^{2+}-binding residues mentioned above for the *P. furiosus* enzyme and presumably form the active-site residues in all of these enzymes. Thus, of the five residues in *P. furiosus* enzyme that are proposed to bind Co^{2+} ions, all of them are conserved in the other prolidases (Fig. 5).

The prolidase from *P. furiosus* represents the first such enzyme to be purified from either an archaeon or a hyperthermophile. All other prolidases are from mesophilic sources (10, 12, 24, 25, 27, 46, 52) and are maximally active at temperatures up to 55°C. As might be expected, the *P. furiosus* enzyme is by far the most thermostable example of a prolidase, with a temperature optimum above 100°C and no loss of activity after 12 h at this temperature. Indeed, it is one of the most thermostable enzymes known of any type (1). Like the *P. furiosus* enzyme, the prolidases from *X. maltophilia* (46) and from eucaryotic sources (guinea pig brain [12], human erythrocytes [24], and bovine intestine [52]) are dimers (although their subunits are larger, 50 to 58 kDa), whereas the enzymes from *Lactobacillus lactis* (10) and *L. casei* (25) are monomers (~42 kDa). So far, however, *P. furiosus* prolidase is the only one that has been shown to contain cobalt as an integral part of the protein. The enzymes from *L. casei*, *X. maltophilia*, and human beings are activated by Mn^{2+} although their metal contents have not been reported, while it is not known if the prolidases from *Aureobacterium esteraromaticum* (27), *L. lactis*, guinea pig brain and bovine intestine contain a metal center or if they are activated by any metal ion. However, the high similarities in the sequences of these enzymes (Fig. 5), and the conservation of the five putative Co-binding residues found in *P. furiosus*, suggest that they all contain a similar and presumably binuclear metal center, even though the nature of the metal (Mn^{2+} or Co^{2+}) may not be the same.

On the other hand, there are differences in substrate specificities of the various prolidases. For example, the *P. furiosus* enzyme is the only one which utilizes only dipeptides with proline at the C (but not the N) terminus. The prolidases of *L. lactis* and *A. esteraromaticum* hydrolyze dipeptides with Pro at either the N- or C-terminal position, and the enzymes from *L. casei* and guinea pig brain efficiently cleave some dipeptides with no prolyl residue. Similarly, the mesophilic prolidases (with the exception of the *L. casei* enzyme) are inhibited by cysteine protease inhibitors such as *N*-ethyl maleimide and *p*-chloromercuribenzoate, suggesting that a reactive cysteine is required for catalysis. However, the *P. furiosus* enzyme was not inhibited by these reagents. Only one Cys residue is present in this prolidase, and this residue is not conserved in any of the

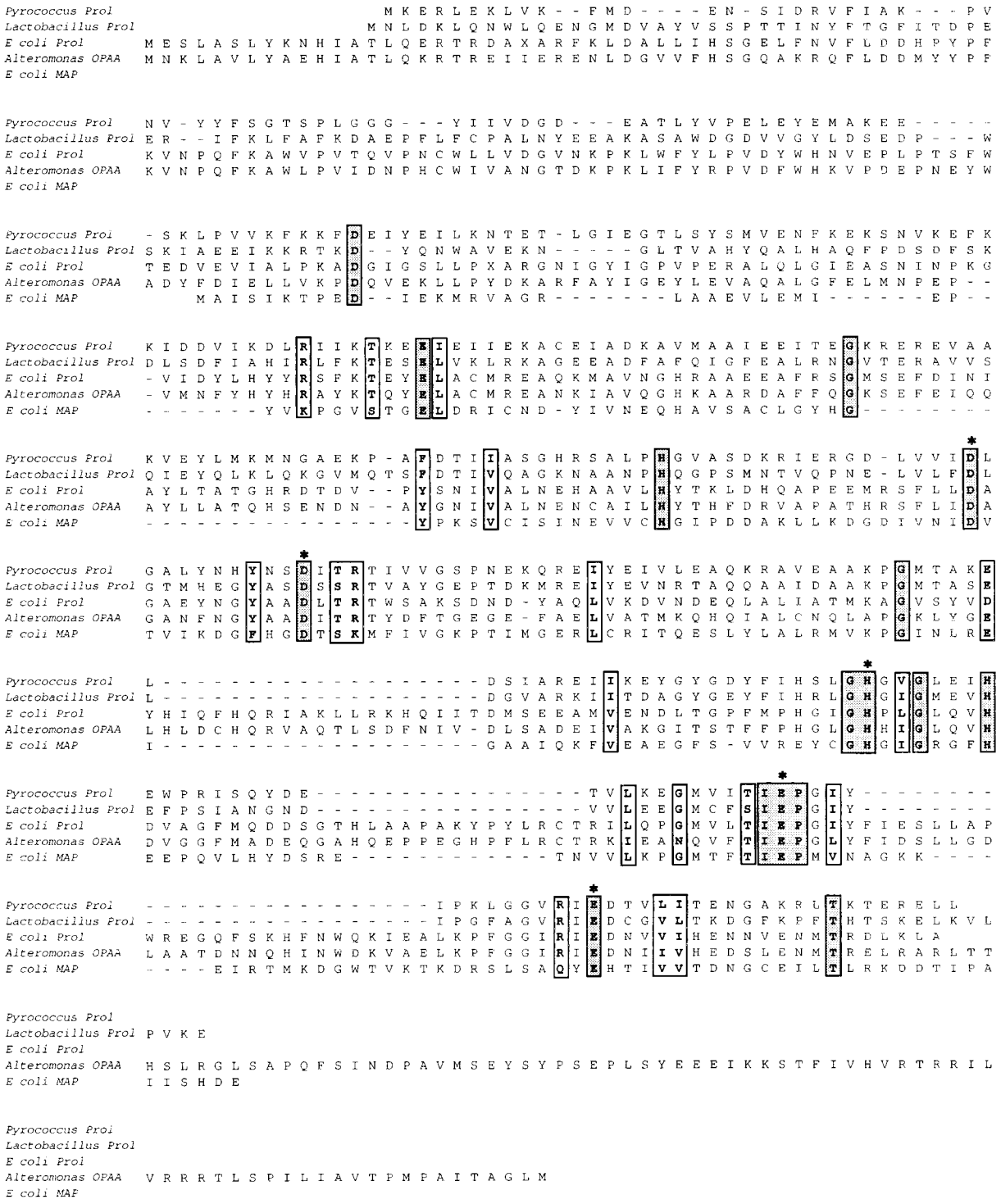

FIG. 5. Alignment of the amino acid sequence of *P. furiosus* prolidase with other prolidases (Prol), *Alteromonas* OPAA, and *E. coli* methionine aminopeptidase (MAP). The GenBank accession numbers for the prolidases other than the one sequenced in this work are as follows: P46545, *L. delbrueckii* prolidase; P15034, *E. coli* prolidase; U56398, *Alteromonas* OPAA; and P07906, *E. coli* methionine aminopeptidase. Identical residues are designated by the gray shading while similar residues are boxed. The five residues that are ligands to the binuclear cobalt site in the subunit of *E. coli* methionine aminopeptidase are indicated by asterisks.

mesophilic prolidases, which contain between 2 (*M. jannaschii*) and 12 (human) Cys residues per subunit. Thus, it would seem unlikely that a Cys residue is directly involved in the catalytic mechanism of any of these prolidases.

The only other enzyme with which the *P. furiosus* prolidase showed significant sequence similarity was that of OPAA from *Alteromonas* (51% similarity and 24% identity). This enzyme is capable of hydrolyzing a variety of toxic organophosphorus

compounds (17). Although OPAA is a monomeric enzyme, in contrast to dimeric *P. furiosus* prolidase, its activity is dependent upon Mn^{2+} ions. Moreover, the five residues proposed to coordinate the binuclear metal center in the *P. furiosus* enzyme are also conserved in the sequence of OPAA (Fig. 4). Thus, while OPAA has a broad substrate specificity and is capable of hydrolyzing P-F, P-C and P-O bonds (17), it exhibits comparable activity with prolidase-type dipeptides such as Leu-Pro and Ala-Pro. Furthermore, like the *P. furiosus* enzyme, it does not hydrolyze tri- or tetrapeptides or dipeptides with Pro at the N terminus (18). Clearly, these two enzymes are closely related, and the effectiveness of the *P. furiosus* enzyme in degrading organophosphorus compounds is currently being explored.

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