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Purification and characterization of a cobalt-activated carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus*

TIMOTHY C. CHENG,[1] VIJAY RAMAKRISHNAN,[1] AND SUNNEY I. CHAN[1,2]

[1]Noyes Laboratories 127-72, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125
[2]Institute of Chemistry, Academia Sinica, Taipei, 11529 Taiwan

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Abstract

A novel metallocarboxypeptidase (PfuCP) has been purified to homogeneity from the hyperthermophilic archaeon, *Pyrococcus furiosus*, with its intended use in C-terminal ladder sequencing of proteins and peptides at elevated temperatures. PfuCP was purified in its inactive state by the addition of ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) to purification buffers, and the activity was restored by the addition of divalent cobalt ($K_d = 24 \pm 4$ μM at 80 °C). The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) had no effect on the activity. The molecular mass of monomeric PfuCP is 59 kDa as determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 58 kDa by SDS-PAGE analysis. In solution, PfuCP exists as a homodimer of ~128 kDa as determined by gel filtration chromatography. The activity of PfuCP exhibits a temperature optimum exceeding 90 °C under ambient pressure, and a narrow pH optimum of 6.2–6.6. Addition of Co^{2+} to the *apo*PfuCP at room temperature does not alter its far-UV circular dichroism (CD) or its intrinsic fluorescence spectrum. Even when the CoPfuCP is heated to 80 °C, its far-UV CD shows a minimal change in the global conformation and the intrinsic fluorescence of aromatic residues shows only a partial quenching. Changes in the intrinsic fluorescence appear essentially reversible with temperature. Finally, the far-UV CD and intrinsic fluorescence data suggest that the overall structure of the holoenzyme is extremely thermostable. However, the activities of both the *apo* and holo enzyme exhibit a similar second-order decay over time, with 50% activity remaining after ~40 min at 80 °C. The N-blocked synthetic dipeptide, N-carbobenzoxy-Ala-Arg (ZAR), was used in the purification assay. The kinetic parameters at 80 °C with 0.4 mM $CoCl_2$ were: K_m, 0.9 ± 0.1 mM; V_{max}, 2,300 ± 70 U mg^{-1}; and turn over number, 600 ± 20 s^{-1}. Activity against other ZAX substrates (X = V, L, I, M, W, Y, F, N, A, S, H, K) revealed a broad specificity for neutral, aromatic, polar, and basic C-terminal residues. This broad specificity was confirmed by the C-terminal ladder sequencing of several synthetic and natural peptides, including porcine N-acetyl-renin substrate, for which we have observed (by MALDI-TOF MS) stepwise hydrolysis by PfuCP of up to seven residues from the C-terminus: Ac-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser.

Keywords: C-terminal sequencing; carboxypeptidase; extremozyme; hyperthermophile; *Pyrococcus furiosus*

Hyperthermophiles are microorganisms which grow optimally at temperatures 80 °C and above (Huber & Stetter, 1998). A majority of these organisms fall under the newly identified domain, *archaea* (Woese et al., 1990). One of the most extensively studied hyperthermophilic archaea is *Pyrococcus furiosus* (Pfu). This microorganism has a growth optimum of 100 °C (Fiala & Stetter, 1986). It is a heterotrophic, anaerobic archaeon, and utilizes complex carbohydrates and peptides/proteins as carbon and energy sources (Adams & Kletzin, 1996). The metabolic end products are organic acids, alanine, CO_2, and H_2 (Kengen & Stams, 1994; Kengen et al., 1996). Additional energy is generated when excess redox equivalents are channeled to elemental sulfur (Schicho et al., 1993).

Not surprisingly, most of the proteins isolated from these hyperthermophiles exhibit a temperature optimum of at least 80–100 °C or above (Adams et al., 1995; Adams & Kelly, 1998). Accordingly, there is much interest in exploiting these proteins for biotechnological applications, as they are able to perform biochemical reactions under harsh conditions, such as high temperatures, organic solvents, and denaturants (Adams et al., 1995). *P. furiosus*

Reprint requests to: Sunney I. Chan, Noyes Laboratories 127–72, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125; e-mail: chans@cco.caltech.edu.

Abbreviations: Pfu, *Pyrococcus furiosus*; Pho, *Pyrococcus horikoshii i*; CP, carboxypeptidase; DT, dithionite; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; ZAR, N-Cbz-Alanyl-Arginine; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; UV-CD, ultraviolet circular dichroism; KMES, potassium 2-[N-morpholino]ethanesulfonic acid; HEPES, (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]); MOPS, (3-[N-morpholino] propanesulfonic acid); NARS, N-acetyl-renin substrate; PMSF, phenylmethylsulfonyl fluoride; TIM, triose phosphate isomerase; TFA, trifluoroacetic acid.

has been the source of many of these biotechnologically important proteins, including DNA polymerase (*Pfu* polymerase) (Lundberg et al., 1991), α-amylase (Laderman et al., 1993), and proteases (Voorhorst et al., 1996; Harwood et al., 1997).

Carboxypeptidases sequentially hydrolyze peptide bonds from the C-terminus of proteins and peptides. They are ubiquitous in animals, plants, and microorganisms; many carboxypeptidases have been characterized based on their substrate specificity and mechanism (serine- vs. metallo-carboxypeptidase) (Skidgel & Erdos, 1998). Carboxypeptidases have been implicated in physiological roles such as protein degradation/turnover, processing of precursor proteins (Steiner, 1998), and in the metabolism of proteins and peptides as carbon or energy sources. Most of the purified carboxypeptidases have a temperature optimum below 40 °C. However, three moderately thermostable carboxypeptidases have been purified from the bacteria *Thermoactinomyces vulgaris* (Stepanov, 1995) and *Thermus aquaticus* (Lee et al., 1992), and the archaeon *Sulfolobus solfataricus* (Colombo et al., 1992), with temperature optima of 60, 80, and 85 °C, respectively. Carboxypeptidase activity has not yet been reported from *P. furiosus*.

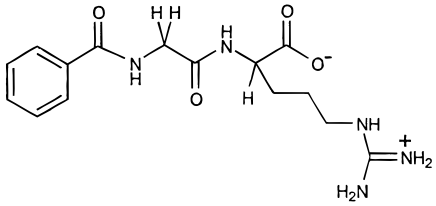
Protein sequencing is an integral component of modern biochemical research. Edman degradation is useful for N-terminal sequencing, but it fails when the amino terminus is chemically protected. Aside from endoproteolytic fragmentation, another way to obtain sequence information from proteins is to sequence from the C-terminus. Various C-terminal sequencing methods have been developed: chemical cleavage analogous to Edman degradation (Hardeman et al., 1998) and enzymatic digestion by carboxypeptidases (Thiede et al., 1997). A particularly powerful approach is enzymatic ladder sequencing, in which a carboxypeptidase is used to generate a set of differentially cleaved peptides that can be visualized in a mass spectrum; mass differences between adjacent peaks correspond to the molecular masses of individual amino acids that have been released. Enzymatic protein ladder sequencing has the potential to sequence as far as the enzyme can cut. However, a number of difficulties have limited the applicability of this approach: (1) the limited specificities of a given carboxypeptidase toward the 20 common amino acids and (2) the resistance of native protein molecules to digestion at mesophilic temperatures. Both of these problems can be overcome by the use of a thermostable carboxypeptidase in C-terminal sequencing of proteins. First, a hyperthermophilic carboxypeptidase is catalytically active at temperatures where the tertiary structures of most mesophilic proteins are denatured. Second, there is evidence that specificities of thermophilic carboxypeptidases are broader than their mesophilic counterparts (Colombo et al., 1992; Lee et al., 1992; Stepanov, 1995).

In this paper, we report the purification and characterization of a novel metallocarboxypeptidase from *P. furiosus*. The activity of the PfuCP shows a temperature optimum exceeding 90 °C; accordingly, this PfuCP might find applications in C-terminal sequencing of peptides and proteins. The first C-terminal sequencing by a hyperthermophilic carboxypeptidase at elevated temperatures is demonstrated here.

Results and discussion

Purification

Initial studies on substrate hydrolysis showed that PfuCP was active toward hippuryl-arginine (N-benzoyl-Gly-Arg, Scheme 1A), the substrate that was used to isolate a carboxypeptidase from



hippuryl arginine

Scheme 1A.

S. solfataricus (Colombo et al., 1992). However, the Bz-Gly bond was susceptible to hydrolysis as evidenced by activity toward hippuric acid (Bz-Gly). Hence, an alternate set of substrates (ZAX) with a stable N-terminal amide bond was chosen to monitor activity. A control experiment showed no hydrolysis of ZA (N-Cbz-Ala), thus localizing PfuCP hydrolysis of ZAR to the C-terminal alanyl-arginine peptide bond, unequivocally demonstrating carboxypeptidase activity.

Preliminary small-scale experiments were performed to standardize the purification conditions, during which it was noted that PfuCP did not bind to the affinity matrix arginine-Sepharose. To test for the presence of multiple carboxypeptidases, *P. furiosus* crude extract was passed through a QFF column and then the eluting carboxypeptidase activity was monitored with various ZAX substrates. Maximum activity was found in the same fraction for all of the substrates tested, suggesting that there is one distinct carboxypeptidase in *P. furiosus*. All activities of representative ZAX substrates (aliphatic, X = Ile; basic, X = Arg and Lys; aromatic, X = Phe and Trp; polar, X = Asn, Ser, and His) eluted in the same major peak. Significant loss of activity was observed in purification fractions over the course of 3–4 days at room temperature. Preliminary studies had shown that DTT and EDTA completely abolished carboxypeptidase activity in crude extract. To inhibit proteolytic and carboxypeptidase activity, 2 mM DTT, 1 mM EDTA, and 1 mM PMSF were added to purification buffers, hence PfuCP was purified in a metal-depleted, catalytically inactive form. Other observed losses in activity due to freeze-thawing of purification samples were prevented by the addition of 10% glycerol to samples stored at −20 °C. Subsequent large-scale purification steps involving anion-exchange, hydroxyapatite, hydrophobic and gel filtration chromatographic procedures yielded 11 mg PfuCP from 21 g crude protein with a 174-fold purification and 9% recovery (Table 1). SDS-PAGE of the most active fractions in each purification step is shown in Figure 1.

Molecular weight and oligomeric state of PfuCP

Reducing and denaturing SDS-PAGE analysis (2 mM DTT and 80 °C pre-incubation for 1 min) showed a single band for purified monomeric PfuCP at a molecular mass of 58 kDa (Fig. 1). However, without heating at 80 °C, PfuCP exists in two homodimeric forms (D_1, D_2 at ~85 kDa) of slightly different apparent molecular masses, both in the presence and absence of DTT (Fig. 2A, lanes 1 and 3). D_1 appears to be a DTT-sensitive dimeric form. When purified PfuCP is heated at 80 °C in the presence of 2 mM DTT, D_1 is monomerized along with D_2 (Fig. 2A, lane 2). However, when purified PfuCP is heated at 80 °C in the absence of DTT, D_1

Table 1. *Purification of PfuCP from P. furiosus*

Purification step	Total protein (mg)	Units (U mL^{-1})	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude extract	21,404	171,250	8	1	100
Q FastFlow	2,398	110,862	46	6	65
Hydroxyapatite	750	99,696	133	17	58
Butyl Sepharose	183	82,765	452	57	48
Q high performance	14	15,831	1,131	141	9.2
Superdex	13	16,941	1,303	163	9.9
Mono-Q	11	15,306	1,391	174	8.9

remains intact while D_2 is monomerized to the 58 kDa band (Fig. 2A, lane 4). Interestingly, PfuCP is not expected to have cysteines (hence disulfides) based on its close homology to a putative, cysteine-less carboxypeptidase from the genome of the hyperthermophilic archaeon, *P. horikoshii* (see below). One possible explanation for the DTT-sensitive dimeric form is that residual metal ions in the purification buffers may act to stabilize D_1; this stabilization is destroyed when DTT acts to chelate these ions. When PfuCP is heated at 80 °C either in the presence of 1 mM EDTA or following treatment of the enzyme by EDTA then ultrafiltration in Chelex 100-treated buffers, D_1 was observed to monomerize.

Gel filtration at room temperature confirmed a dimeric state of approximate molecular weight 128 kDa for PfuCP in the presence or absence of DTT (data not shown); however, D_1 could not be distinguished from D_2. MALDI-TOF MS showed a predominantly monomeric PfuCP at 59 kDa with a trace of dimer at 118 kDa, in the presence or absence of DTT (Fig. 2B). Monomerization may have been induced by the conditions of the MALDI-TOF MS experiment (e.g., laser irradiation, organic solvent in the matrix solution). N-terminal sequencing suggests a homogeneous preparation of purified PfuCP.

Molecular mass characterization studies on several hyperthermophilic proteins have shown that self-association (into higher states) is a ubiquitous phenomenon. Phosphoribosyl anthranilate isomerase (tPRAI) from *Thermotoga maritima* (Sterner et al., 1996) and 3-phosphoglycerate kinase (PGk) from *P. furiosus* and *Methanothermus fervidus* (Hess et al., 1995) exist as homodimers, whereas they are monomeric in mesophiles. Similarly, triose phosphate isomerase (TIM) is a tetramer in *P. furiosus*, but a dimer in all mesophiles (Kohloff et al., 1996). Furthermore, ornithine carbamoyltransferase (OTCase) from *P. furiosus* exists as a dodecamer while the mesophilic OTCases isolated to date have been trimeric (Legrain et al., 1997). These results have led to suggestions that quaternary structure in hyperthermophilic proteins may play an important role in thermostability. Additional structural investigations are underway to determine if this is the case for PfuCP.

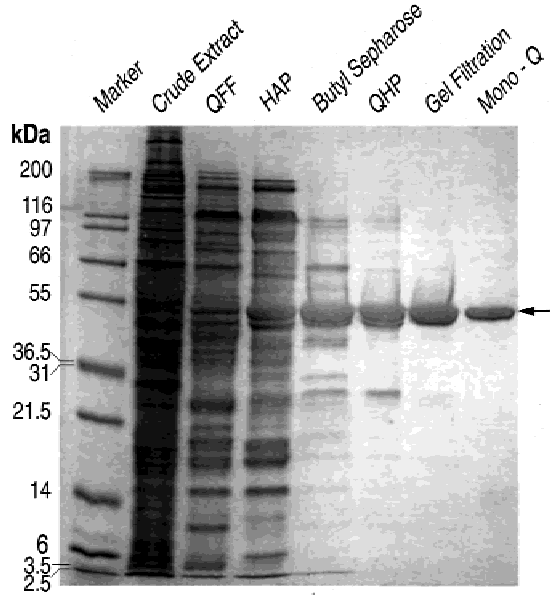


Fig. 1. Purification of PfuCP from *P. furiosus* crude extract. The fractions containing maximum activity from each purification step were analyzed by denaturing and reducing SDS-PAGE. Samples were pre-incubated at 80 °C for 1 min in 0.1% SDS and run on a 12% Tris-glycine gel.

Sequence analysis

Edman sequencing of the N-terminus of PfuCP yielded 30 residues (Pfu-N), while sequencing of two Endo-Lys C peptide fragments yielded two internal amino acid sequences of 32 and 16 residues, respectively (Pfu-I and Pfu-II) (Fig. 3). A BLAST search of all three sequences revealed no significant homology to any known entries in protein databases. However, all three fragments matched with 90–100% sequence similarity to a putative carboxypeptidase sequence (PhoCP) from the genome of *P. horikoshii* (Kawarabayasi et al., 1998). No potential glycosylation sites were found. Comparison of the PfuCP N-terminal sequence with that of the deduced PhoCP shows that PfuCP may also have a pro-sequence of 16 residues. This pro-sequence does not correspond to any of the currently known membrane-spanning or secretory signal sequences. PfuCP does not contain this sequence, which suggests that it undergoes post-translational processing. Studies on the pyrolysin from *P. furiosus* showed the occurrence of a 26-residue signal sequence (Voorhorst et al., 1996). Interestingly, pyrolysin is converted from a high molecular weight form (150 kDa) to a low molecular weight form (130 kDa) by autodigestion; this type of maturation is not likely in PfuCP as the molecular weight of PfuCP corresponds well with the size of the deduced PhoCP.

In the *T. aquaticus* carboxypeptidase, an HEXXH motif (where X is a nonconserved amino acid) has been found at positions 276–280, and both histidines have been determined to be catalytically

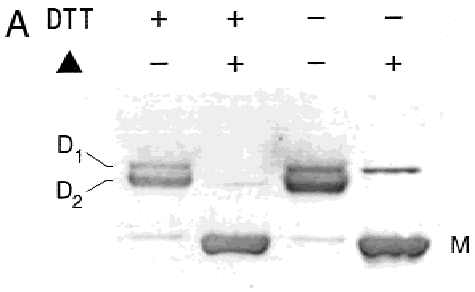
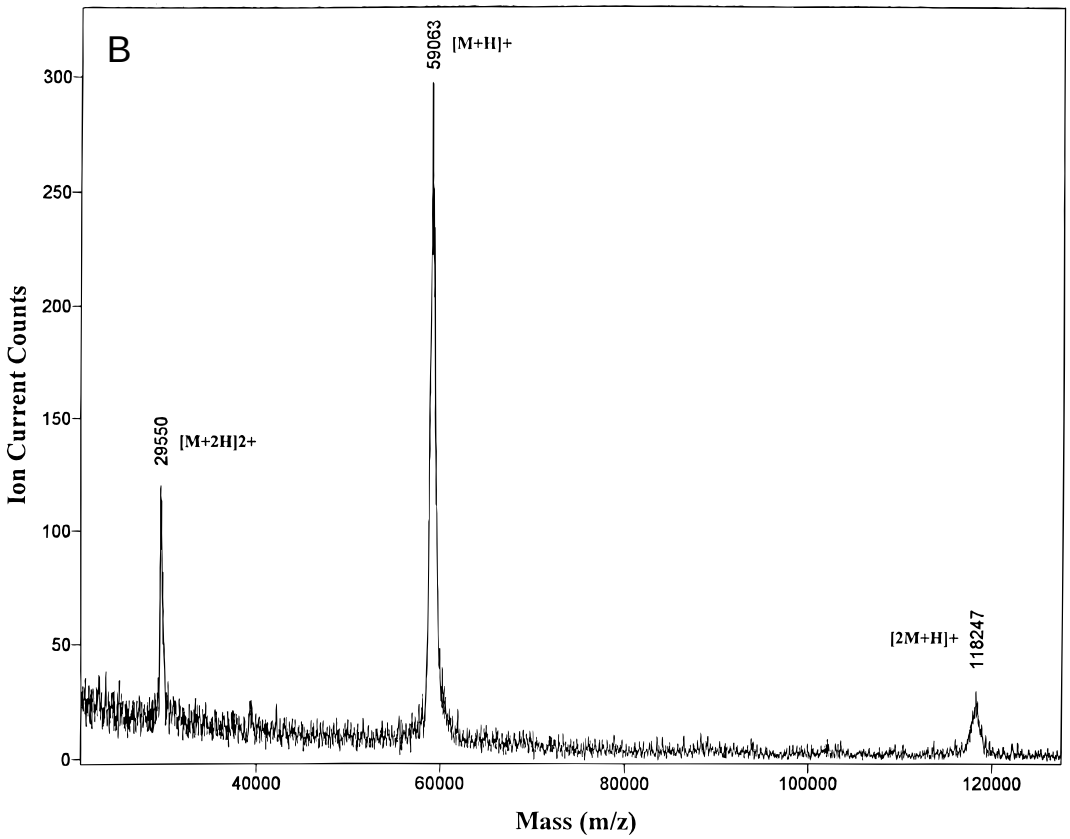


Fig. 2. Oligomeric behavior of PfuCP. **A:** Purified *apo*PfuCP was examined by SDS-PAGE under reducing or nonreducing conditions (in the presence or absence of 2 mM DTT), with or without preheating (Δ) at 80 °C. Dimer bands, D_1 and D_2, appear at an apparent molecular mass of 85 kDa while monomeric PfuCP (M) appears at 58 kDa. **B:** Purified enzyme was exchanged twice with deionized water by ultrafiltration and then analyzed by MALDI-TOF MS in the presence or absence of 2 mM DTT.



relevant by mutagenesis and Zn^{2+} binding studies (Lee et al., 1996). As the HEXXH motif typically occurs in endopeptidases and aminopeptidases, the carboxypeptidase from *T. aquaticus* was proposed to be the first member of a new class of carboxypeptidases. The presence of the same HEXXH motif in a similar position in PhoCP (residues 285–289) suggests that PfuCP may represent the second member of this new class of carboxypeptidases. Cloning and expression studies of the PfuCP gene are underway to further examine these issues.

Metal ion reactivation

Although the buffers used in these metal activation studies were passed through a Chelex 100 column and the enzyme treated with up to 50 mM EDTA followed by ultrafiltration, the "*apo*PfuCP" exhibited a consistent residual activity (~200 U mg^{-1}) prior to the addition of Co^{2+}. These observations suggest that there may be one or more tightly bound catalytically essential metal ions that are not lost during purification or EDTA treatment; thus, *apo*PfuCP is taken to mean metal-depleted enzyme. Re-activation of *apo*PfuCP by Co^{2+} followed simple hyperbolic behaviour (Fig. 4A). When the background activity of 200 U mg^{-1} was subtracted from the data, a linear reciprocal plot gave a binding constant (K_d) at 80 °C of 24 ± 4 μM for Co^{2+} and is consistent with binding to a single site on the enzyme (Fig. 4B). PfuCP activity was not observed in the presence of either 1 mM EDTA or 2 mM DTT. Even after extended incubation (up to 24 h), the inhibition was found to be fully reversible upon 50-fold dilution and re-activation by Co^{2+}. The serine protease inhibitor PMSF had no effect on activity at a concentration of 1 mM. Thus, PfuCP is a metallocarboxypeptidase. Similar to *apo*PfuCP, *apo*carboxypeptidase purified from *T. aquaticus* is activated by Co^{2+} and not by Zn^{2+} (Lee et al., 1992). However, when the *T. aquaticus* carboxypeptidase was expressed in *Escherichia coli*, it was found to contain one g atom of Zn^{2+}

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Pfu-N          ..............MEEVFQNETIKQILAKYRRIWAIGHAQRVL
                              ||||||.|:|||:||.|||||||:||||.||
Pho          1 METVLRRLHLEKSGETMEEVFRNDTIKEILQKYRRIWALGHAQSVL 46

Pfu-I          EGILERSVAQGELSVLSHELLLHPEFVNLVEK
               |||||||||||||||||:|||||.||||:||||
Pho         57 EGILERSVAQGELSVLSQELLLKPEFVELVEK 88

Pfu-II         AIGEDMDAEYFVRWVK
               |||||::|||||||:|
Pho        496 AIGEDVNAEYFVRWIK 511
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Fig. 3. Amino acid sequence analysis of PfuCP. The amino acid sequences of the N-terminus (Pfu-N) and two Endo-Lys C fragments of PfuCP (Pfu-I and Pfu-II) were aligned with the sequence of the putative *P. horikoshii* carboxypeptidase (PhoCP) using the GAP program from GCG software v.8. The gap weight was 3.0 and the extension penalty was 0.1. The N-terminal extension of the PhoCP gene product is also shown.

mol^{-1} enzyme (Lee et al., 1994). Further studies are underway to examine the nature of metal ion site(s) in native PfuCP and the effects of other divalent metal ions on activity.

Optimal temperature and pH range for activity

The activity of PfuCP exhibits a linear temperature dependence from 40–100 °C (Fig. 5A), and the optimum temperature observed is 90–100 °C. The activation energy (E_a) and Q_{10} value calculated from activity data between 59 and 93 °C were 47.8 $kJ \cdot mol^{-1}$ and 1.19, respectively (Fig. 5B). Activity was not measured above 100 °C, and no activity was observed at temperatures less than 40 °C under standard conditions. However, C-terminal ladder sequencing studies showed that PfuCP is active against peptide substrates at room temperature, suggesting that the ninhydrin assay was simply not sensitive enough to detect low rates of substrate turnover. The carboxypeptidases reported from *S. solfataricus* and *T. aquaticus* have temperature optima at 85 and 80 °C, respectively (Colombo et al., 1992; Lee et al., 1992). Thus, PfuCP has the highest optimal temperature for activity of any carboxypeptidase purified to date.

Unlike the hydrogenase (Bryant & Adams, 1989) from *P. furiosus* that exhibits an abrupt transition in the temperature profile from 60–70 °C, the linear temperature dependence of PfuCP activity suggests that the enzyme does not undergo drastic conformational changes as the temperature is increased. Such linear temperature dependence has been observed with other proteolytic enzymes purified from *P. furiosus*, including prolyl endopeptidase (Harwood et al., 1997) and prolidase (Ghosh et al., 1998). This reflects the mechanistic simplicity of proteases in *P. furiosus* in comparison to its other enzymes. Furthermore, the pH optimum for PfuCP activity is between 6.2–6.5 using the substrate ZAR in the standard assay; the activity decreases sharply on either side of the pH profile (Fig. 5C).

Structural thermal stability

At room temperature, both the intrinsic fluorescence intensity and far-UV CD spectrum of PfuCP remained unchanged following the

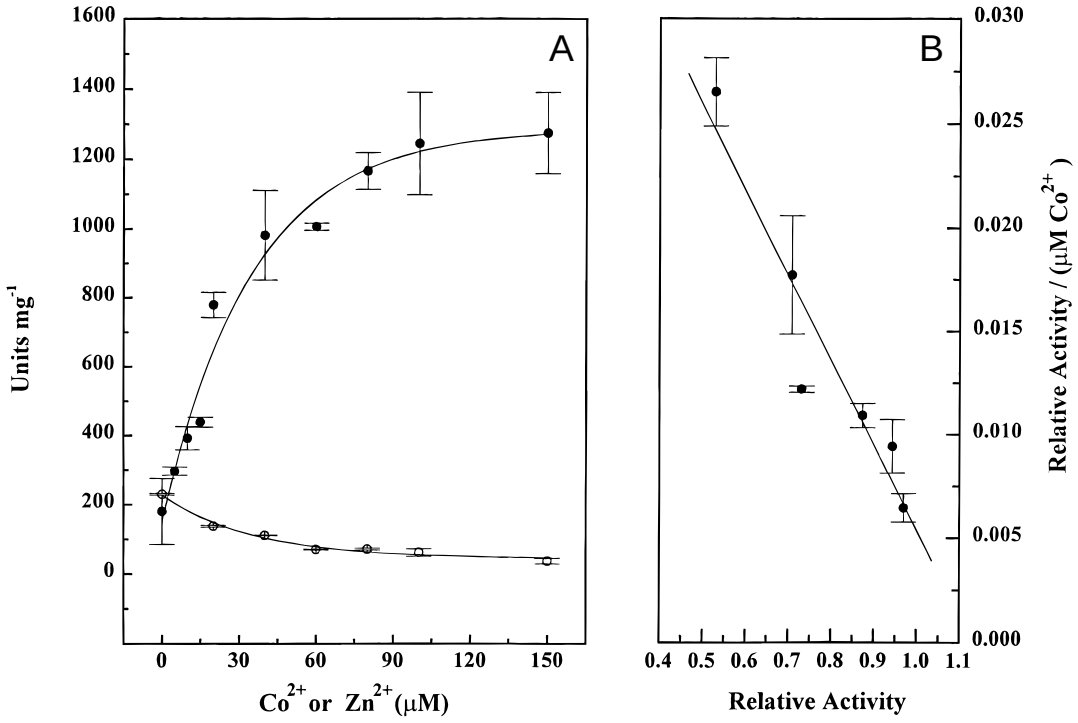


Fig. 4. Divalent metal ion dependence of PfuCP. **A:** Activity assays were performed under standard conditions while the concentration of reconstituting divalent metal ion was varied: Co^{2+} (●); Zn^{2+} (○). Samples were pre-incubated on ice with metal ions for 1 h. Data shown represent the average of duplicate values. **B:** Linear reciprocal plot of binding data for Co^{2+}. Error limits represent one standard deviation.

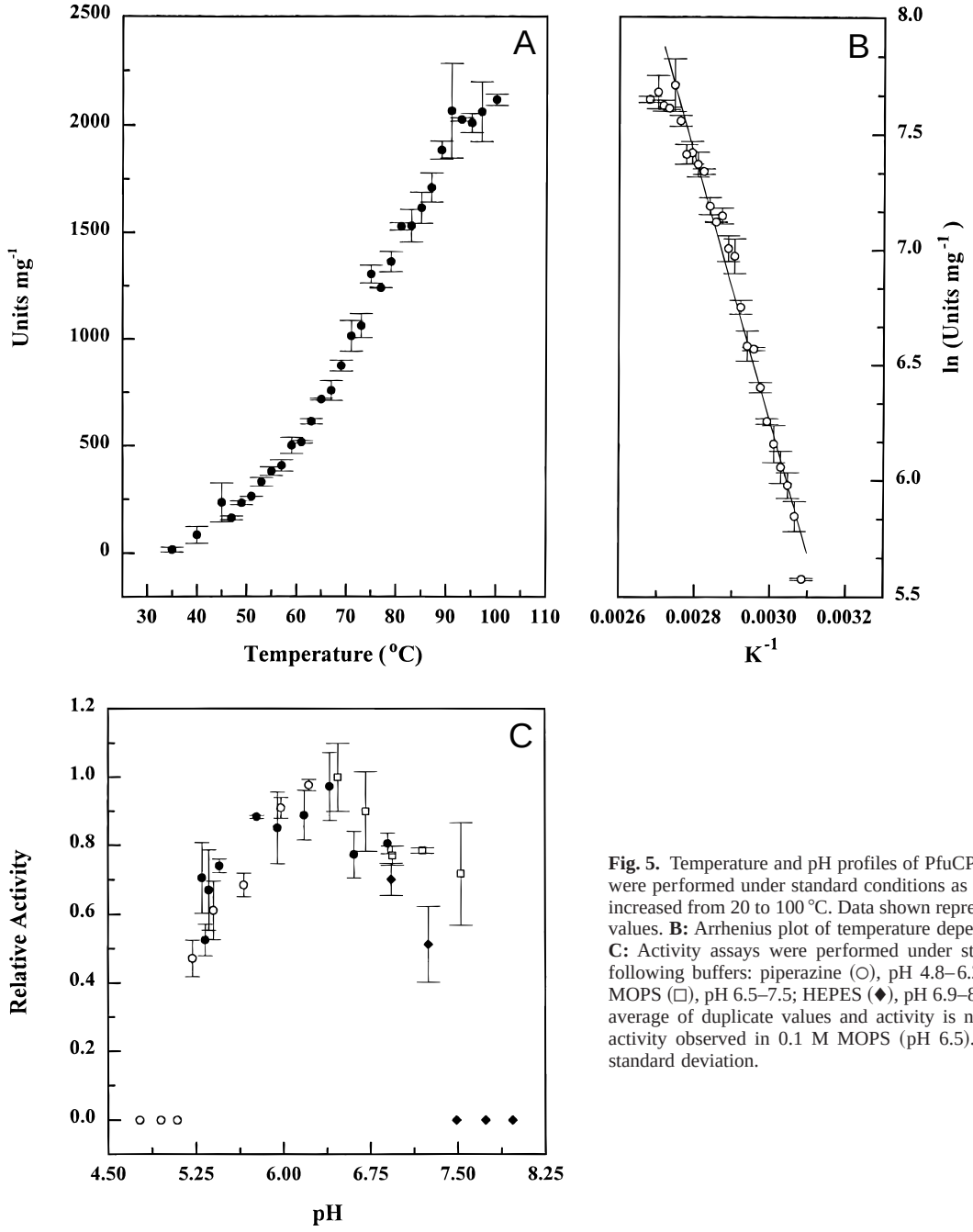


Fig. 5. Temperature and pH profiles of PfuCP activity. **A:** Activity assays were performed under standard conditions as the sample temperature was increased from 20 to 100 °C. Data shown represent an average of duplicate values. **B:** Arrhenius plot of temperature dependence data from 59–93 °C. **C:** Activity assays were performed under standard conditions with the following buffers: piperazine (○), pH 4.8–6.2; KMES (●), pH 5.3–6.9; MOPS (□), pH 6.5–7.5; HEPES (♦), pH 6.9–8.0. Data shown represent an average of duplicate values and activity is normalized to the maximum activity observed in 0.1 M MOPS (pH 6.5). Error limits represent one standard deviation.

addition of 1 mM Co^{2+} to *apo*PfuCP, suggesting that the binding of Co^{2+} had no significant effect on either global secondary structures or on local conformations of residues at the metal binding site (Figs. 6, 7A). Carboxypeptidase A, a mesophilic metallocarboxypeptidase, also shows little difference between the native Zn^{2+} containing enzyme and its *apo* form, the only difference being that the metal ligand, His196, in the apoenzyme is rotated 110° about χ^2 to salt bridge with Glu270, another catalytically essential residue (Feinberg et al., 1993). As the temperature was increased to 85 °C, the far-UV CD spectrum of PfuCP remained essentially the same in terms of its overall features, with only a slight decrease in negative ellipticity from 205–230 nm. However, the intensity of the intrinsic fluorescence decreased linearly with increasing temperature (Δ −17 °C^{-1}) without a noticeable shift in the wavelength of the emission maximum at 344 nm (Fig. 7B). This gradual quenching of the fluorescence follows the observed linear temperature dependence of activity (see above), indicating a smooth change in activity as opposed to a "switch" operating at a threshold temperature. In hyperthermophilic enzymes, a gradual increase in activity with temperature has been correlated to increased flexibility in the overall structure (Laderman et al., 1993). In addition, the quenching of fluorescence is essentially reversed upon cooling to room temperature (data not shown). The invariance in CD spectra and reversible quenching of intrinsic fluorescence suggest that PfuCP is structur-

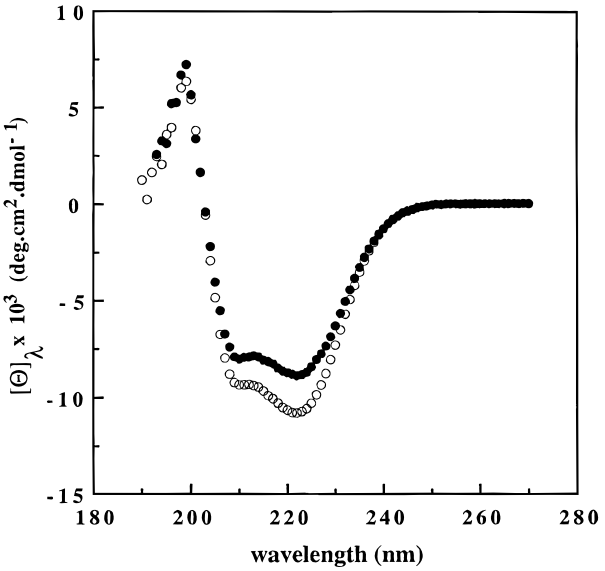


Fig. 6. Far-UV CD spectra of PfuCP. Potential changes in the secondary structure of the purified PfuCP were monitored in 10 mM potassium phosphate buffer (pH 7.0). Spectra were recorded for the following: (1) *apo*PfuCP at room temperature (○); (2) the Co^{2+}-incubated sample at 80 °C (●). Concentration of PfuCP was 15 μM. The spectra had two ellipticity minima at 222 and 210 nm, and a maximum at 199 nm.

ally thermostable in the presence of Co^{2+}. Studies are underway to characterize the thermal stability and folding of the *apo*PfuCP.

Thermal inactivation

In contrast to the apparent structural thermostability of Co^{2+}-reconstituted PfuCP, the enzymatic activity showed an irreversible second-order decay during incubation at 80 °C; 50% activity remained after ~40 min and the second order decay constant was $2.0 \times 10^{-5} \pm 2 \times 10^{-6}$ s^{-1} mg U^{-1} (Fig. 8). The mechanism of inactivation remains unclear. However, the kinetics do not appear to be first order, which might be expected if the rate-determining step of activity loss were solely "metal-ion escape" from the binding site or oxidation of Co(II) to Co(III). Furthermore, the activity decay of CoPfuCP (data not shown) is essentially identical to that of the *apo*PfuCP (Fig. 8). It is also possible that in vivo, PfuCP is stabilized by solutes such as *cyclic*-2,3-diphosphoglycerate in methanogens (Hensel & Konig, 1988) or di-*myo*-inositol-1,1′-phosphate and mannosyl glycerate in *P. furiosus* (Martins & Santos, 1995). PfuCP heated at 80 °C in the presence of 0.4 mM Co^{2+} for an hour shows no obvious fragmentation or autoproteolysis as determined by MALDI-TOF MS and SDS-PAGE (data not shown). Future experiments, including electronic absorption spectroscopy under anaerobic conditions, will explore the nature of this slow but gradual inactivation.

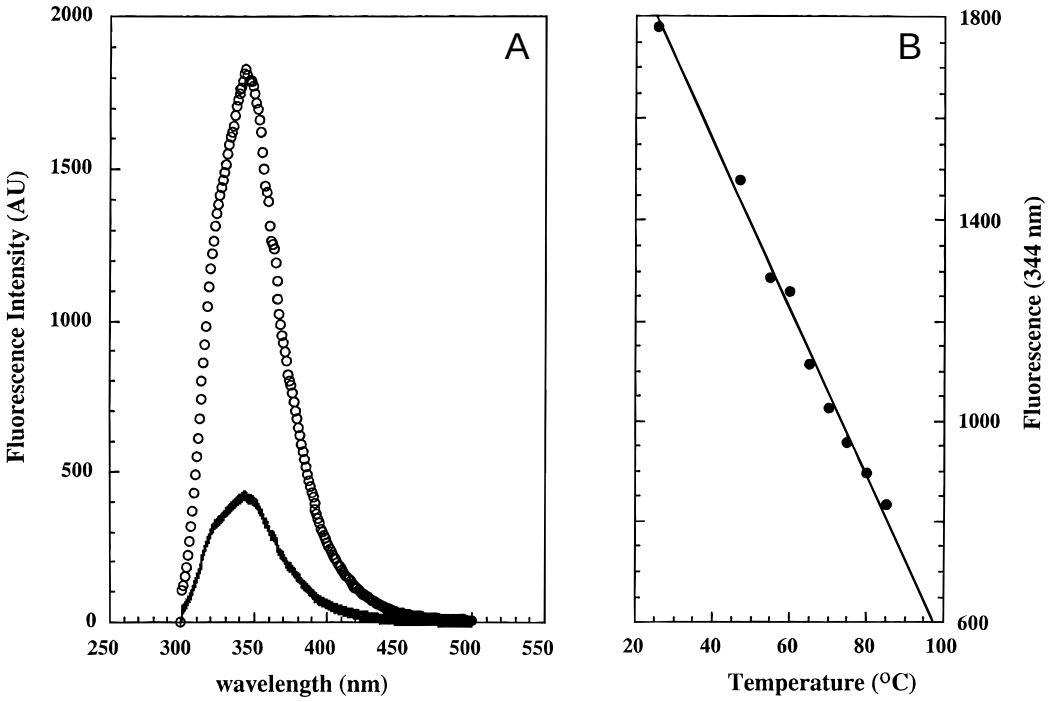


Fig. 7. Intrinsic fluorescence of PfuCP. **A:** Comparison of emission spectra at 22 and 80 °C. The following fluorescence spectra were recorded: (1) *apo*PfuCP at 22 °C (○); (2) the Co^{2+}-incubated sample at 80 °C for 5 min (|). **B:** Fluorescent intensity as a function of temperature. Emission spectra were also recorded for the Co^{2+}-incubated sample at the following temperatures (°C): 22, 47, 55, 60, 65, 70, 75, 80, and 85. The maximum excitation and emission wavelengths were 290 and 344 nm, respectively, and the bandwidth was 8 nm. Concentration of PfuCP for all fluorescence experiments was 15 μM.

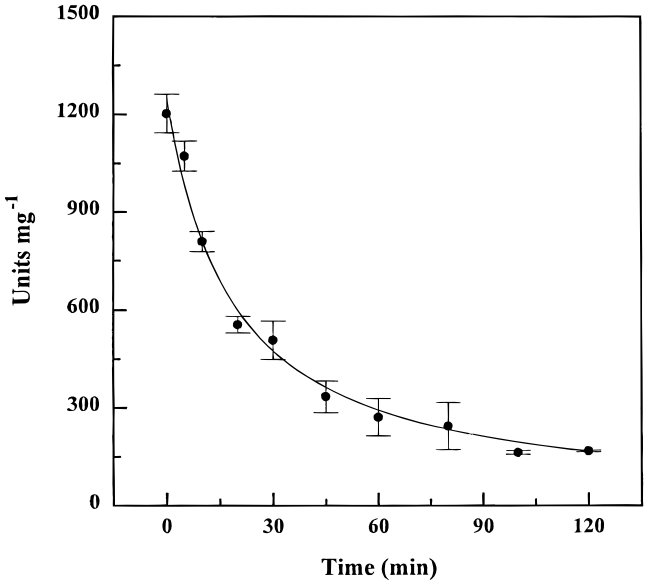


Fig. 8. Thermal inactivation of a*po*PfuCP. *Apo*PfuCP (730 nM in 0.1 M KMES, pH 6.5) was pre-incubated at 80 °C for various time points and then residual activity was assayed under standard conditions. Data points represent the average of duplicate values. Error limits represent one standard deviation.

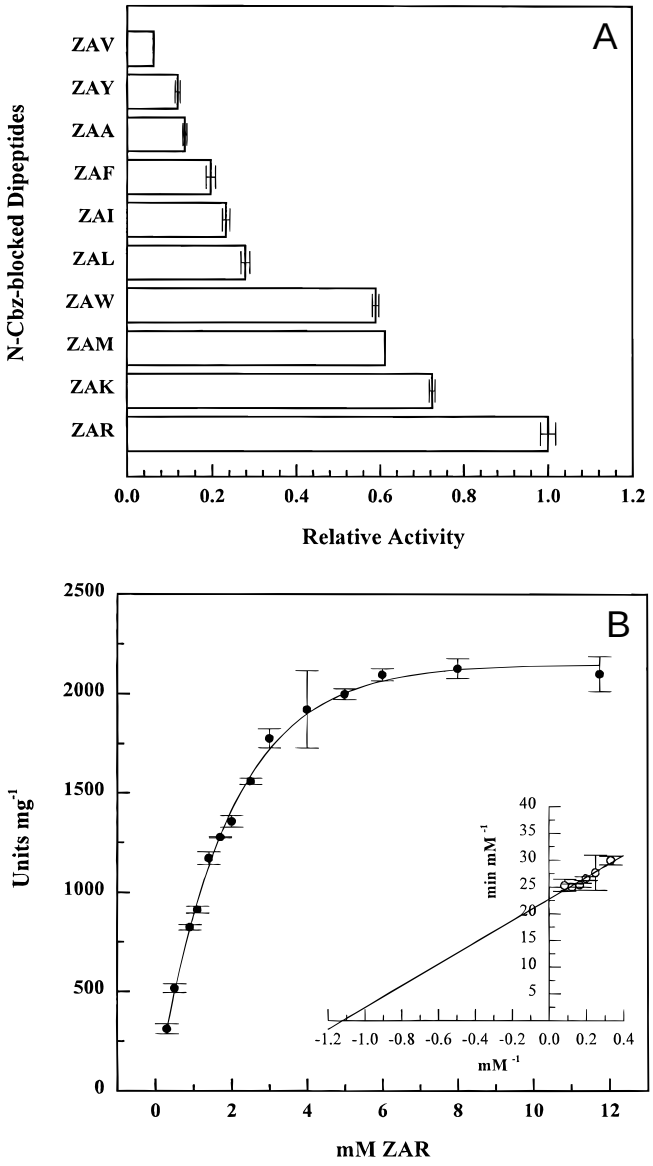


Fig. 9. C-terminal specificities of PfuCP. **A:** Activity assays were performed under standard conditions for the hydrolysis of a series of synthetic N-Cbz-alanyl-Xaa (ZAX) dipeptides and data was normalized to the maximum activity observed against ZAR. PfuCP shows a preference for basic, aliphatic, and aromatic C-terminal amino acids. Activity against ZAS, ZAH, and ZAN was observed at longer digestion times (30 min). Data shown represent the average of triplicate values. **B:** Activity assays were performed under standard conditions while varying the concentration of the N-blocked dipeptide ZAR. Data shown represent the average of duplicate values. Error limits represent one standard deviation.

PfuCP C-terminal specificities

PfuCP exhibits a relatively broad specificity for neutral, aliphatic, basic, polar, and aromatic C-terminal residues under standard assay conditions against synthetic N-Cbz-blocked dipeptide substrates; maximum activity occurs with a C-terminal arginine (Fig. 9A). This broad specificity of PfuCP may reflect the phylogenetic "primitiveness" of *P. furiosus* in comparison to higher organisms whose carboxypeptidases have more specific purposes; for instance, human carboxypeptidase N deactivates bioactive peptides by hydrolyzing only basic C-terminal residues (Skidgel, 1995). Although ZAS, ZAH, and ZAN were hydrolyzed, the assay was not sensitive enough to detect hydrolysis under standard conditions; hence, digestion times were increased to 30 min to detect activity that was less than 17 U mg^{-1}. However, no activity was seen under standard conditions against ZAG, ZAP, ZAD, or ZAE; mutagenesis experiments are planned to engineer in these remaining specificities and improve the versatility of PfuCP as a sequencing tool. Although the substrates ZAC, ZAQ, and ZAT were not commercially available, the observed activities of ZAS and ZAN were taken to be representative of ZAC/ZAT and ZAQ to a first approximation. The kinetic parameters of PfuCP activity at 80 °C in the presence of 0.4 mM Co^{2+} with ZAR as substrate were determined from Lineweaver–Burke analysis: K_m, 0.9 ± 0.1 mM; V_{max}, 2,300 ± 70 U mg^{-1}; and turnover number, 600 ± 20 s^{-1} (Fig. 9B).

C-terminal ladder sequencing

Sequential C-terminal hydrolysis of the peptide N-acetyl-renin substrate (NARS) by PfuCP, followed by MALDI-TOF MS detection, offered unambiguous proof of carboxypeptidase activity (Fig. 10). At 80 °C, PfuCP was able to sequence up to seven residues from the C-terminus of NARS releasing leucine, valine, phenylalanine, tyrosine, serine, and histidine residues. Sequencing of NARS at room temperature yields a similar mass spectrum; however, the intensity is greater for mass peaks that are closer to the C-terminus (right side of the mass spectrum), indicating a less complete digestion. Several other natural and synthetic peptides were also digested by PfuCP (data not shown); digestion conditions are currently being optimized to show complete ladders. These sequencing experiments confirm the broad specificity of PfuCP demonstrated in the experiments with N-blocked ZAX dipeptides and represent

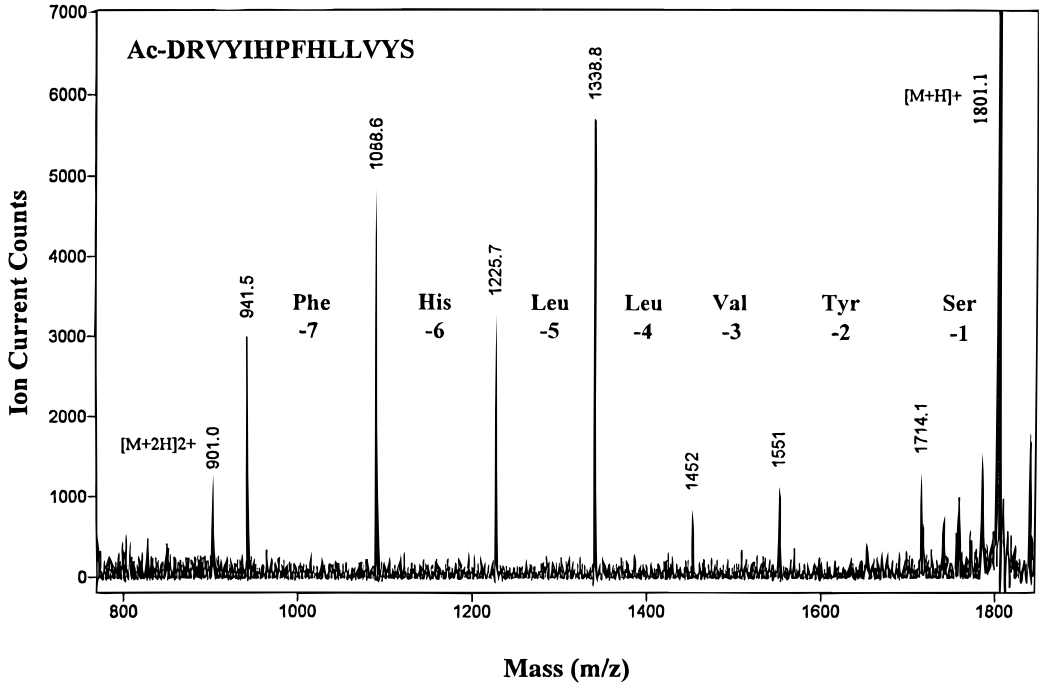


Fig. 10. C-terminal ladder sequencing of N-acetyl-renin substrate by PfuCP. The digestion was performed in 5 mM KMES buffer (pH 6.5) with 50 μM N-acetyl-renin substrate, 0.4 mM $CoCl_2$, and 150 nM purified *apo*PfuCP (total volume 5 μL). The reaction was carried out for 1 min at 80 °C followed by quenching on ice and the addition of 0.1% TFA. The sample was subsequently analyzed by MALDI-TOF MS, and the monoisotopic masses were recorded.

the first reported instance of high-temperature C-terminal sequencing. Studies are underway to sequence larger proteins.

Summary

An inactive metallocarboxypeptidase (PfuCP) has been purified from the hyperthermophilic archaeon *P. furiosus* and its activity has been restored by the addition of Co^{2+} but not Zn^{2+}. Careful choice of substrate for the purification assay was necessary to ensure true carboxypeptidase activity, as many carboxypeptidase substrates are susceptible to hydrolysis by endoproteases or enzymes with mixed specificities (Shibata & Doi, 1984; Zheng et al., 1998). PfuCP has the highest optimal temperature of any carboxypeptidase purified to date; furthermore, its wide temperature range for activity (down to at least 20 °C) will conveniently allow many aspects of mechanism and thermoactivation to be examined.

Further characterization is necessary to understand the nature of the apparent structural thermostability of Co^{2+}-reconstituted PfuCP at high temperatures despite its irreversible thermal inactivation under similar conditions. The identification of the molecular determinants of thermal stability in hyperthermophilic proteins is currently an active field of investigation. Surprisingly, while most proteins isolated from hyperthermophiles are optimally active at temperatures 80 °C or above, several proteins have been shown to be unstable beyond 10–20 min. To date, no unique cytoplasmic factors have been identified in these organisms, which might serve as "universal thermoprotectants" (Ramakrishnan et al., 1997). Aside from a few reports, most of the stability studies have been directed toward small proteins such as rubredoxin (Cavagnero et al., 1995) and ferredoxin (MacedoRibeiro et al., 1996; Pfeil et al., 1997). Structural studies are underway to investigate the structure-function relationships and the mechanisms of thermal stabilization of PfuCP as the crystal structures for several mesophilic carboxypeptidases are available (Rees et al., 1983; Coll et al., 1991; Rowsell et al., 1997). CoPfuCP has already been crystallized in a number of crystal forms and the determination of its three-dimensional structure is in progress (M.K. Chan, unpubl. data). Furthermore, crystallographic studies might elucidate the nature of the observed dimerization behavior and identify the catalytically essential residues, confirming if PfuCP is in fact the second member of a new class of carboxypeptidases.

The broad specificities of PfuCP against synthetic and natural peptide substrates suggest a role for PfuCP in digestion and protein turnover as opposed to specific post-translational modification seen in higher organisms. However, it is noteworthy that *P. furiosus* cannot grow on free amino acids alone as the sole carbon and nitrogen source (Snowden et al., 1992), so the role of carboxypeptidase in nutritive metabolism has yet to be established. Finally, the high temperature C-terminal enzymatic ladder sequencing reported here illustrates the inherent biotechnological potential of enzymes from hyperthermophiles.

Materials and methods

Chemicals and reagents

All chemicals were of reagent grade and were obtained from the following sources: yeast extract and tryptone from Difco (Detroit, Michigan); lysozyme, DNase-I, and PMSF from Sigma (St. Louis, Missouri); glycerol and DTT from ICN Biomedical (Aurora, Ohio);

column chromatographic resins from Amersham Pharmacia Biotech (Piscataway, New Jersey); hydroxyapatite from American International Company (Natick, Massachusetts); argon gas from Air Liquide (Houston, Texas); Amicon YM10 membranes and Microcon 3 ultrafiltration units from Millipore (Bedford, Massachusetts); SDS-PAGE gels, Mark-12 marker proteins, and PVDF membrane from Novex (San Diego, California); Tris-Cl base from Boehringer Mannheim (Indianapolis, Indiana); ammonium sulfate from Mallinckrodt (Paris, Kentucky); Na_2EDTA dihydrate from Fluka (Ronkonkoma, New York); and NaCl from Fischer (Fair Lawn, New Jersey). Amido black, KMES buffer, free amino acids, $CdCl_2$, ninhydrin, Chelex-100, and N-acetyl renin substrate were also purchased from Sigma. All ZAX N-blocked dipeptides were purchased from Bachem (Torrance, California). $CoCl_2$ and TFA were obtained from Mallinckrodt. Polypropylene microcentrifuge tubes were purchased from Eppendorf (Westbury, New York). Bovine serum albumin standard solution was purchased from Pierce (Rockford, Illinois). $ZnCl_2$ was purchased from EM Science (Gibbstown, New Jersey).

Archaeal strain and culture conditions

Pyrococcus furiosus strain DSM 3638 was obtained as a lyophilized culture from the Deutsche Sammlung von Microorganismen und Zellkulturen (Braunschweig, Germany). *P. furiosus* was grown at 90 °C under strictly anaerobic conditions (Fiala & Stetter, 1986). The archaeon was revived on a complex medium and scaled up in a 450 L fermentor at the Fermentation Facility, University of Wisconsin (Madison, Wisconsin). After sterilization, the fermentor was cooled down to 90 °C. Solid cysteine hydrochloride was added a few hours before inoculation at a final concentration of 0.4 g L^{-1} to pre-reduce the medium. Growth was monitored by measuring the optical density at 600 nm. Cells were harvested at late log phase (Δ O.D. ~0.6 in 12 h) with a Sharples continuous centrifuge. The cell paste was frozen immediately in liquid N_2 and stored at −80 °C. The approximate cell yield (wet weight) was 3 g L^{-1}.

Preparation of P. furiosus cell extract

Frozen *P. furiosus* cell paste (~450 g) was thawed out in 2 L of cell lysis buffer (see below) by incubating at room temperature for 30 min with gentle stirring. Then the cell lysate was centrifuged at 12,000 rpm for 30 min at 5 °C in a Sorvall GSA rotor. The supernatant fractions were pooled, degassed, and 2 mM each of dithiothreitol (DTT) and sodium dithionite (DT) were added.

Buffers

The cell lysis buffer was composed of 50 mM Tris-Cl (pH 8.0); DNase-I (10 mg L^{-1}); 5 mM $MgSO_4$; and 1 mM PMSF. Buffer A consisted of 50 mM Tris-Cl (pH 8.0); 1 mM EDTA; 1 mM PMSF; 2 mM DTT and 10% glycerol. Buffer B was the same as buffer A but without DTT. The pH of all buffers was adjusted at room temperature. The buffers were degassed, sparged with argon gas, and maintained under positive argon pressure.

Purification of PfuCP

The *P. furiosus* crude extract (8 U mg^{-1}; 21,400 mg) was loaded at a flow rate of 20% of 9 mL min^{-1} onto a QFF column (10 × 30 cm) previously equilibrated with buffer A. Elution was performed over 700 min at a flow rate of 9 mL min^{-1} with a gradient of 0–0.5 M NaCl in the same equilibration buffer. QFF fractions between 0.25–0.36 M NaCl containing PfuCP activity (46 U mg^{-1}) were pooled and suspended in EDTA-free Tris-Cl buffer (pH 6.5) to a total volume of 1,220 mL. Dilution of EDTA was achieved by Amicon ultrafiltration through a YM10 membrane (10 kDa cutoff), and the volume was reduced to 75 mL. This procedure was repeated twice and then the combined fractions were diluted to a final volume of 1,125 mL and loaded onto a hydroxyapatite column (XK50 column; 50 × 10 cm) that was pre-equilibrated with 50 mM Tris-Cl (pH 6.5) at a flow rate of 5 mL min^{-1}. Elution was performed over 420 min with a linear gradient of 0–0.5 M potassium phosphate in Tris-Cl buffer (pH 8.0) at a flow rate of 5 mL min^{-1}. Fractions with the highest activity were pooled (133 U mg^{-1}; 750 mg) and diluted with buffer A. Ammonium sulfate was then added to a final concentration of 10% (w/v) in a final volume of 500 mL and loaded at 50% of 5 mL min^{-1} onto a butyl-sepharose column (XK50 column; 50 × 12 cm) previously equilibrated with the same buffer. Elution was performed at a flow rate of 5 mL min^{-1} over 280 min with a gradient of 7–0% ammonium sulfate. Fractions with a specific activity of 452 U mg^{-1} (183 mg) were pooled and diluted in buffer A to a final volume of 780 mL and loaded at 50% of 6 mL min^{-1} onto a 50 mL Q-Sepharose High-Performance Column (QHP; XK26/10) previously equilibrated with buffer A. Elution was performed at 6 mL min^{-1} over 65 min with a gradient of 0.1–0.5 M NaCl in buffer A. QHP fractions containing highest activity were pooled (1,131 U mg^{-1}; 14 mg) and exchanged with buffer B. The protein sample was concentrated by Amicon filtration and loaded onto a Superdex G-200 gel filtration column (XK60 column; 26 × 58 cm) at a flow rate of 2 mL min^{-1} in buffer A containing 0.25 M NaCl. Elution was monitored by the UV absorbance at 280 nm. The fractions with highest activity (1,303 U mg^{-1}; 13 mg) were pooled and loaded onto a MonoQ column pre-equilibrated with buffer B. Elution was performed at 1 mL min^{-1} over 60 min with a gradient of 0–0.35 M NaCl in equilibration buffer. Pure PfuCP fractions (1,391 U mg^{-1}; 11 mg), as determined by SDS-PAGE, were pooled and stored at −80 °C.

Assay of enzyme activity

Unless stated otherwise, enzymatic activity was assayed using 4 μL of enzyme sample in a total volume of 100 μL of 0.1 M KMES buffer (pH 6.5), starting with 2 mM ZAR in the presence of 0.4 mM $CoCl_2$. Polypropylene microcentrifuge tubes were used. The enzyme and substrate were mixed quickly at room temperature and then brought to 80 °C; the equilibration time for the reaction mixture to reach 80 °C was considered negligible relative to the timescale of the assay. The reaction mixture was incubated at 80 °C in a water bath (Neslab EX-200DD) for 10 min followed by quenching on ice. Subsequently, 750 μL of cadmium-ninhydrin reagent (Doi et al., 1981) was added, and the samples were incubated for an additional 5 min at 80 °C for color development followed by quenching on ice. The absorbance was read at 500 nm (HP 8452A diode-array spectrophotometer) with an unheated assay mixture containing Cd-ninhydrin reagent serving as the blank. Absorbances were compared with an arginine standard curve in order to calculate specific activities. Stock solutions of ZAR and $CoCl_2$ were prepared in 0.1 M KMES buffer (pH 6.5), and when necessary, dilution of enzyme samples was done in 50 mM Tris-Cl buffer (pH 8.0). All pH adjustments were performed at room tem-

perature. The hydrolysis of ZAR by PfuCP is shown in Scheme 1B. The free amine of the liberated arginine reacts with ninhydrin to yield the chromophore, Ruhemann's purple (absorbance at 500 nm).

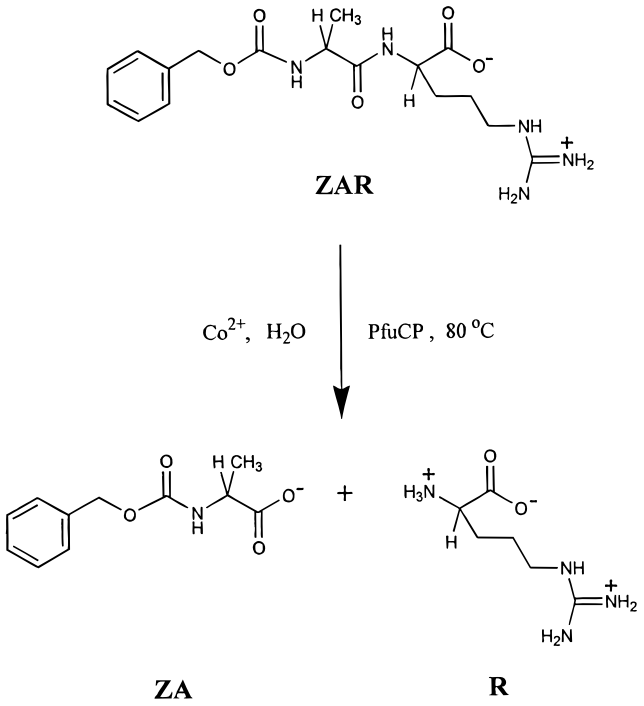
One unit of activity is defined as the amount of enzyme required to generate 1 μmol of arginine from ZAR in 1 min at 80 °C. Activity assays for other ZAX amino acid substrates (X = A, D, E, F, G, H, I, K, L, M, N, P, R, S, V, W, Y) were performed under otherwise identical assay conditions; appropriate amino acids were used to prepare standard curves. Spontaneous hydrolysis of the synthetic substrates was not observed at these temperatures. Protein concentrations were quantitated by the Bradford assay using bovine serum albumin as the standard (Bradford, 1976).

SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed in 12% Tris-glycine gels according to established procedures (Laemmli, 1970). For reducing conditions, samples were prepared in 0.1% SDS sample buffer containing 2 mM DTT; for reducing and denaturing conditions, samples were also pre-incubated at 80 °C for 1 min prior to electrophoresis at 125 V cm^{-1} for ~2 h. The gels were stained overnight in colloidal Coomassie stain and destained in distilled water. Mark-12 protein markers were used to calibrate the gels, and a second-order polynomial function was used to calculate the apparent molecular weights.

Gel filtration

The native molecular weight of PfuCP was determined by gel filtration at room temperature using a Superdex 200 column (1.6 × 60 cm; Pharmacia-LKB) connected to an FPLC. The column was calibrated with the following standards: blue dextrin, thyroglobulin, ferritin, catalase, aldolase, bovine serum albumin, and carbonic anhydrase. To avoid anomalous migration behavior of proteins within the gel matrix, gel filtration was performed in the presence of 0.2 M NaCl in 50 mM Tris-Cl buffer (pH 8.0) at a flow rate of 0.5 mL min^{-1}.



Scheme 1B.

MALDI-TOF MS

The molecular weight of the purified *apo*PfuCP was determined by matrix-assisted laser desorption ionization TOF mass spectrometry. Purified PfuCP in Tris buffer was exchanged three times with distilled water through Microcon ultrafiltration. Sinapinic acid was used as the matrix and 4.5 pmol of *apo*PfuCP was loaded. The ELITE mass spectrometer (PerSeptive Biosystems Inc., Framingham, Massachusetts) was operated in linear mode with an acceleration voltage of 25 kV using delayed extraction; 63 scans were averaged.

Protein sequence analysis

Following reducing and denaturing SDS-PAGE, the monomeric *apo*PfuCP band was electroblotted onto a 0.2 μm polyvinylidene-difluoride (PVDF) membrane for 3 h at 38 V. The membrane was stained with amido black for ~5 min and then destained in deionized water; the PfuCP band was cut out and subjected to N-terminal sequencing (Perkin Elmer 476; Applied Biosystems, Foster City, California). In situ Endo-Lys C digestion was performed on the PVDF membrane according to the established procedures (Fernandez et al., 1994). The lyophilized digest was resuspended in minimal buffer (2% acetonitrile, 0.057% TFA) and injected onto a Reliasil C18 column (0.5 × 100 mm) at 35 °C; a linear gradient of 9–54% B (90% acetonitrile, 0.04% TFA) was performed at a flow rate of 10 μL min^{-1} over 45 min; peptides were monitored at 200 nm. Two major peaks collected at 5–10% gradient were then subjected to MALDI-TOF MS analysis followed by N-terminal sequencing.

Re-activation by metal ions

The divalent metal ion dependence of PfuCP activity was examined by performing the enzymatic assay at various concentrations of Co^{2+} and Zn^{2+} under otherwise standard conditions. The metal chlorides were prepared in 0.1 M KMES buffer (pH 6.5), which had been passed through a Chelex 100 column (23 × 25 mm) according to established procedures (technical notes, Biorad, Hercules, California). Prior to use, purified PfuCP was diluted 500-fold in the same buffer. De-metallizing PfuCP involved the addition of 50 mM EDTA followed by ultrafiltration to remove EDTA and adventitious metal ions. Data fitting for these and other experiments was done using Microcal Origin 5.0 (Northampton, Massachusetts).

Temperature and pH optima of PfuCP activity

The temperature optimum of PfuCP was determined by measuring the activity at various temperatures from 20 to 100 °C with saturating concentrations of ZAR (11.75 mM) but under otherwise standard assay conditions. Similarly, the pH optimum of the activity was determined by measuring the activity at various pH ranges using the following buffers: piperazine, 4.8–6.2; KMES,

5.3–6.9; MOPS, 6.5–7.5; and HEPES, 6.9–8.0. The pH of all buffers was adjusted at room temperature.

Far UV-CD spectroscopy

Changes in the secondary structures of PfuCP were monitored in the far-UV range from 190–300 nm in 10 mM potassium phosphate buffer (pH 7.0) on an Aviv Circular Dichroism Spectrometer (model 62A-DS). Room temperature experiments were performed at 26 °C on *apo*PfuCP (15 μM) and after reconstitution with 1 mM $CoCl_2$. The spectrum of the Co^{2+}-treated sample was also examined at 80 °C. The results were expressed as molar residue ellipticity (deg cm^2 dmol^{-1}).

Fluorescence spectroscopy

The intrinsic fluorescence properties of PfuCP were determined using a Hitachi F-4500 fluorescence spectrophotometer. The excitation wavelength used was 290 nm, and the emission spectrum was monitored from 300–450 nm. The scan speed was 60 min^{-1} and the photomultiplier voltage was set at 700. All the experiments were performed in 10 mM potassium phosphate buffer (pH 7.0). After measuring the fluorescence intensity of *apo*PfuCP at room temperature, Co^{2+} was added to a final concentration of 1 mM and incubated for 5 min; measurements were then made at both room temperature and 80 °C. In a separate experiment, the reversibility of the temperature-dependent changes was examined by monitoring the fluorescence intensity as CoPfuCP was heated from 25 to 85 °C and then after cooling back down to room temperature.

Thermal inactivation

PfuCP (730 nM in 0.1 M KMES buffer, pH 6.5) was incubated in the presence and absence of 0.4 mM Co^{2+} at 80 °C for various time points up to 120 min. Residual activity was then measured under standard assay conditions.

C-terminal ladder sequencing of N-acetyl renin substrate with PfuCP

The sequencing reaction was carried out in polypropylene microcentrifuge tubes with the following reaction mixture in a total volume of 5 μL: 5 mM KMES buffer (pH 6.5), 50 μM porcine N-acetyl-renin substrate (NARS), 0.4 mM $CoCl_2$, and 150 nM PfuCP. The samples were incubated at 80 °C in a water bath for 1 min followed by quenching on ice and the addition of 0.1% TFA. Samples were analyzed by MALDI-TOF MS, and the peaks assigned to within 0.3 Da of their monoisotopic masses. The mass differences between adjacent peaks were correlated with the molecular masses of released amino acids; although for an unknown sequence, leucine and isoleucine would be indistinguishable in the spectrum. The amino acid sequence of NARS (MW 1,801.3) is: Ac-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser. The amount of digested NARS loaded was approximately 13 pmol, and the matrix used was α-cyanohydroxycinnamic acid. The acceleration voltage was 20 kV and 99 scans were averaged. Room temperature sequencing experiments were carried out at 20 °C.

Acknowledgments

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