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Purification and Characterization of Two Functional Forms of Intracellular Protease PfpI from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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The hyperthermophilic archaeon *Pyrococcus furiosus* grows optimally at 100°C by the fermentation of peptides and carbohydrates. From this organism, we have purified to homogeneity an intracellular protease, previously designated PfpI (*P. furiosus* protease I) (S. B. Halio, I. I. Blumentals, S. A. Short, B. M. Merrill, and R. M. Kelly, J. Bacteriol. 178:2605–2612, 1996). The protease contains a single subunit with a molecular mass of approximately 19 kDa and exists in at least two functional conformations, which were purified separately. The predominant form from the purification (designated PfpI-C1) is a hexamer with a molecular mass of 124 ± 6 kDa (by gel filtration) and comprises about 90% of the total activity. The minor form (designated PfpI-C2) is trimeric with a molecular mass of 59 ± 3 kDa. PfpI-C1 hydrolyzed both basic and hydrophobic residues in the P1 position, indicating trypsin- and chymotrypsin-like specificities, respectively. The temperature optimum for Ala-Ala-Phe-7-amido-4-methylcoumarin (AAF-MCA) hydrolysis was ~85°C both for purified PfpI-C1 and for proteolytic activity in *P. furiosus* cell extract. In contrast, the temperature optimum for PfpI prepared by incubating a cell extract of *P. furiosus* at 98°C in 1% sodium dodecyl sulfate for 24 h at 95 to 100°C (I. I. Blumentals, A. S. Robinson, and R. M. Kelly, Appl. Environ. Microbiol. 56:1255–1262, 1990), designated PfpI-H, was ~100°C. Moreover, the half-life of activity of PfpI-C1 at 98°C was less than 30 min, in contrast to a value of more than 33 h measured for PfpI-H. PfpI-C1 appears to be a predominant serine-type protease in cell extracts but is converted in vitro, probably in part by deamidation of Asn and Gln residues, to a more thermally stable form (PfpI-H) by prolonged heat treatment. The deamination hypothesis is supported by the differences in the measured pI values of PfpI-C1 (6.1) and PfpI-H (3.8). High levels of potassium phosphate (>0.5 mM) were found to extend the half-life of PfpI-C1 activity towards AAF-MCA by up to 2.5-fold at 90°C, suggesting that compatible solutes play an important role in the in vivo function of this protease.

Pyrococcus furiosus is a hyperthermophilic, fermentative archaeon that grows optimally near 100°C (16). Like most heterotrophic hyperthermophiles, it utilizes peptides and various carbohydrates as growth substrates and also facultatively reduces elemental sulfur to H_2S (25, 36, 37). Several enzymes have been purified and characterized from this organism (1), including those implicated in functions ranging from carbohydrate metabolism (8, 9, 24) and bioenergetics (3, 6, 34) to DNA modification (29). *P. furiosus* grows on peptide-based media (39), and several enzymes involved in amino acid metabolism have also been purified (19, 28, 30). Proteases are presumably key enzymes in peptide utilization for nutritional purposes by hyperthermophilic organisms (13, 39), although, as yet, such enzymes from *P. furiosus* have not been extensively characterized. To date, proteases from other members of the *Thermococcales* have been examined, including two extracellular proteases purified from a *Pyrococcus* species (33), several intracellular proteases located in cell extracts of *Thermococcus* species (26), and a serine protease purified from *Thermococcus stetteri* (27).

Preliminary studies with cell extracts of *P. furiosus* and culture supernatants showed that both contained a variety of proteolytically active species with molecular masses, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of between 65 and 140 kDa (12, 15). Blumentals et al. (7) described an unusual purification procedure for an intracellular protease from *P. furiosus*. After heating cell extracts at 98°C for 24 h with SDS (1%, wt/vol), one of the few remaining proteins had proteolytic activity. This protease, now designated PfpI (for *P. furiosus* protease I [18]), was further purified by preparative gel electrophoresis. PfpI contained two major protein species, both of which retained activity after SDS-PAGE, and these had apparent molecular masses of 86 and 66 kDa. Furthermore, they had the same amino-terminal amino acid sequence, indicating that PfpI existed as two homomultimeric assemblies. The proteolytic activity remaining after heat treatment was extremely thermostable, with a half-life of activity at 98°C of more than 30 h. Using amino acid sequence information from PfpI, Halio et al. (18) isolated the corresponding gene and showed that it coded for a protein of 18.8 kDa. The PfpI gene was expressed in *Escherichia coli* to yield an active protease, but this protease had a half-life of only 19 min at 95°C. This was much less thermostable than the enzyme purified from *P. furiosus* by the SDS-heat treatment procedure (18).

PfpI purified by SDS-heat treatment was also shown to be immunologically related to the proteasome complex found in eukaryotes (39). However, the sequence deduced from the PfpI gene showed no similarity to any known protease or proteasome complex (18). These included proteins such as La (43), ClpP (23, 31, 32), and Ti (22) from *E. coli*, the archaeal

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proteasome from *Thermoplasma acidophilum* (14), and the 20S proteasome, the 26S complex, and multipain from eukaryotes (17), all of which have broad specificities and participate in the degradation of cytoplasmic proteins (4, 40, 41). PfpI might have a similar function in *P. furiosus*, as suggested by its apparent ability to hydrolyze most other proteins in cell extracts upon heat treatment. A fundamental question, therefore, concerns the precise nature of PfpI within the cell. Does it correspond to the extremely thermostable SDS-heat-prepared form, or is this species an artifact of the harsh purification procedure? If so, untreated PfpI might be more thermally labile, like the recombinant form produced in *E. coli*, and is, perhaps, part of a high-molecular-weight proteolytic species. To address these issues, we have purified two active forms of the PfpI protease from cell extracts of *P. furiosus* by column chromatography under nondenaturing conditions. Possible reasons for the observed differences between the activities and thermal stabilities of the functional forms of the PfpI protease are discussed.

MATERIALS AND METHODS

Culture of *P. furiosus* and preparation of cell extract. *P. furiosus* (DSM 3638) was obtained from Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. Cultures were grown in a 600-liter, batch fermentor as described previously (10). Cells were harvested at the end of log phase and immediately frozen in liquid N_2 and stored at −80°C. A cell extract was prepared by thawing cells (480 g, wet weight) in 1,300 ml of 50 mM Tris-HCl (pH 8) containing 2 mM sodium dithionite, 10% (vol/vol) glycerol, 1 mg of lysozyme per ml, and 1 mg of DNase (DN25; Sigma Chemical, St. Louis, Mo.) per ml, followed by incubation for 2 h at 37°C and centrifugation at 50,000 × *g* for 1.5 h at 4°C in a Beckman L8-70 ultracentrifuge.

Purification of protease. All purification steps were carried out at room temperature with a standard fast-performance liquid chromatography system (Pharmacia LKB, Piscataway, N.J.). Because several other enzymes, many of which are very oxygen sensitive (6, 10, 30, 34), were purified from the same batch of cells, the procedure was carried out under anaerobic conditions (10), and, where indicated, sodium dithionite and/or dithiothreitol (DTT) were added as scavengers of contaminating oxygen.

DEAE-Sepharose fast-flow chromatography. The cell extract was applied directly to a column (20 by 10 cm) of DEAE-Sepharose (Pharmacia LKB). After the column was washed with 7 liters of buffer (50 mM Tris-HCl [pH 8] containing 2 mM sodium dithionite–10% [vol/vol] glycerol), the adsorbed proteins were eluted in 100-ml fractions with a linear gradient from 0 to 11.5% NaCl (90 ml) and 11.5 to 37.6% NaCl (5,000 ml) at 12 ml/min. Protease activity eluted between 21.5 and 24.3% NaCl. These fractions were pooled and concentrated by a 30,000-molecular-weight-cutoff membrane (Amicon PM30), following which DTT (to a final concentration of 2 mM) was added.

Hydroxyapatite chromatography. The concentrated fractions from the previous column were applied to a column (10 by 15 cm) of hydroxyapatite (Bio-Rad, Hercules, Calif.) previously equilibrated at 10 ml/min. Adsorbed proteins were eluted with a linear gradient of potassium phosphate buffer, pH 8, from 0 to 200 mM at 10 ml/min. Protease activity eluted between 127 and 191 mM. These fractions were pooled and dialyzed in hydrophobic interaction chromatography (HIC) loading buffer (100 mM sodium phosphate, pH 7, containing 243 g of ammonium sulfate per liter and 2.5% glycerol).

Phenyl-Sepharose chromatography. One-tenth of the dialyzed pool from the hydroxyapatite step was applied to a column (5 by 50 cm) of Phenyl-Sepharose 650M (Toso Haas, Montgomeryville, Pa.), previously equilibrated with HIC loading buffer at 10 ml/min. After the column was washed with 600 ml of HIC loading buffer, adsorbed proteins were eluted with a step gradient of 100 ml of 25% HIC loading buffer, 100 ml of 15% HIC loading buffer, and 100 ml of 7.5% HIC loading buffer at 10 ml/min. Protease activity eluted at 7.5% HIC loading buffer. These fractions were pooled, concentrated, and dialyzed in 100 mM sodium phosphate buffer, pH 8.

Mono Q chromatography. One-half of the dialyzed pool from the phenyl-Sepharose step was applied to a column (HR 5/5; Pharmacia LKB) of Mono Q (Pharmacia LKB), previously equilibrated with 100 mM sodium phosphate buffer, pH 8, at 1 ml/min. After the column was washed with 10 ml of 100 mM sodium phosphate buffer, pH 8, adsorbed proteins were eluted with a linear gradient of 0 to 600 mM NaCl at 1 ml/min. The gradient was held at 380 mM NaCl, where protease activity eluted. The fractions containing protease activity were concentrated by a Centriprep-10 (Amicon, Beverly, Mass.) to 0.5 ml.

Gel filtration chromatography. The concentrated fraction from the Mono Q step (0.5 ml) was applied to a HiLoad 16/60 Superdex 200 column (Pharmacia LKB) which had been equilibrated with 50 mM Tris buffer, pH 8, at 0.5 ml/min. Proteins were eluted in 50 mM sodium phosphate buffer, pH 7, at 0.3 ml/min.

Enzyme assays. Proteolytic activity was detected by cleavage of 7-amido-4-methylcoumarin (MCA)-linked peptides (Sigma Chemical) on a CytoFluor 2350 fluorimeter (Millipore Corp., Bedford, Mass.). Temperature, pH, and substrate specificity assays were carried out by heating both the enzyme or buffer (for blanks) and the substrate in the appropriate buffer to the assay temperature in a thermocycler (Perkin-Elmer, Norwalk, Conn.). Each assay tube contained 0.5 μl of substrate stock (100 mM in dimethyl sulfoxide), 12 μl of diluted enzyme sample, and 107.5 μl of 50 mM sodium phosphate buffer, pH 7, except for the assay to determine optimum pH. For measurements of pH influence on activity, the following buffers were used: 100 mM sodium acetate–acetic acid buffer (pH 4 to 5.6), 100 mM sodium phosphate buffer (pH 5.6 to 8), and 100 mM glycine–NaOH buffer (pH 7.9 to 9.5). The pH of each buffer was adjusted at temperatures between 80 and 90°C. The assay tubes were incubated in the thermocycler for 5 min, chilled on ice, and centrifuged. Microtiter plates (Corning, Corning, N.Y.) were filled with 100 μl from each assay tube, and fluorescence was measured at 360 ± 40 nm excitation and 460 ± 40 nm emission. The values for temperature and pH optima were calculated from the second derivative of the polynomial fit to the data. Fluorescence units measured were converted to picomoles of MCA released by using a standard curve prepared with known dilutions of MCA in 5% dimethyl sulfoxide in 50 mM sodium phosphate, pH 7. When units are indicated they refer to the numerical report of fluorescence by a CytoFluor 2350 fluorimeter (Millipore) on sensitivity setting three.

Stability assays were carried out by incubating enzyme samples in screw-cap Microfuge tubes sealed with O rings (USA/Scientific Plastics, Milton Keynes, United Kingdom). The sample was overlaid with Ampliwax (Perkin-Elmer) to prevent evaporation. Residual activity was measured by adding 5 μl of sample at each time point to a microtiter plate, together with 95 μl of substrate, diluted in 50 mM sodium phosphate buffer, pH 7, to 500 μl. The plates were incubated at 85°C for 5 min prior to recording fluorescence. The effects of various salts and other chemicals on protease activity were also measured directly in microtiter plates similar to those used for the stability assays, with the exception that each sample contained the appropriate dilution of the given salt or chemical in the buffer. In all assays, the fluorescence of a blank containing only 50 mM sodium phosphate buffer, pH 7, and substrate was subtracted. Spontaneous hydrolysis of the MCA-linked peptides at the assay temperature typically accounted for less than 20% of the total fluorometric units. Standardization experiments with each substrate ensured that assays were not carried out under substrate limitation.

Total protein assays. Total protein concentration was determined with a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Ill.). Samples were diluted to the linear range (where A_{595} = 0.1 to 1) and incubated with reagent at 37°C for 30 min. A_{595} was determined by using an EL 340 microplate reader (Bio-Tek Instruments, Winooski, Vt.) with albumin used as a standard.

Electrophoresis and gelatin overlay assays. SDS-polyacrylamide slab gels were run by standard procedures (35). Gelatin (0.1%) was copolymerized in a 10% acrylamide gel different from that containing the enzyme. Proteolytic activity was indicated by cleared zones in the stained gelatin of the overlay of the SDS-PAGE-separated cell extracts. Protein samples containing 1 to 50 μg of protein were electrophoresed, washed, overlaid for 6 to 12 h at 75°C in 50 mM sodium phosphate buffer (pH 7.5), and stained as described previously (7). Nondenaturing PAGE, SDS-PAGE, and isoelectric focusing were also carried out on a Phast System (Pharmacia LKB), according to the manufacturer's protocols and using a broad pI calibration kit (Pharmacia Biotech, Piscataway, NJ).

Estimation of molecular mass. A HiLoad 16/60 Superdex 200 column (Pharmacia LKB) equilibrated with 50 mM sodium phosphate buffer, pH 7, was used at 0.5 ml/min to estimate molecular mass. The column was calibrated with the following proteins (obtained from Sigma Chemical): thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and α-lactalbumin (14.2 kDa).

RESULTS

Proteolytic species in *P. furiosus* cell extract. As shown in Fig. 1A, upon incubation of *P. furiosus* cell extracts at 98°C in 1% SDS for periods of up to 24 h, intracellular proteases hydrolyze most other proteins present. The apparent size of the proteolytically active species (as determined by clearing zones of gelatin overlays of cell extracts separated by SDS-PAGE) decreases with increasing incubation periods, such that only two major activities remain after 24 h. These have apparent molecular masses of 66 and 86 kDa on SDS-PAGE (Fig. 1B), but since these two species remain catalytically active, their mobilities are unlikely to accurately reflect their native molecular masses. Nevertheless, one can conclude that neither protease species is inactivated after heating at 95°C for 5 min in 1% SDS during preparation for electrophoresis. These two proteins accounted for most of the activity against Ala-Ala-Phe (AAF)-MCA, as determined by SDS-PAGE substrate gel assay

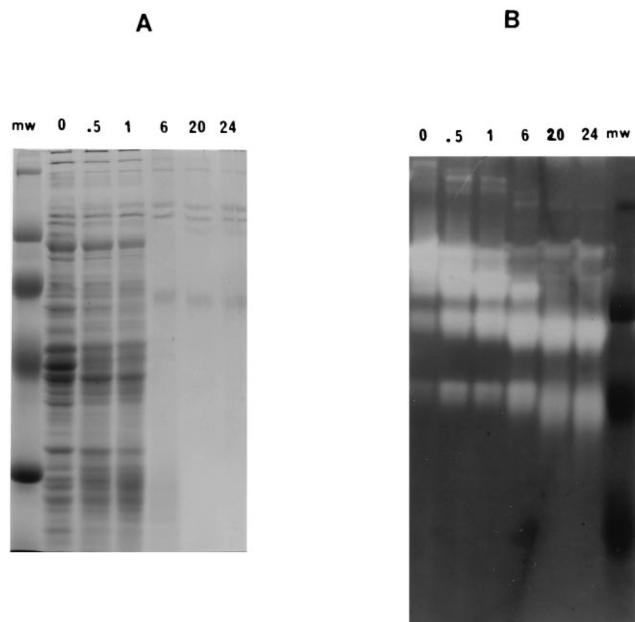

FIG. 1. Changes in protein composition of *P. furiosus* cell extracts upon incubation at 98°C in 1% SDS. (A) Fifteen percent SDS, Coomassie-stained slab gel containing extracts were incubated for the indicated amount of time in hours. Forty micrograms of protein was loaded per lane. Lane mw contains the following molecular mass markers (from top to bottom): myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). (B) Changes in proteolytic activity in *P. furiosus* cell extracts upon incubation at 98°C in 1% SDS. Shown is a gelatin overlay of an SDS slab gel run under similar conditions as described for panel A. Two micrograms of total protein was loaded per lane. Molecular mass markers were identical to those shown in panel A minus carbonic anhydrase (29 kDa).

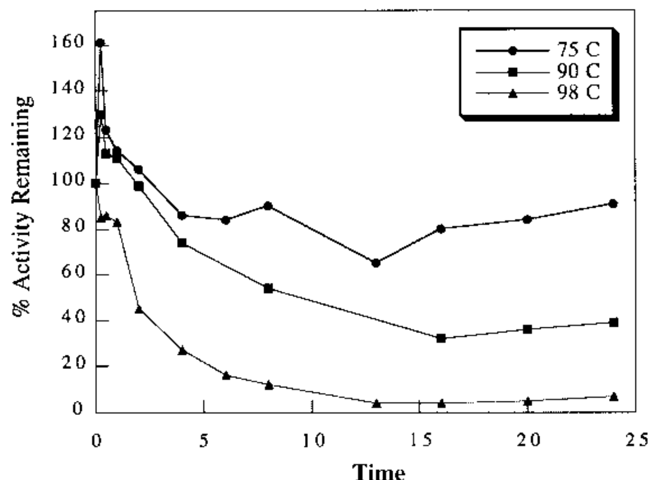

FIG. 2. Stability of proteolytic activity in *P. furiosus* cell extracts at 75, 90, and 98°C as monitored with the fluorometric substrate AAF-MCA. Activity was determined at 85°C after a 5-min incubation of substrate with enzyme.

of cell extracts that had not undergone the SDS-heat treatment procedure (data not shown). In a prior study, the 66- and 86-kDa forms were shown to have the same, nonambiguous amino-terminal amino acid sequence (18). Two minor protein species were also present in preparations of the 66- and 86-kDa forms. These had apparent molecular masses of approximately 47 and 20 kDa (from SDS-PAGE) and the same amino-terminal sequence as the 66- and 86-kDa forms but lacked proteolytic activity (18). Thus, the 66- and 86-kDa proteases prepared by the heat treatment procedure appear to be different assemblies of the same protein, which is now termed PfpI (18). Moreover, this protease represents most of the proteolytic activity in cell extracts of *P. furiosus*, at least with AAF-MCA or gelatin as the substrate (data not shown).

Total proteolytic activity in untreated cell extracts of *P. furiosus* was measured with AAF-MCA as the substrate over a 24-h period at either 75, 90, or 98°C. As shown in Fig. 2, the total activity decreased rapidly over 10 h, but at all temperatures further heating did not lead to significant additional losses of activity. These results indicate that after 24 h at 98°C, the proteolytically active forms that are generated (the 66- and 86-kDa species of PfpI) (Fig. 1) represent approximately 5% of the proteolytic activity (as measured by AAF-MCA hydrolysis) initially present in cell extracts. Figure 2 also shows that once these forms are created, they are extremely stable at high temperature and are much more stable than the higher-molecular-weight proteolytic species seen in the cell extract (Fig. 1). Furthermore, the catalytic activity of PfpI purified by heat treatment (PfpI-H) was found to have a higher temperature optimum (~100°C) than that of untreated cell extract (84°C) (data not shown). Clearly, these results indicate that PfpI-H is extremely stable, and PfpI appears to be present in the cell extract in multiple forms.

Purification of two forms of PfpI. To investigate the nature of PfpI in cell extracts in *P. furiosus*, efforts were directed to purify proteolytically active species under nondenaturing conditions by conventional chromatographic techniques. When cell extracts were applied to an ion-exchange column (DEAE-Sepharose), much of the AAF-MCA-hydrolyzing proteolytic activity bound to the column and was eluted as a single peak. The activity behaved in a similar fashion when subsequently applied to columns of hydroxyapatite and phenyl-Sepharose. Using these procedures, the protease(s) could be purified approximately 45-fold with a yield of activity of 16% (Table 1). Subsequent chromatography of the active fractions on a Mono Q column also yielded a single peak of activity and this contained four protein species (as determined by nondenaturing PAGE) (Fig. 3A, lane 5). AAF-MCA-hydrolyzing proteolytic activity eluted from a Superdex 200 column as three protein peaks (data not shown). The first peak, which we designate

TABLE 1. Purification of protease PfpI from *P. furiosus* cell extract[a]

Column[b]	Total protein (mg)	Sp act (10^6 U/mg)	Total units (10^9)	Fold purification	Yield (%)
Cell extract	1.2×10^4	0.50	6.2	1.0	100
DEAE	1.7×10^3	2.2	3.8	4.5	61
Hydroxyapatite	1.6×10^3	1.4	2.2	3.2	35
Phenyl-Sepharose	45	22	1.0	45	16
Mono Q	7.3	44	0.3	89	5
Superdex 200	1.2	99	0.1	200	2

[a] Protease activity was measured by fluorescence resulting from the cleavage of MCA-linked peptide substrate Ala-Ala-Phe as described in Materials and Methods. Unit is the numerical report of fluorescence measured with a Millipore CytoFluor 2350 fluorimeter at sensitivity setting three (adjusted for spontaneous hydrolysis of the peptide at high temperature).

[b] Ten percent of the material from the hydroxyapatite step was applied to the phenyl-Sepharose column. Results are scaled to 100% for the purpose of consistency in the purification table. Fifty percent of the material from the hydroxyapatite step was applied to the Mono Q column. Results are scaled to 100% for the purpose of consistency in the purification table.

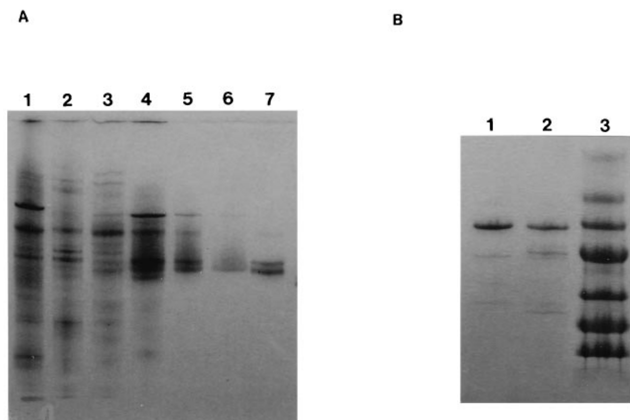

FIG. 3. (A) Protein composition at each stage of purification. Coomassie-stained native PAGE gel (8 to 25%) is shown. Lane 1, *P. furiosus* cell extract; lanes 2 to 5, eluent from DEAE, hydroxyapatite, phenyl-Sepharose, and Mono Q columns, respectively; lane 6, peak 1 eluent from Superdex 200; lane 7, peak 3 eluent from Superdex 200. (B) SDS-gel analysis of PfpI-C1 and PfpI-C2. Lane 1, peak 1 eluent from Superdex 200; lane 2, peak 3 eluent from Superdex 200; lane 3, molecular mass markers as described in legend for Fig. 1A plus β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). Protein loading per lane was 1 to 20 μg.

PfpI-C1 (for chromatographically prepared), contained the majority of protease activity and, after native PAGE, gave rise to a single diffuse protein band (Fig. 3A, lane 6). The third protein peak was also proteolytically active and was termed PfpI-C2. This contained two protein species, one of which appeared to be the same as that in PfpI-C1 (Fig. 3A, lane 7). Overall, PfpI-C1, which represented most of the AAF-MCA-hydrolyzing proteolytic activity obtained by the chromatographic purification procedure, was purified 200-fold (Table 1).

Analyses of PfpI-C1 and PfpI-C2 by SDS-PAGE demonstrated that they contained the same protein species previously seen in PfpI-H (prepared by SDS-heat treatment). As shown in Fig. 3B, PfpI-C1 contained three major species with apparent molecular masses of 66, 40, and 22 kDa. PfpI-C2 contained the same three species, with two additional ones of approximately 42 and 20 kDa (Fig. 3B). In both cases the 66-kDa species is the major protein form, and this was shown to have the same amino-terminal amino acid sequence as the 66-kDa species of PfpI-H purified by SDS-heat treatment. PfpI-C1 and PfpI-C2 also contain the same lower-molecular-weight species (~20 and 45 kDa) seen in the PfpI-H preparation. Thus, PfpI prepared both by column chromatography and by SDS-heat treatment is comprised of the same 18.8-kDa subunit encoded by the gene previously cloned and sequenced (18). Purified PfpI-C1 had a molecular mass of 124 ± 6 kDa as determined by gel filtration, while that of PfpI-C2 was 59 ± 3 kDa. These data indicate that PfpI-C1 is a hexamer of the 18.8-kDa subunit, while PfpI-C2 is a trimer; these proteases correspond to the 86- and 66-kDa species, respectively, shown in Fig. 1. As noted above, the migration of the various forms of PfpI after SDS-PAGE is not a true indication of their molecular size.

Characterization of PfpI-C. The pH optimum for catalysis of PfpI-C1 with AAF-MCA as a substrate was 6.3 (Fig. 4). This compares well with the value (6.5) previously determined for PfpI-H after electroelution (7). However, the pI of PfpI-C1 was 6.1, which is very different from the value of 3.8 determined for PfpI-H. The temperature optimum for PfpI-C1 activity was 86°C with AAF-MCA as the substrate (Fig. 5). LY-MCA and AFK-MCA also served as substrates for PfpI-C1, with temperature optima of 84 and 97°C, respectively (Fig. 5). Thus, like PfpI-H (7), PfpI-C1 has both trypsin- and chymotrypsin-like specificities, with the trypsin-like activity having a higher temperature optimum.

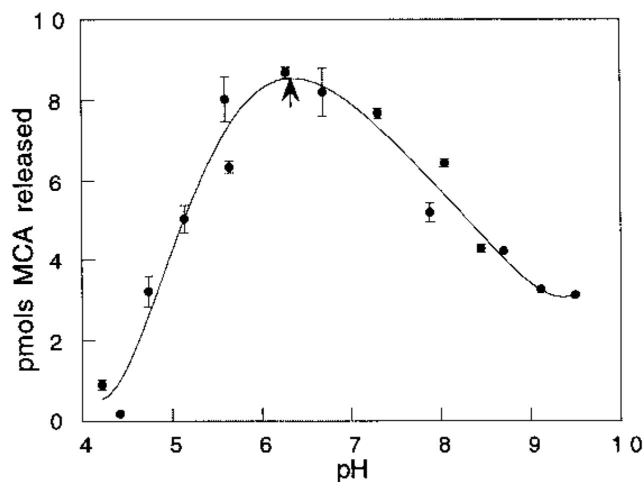

FIG. 4. Effect of pH on proteolytic activity of PfpI-C1 (hexamer) with AAF-MCA as the substrate.

The stability of PfpI-C1 was investigated by incubating samples at 75, 90, and 98°C (Fig. 6). Its half-life, as determined by AAF-MCA hydrolysis, was much lower than the half-life of the AAF-MCA activity of cell extracts. For example, for the purified protease, the values were 45 min at 90°C and <30 min at 98°C, in comparison to values of 9 and 2 h, respectively, for the cell extract. However, the thermal stability of purified PfpI-C1 is similar to that of the recombinant protein produced in *E. coli*, which had a half-life of 19 min at 95°C (18). To determine whether the lower stability of PfpI-C1 in the purified form was due to the absence of certain factors that are present in the cell extract of *P. furiosus*, various compounds were tested to determine whether they affected its thermostability. However, DTT (1 to 100 mM), SDS (0.01 to 1%, wt/vol), Triton X-100 (1 to 10%, vol/vol), glycerol (1 to 10%, vol/vol), mercaptoethanol (1 to 10%, vol/vol), ATP (1 to 10 mM), urea (1 to 4 M), guanine hydrochloride (1 to 4 M), inositol (1 to 20 mM), and $CaCl_2$, $CoCl_2$, $CuCl_2$, EDTA, $FeSO_4$, $KHPO_4$, $MgCl_2$, $MnCl_2$, NaCl, $NaMoO_4$, $NaWO_4$, $NiCl_2$ and $ZnCl_2$ (each at 1 to 100 mM) did

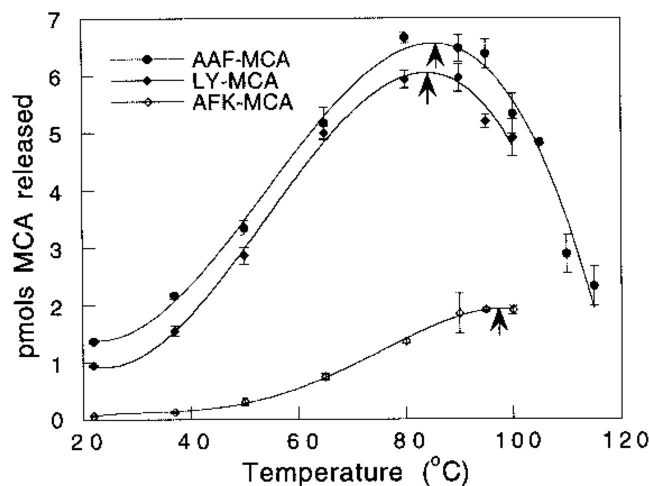

FIG. 5. Effect of temperature on the activity of PfpI-C1 (hexamer) with AAF-MCA, MCA, and LY-MCA as substrates.

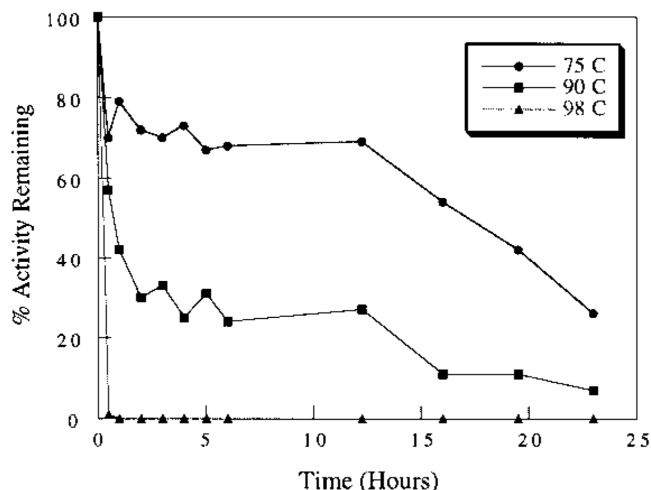

FIG. 6. Stability of PfpI-C1 at 75, 90, and 98°C with AAF-MCA as the substrate.

not cause a significant increase (>10%) in activity but, in most cases, caused inhibition when each was included in the standard assay mixture. On the other hand, NaCl (100 mM) and *N*-lauryl sarcosine (0.01%, vol/vol) increased proteolytic activity by about 50%. However, neither had any stabilizing effect when the enzyme was incubated at 90 or 98°C over prolonged periods (data not shown). Based on previous reports of stabilization of proteins by high levels of intracellular concentrations of K^+ and organic phosphates (~600 mM) in *P. furiosus* (20, 21, 37), the effect of high levels of potassium phosphate on the stability of AAF-MCA activity was investigated. The half-life of PfpI-C1 activity was extended (from 45) to 80 min at 90°C in the presence of 0.5 M $KHPO_4$ and to 110 min at 90°C in the presence of 0.8 M $KHPO_4$, although this is still much less than the 9 h observed for the same activity in the cell extract.

DISCUSSION

It was previously shown that antibodies raised against the 66-kDa form of PfpI-H cross-react with several proteolytically active and inactive species in *P. furiosus* cell extracts (7). These species had electrophoretic mobilities as determined by SDS-PAGE which were both higher and lower than 66 kDa, although no proteolytically active bands were observed at apparent molecular masses lower than 66 kDa (7). Herein, we report the direct purification to homogeneity of two functional forms of protease PfpI that, by SDS-PAGE analyses, have the same composition as PfpI-H. These appear to be hexameric (C1) and trimeric (C2) assemblies of the same 18.8-kDa subunit that constitutes PfpI-H. Blumentals et al. (7) showed that PfpI-H, prepared by incubation of *P. furiosus* cell extracts in 1% SDS for 24 h, was extremely thermostable, with a half-life of over 30 h at 98°C. Figure 2 shows that AAF-MCA-dependent proteolytic activity in cell extracts incubated in the absence of SDS rapidly diminishes at high temperature but stabilizes after approximately 15 h to a low but significant level (approximately 5% residual activity at 98°C). Since earlier efforts focused only on the form of PfpI recovered after heating extracts for 24 h at 98°C, it is now clear why the stability of this sample is much greater than that determined for PfpI purified by using column chromatography. Thus, in Fig. 2, PfpI-C1 is the predominant proteolytic species at the beginning of the experiment, while PfpI-H is the main form produced after approximately 24 h at 98°C. Presumably, the data obtained at intermediate times or at a lower temperature reflect various combinations of both forms.

TABLE 2. Isoelectric points (pI) for PfpI functional forms

Sample	pI	
	Measured	Calculated[a]
18.8-kDa subunit[b]		6.1
18.8-kDa subunit (all Asn→Asp and Gln→Glu)		4.8
PfpI-H (heat treated and electroeluted from SDS-PAGE)	3.9	
PfpI-C2 (trimer)	3.7	
PfpI-C1 (hexamer)	6.1	

[a] Determined by the Genetics Computer Group (University of Wisconsin) protein analysis program, Isoelectric.

[b] The amino acid sequence for the subunit contains the following charged groups as reported by Halio et al. (18) (numbers in parentheses indicate number of residues): Arg (10), Lys (12), His (5), Tyr (6), Cys (1), Glu (16), and Asp (10) in addition to Asn (5) and Gln (1).

The issue, therefore, is to account for the conversion of PfpI-C into PfpI-H and to explain their dramatically different stabilities. Similarly, why is PfpI-C less stable in its purified form than it is in cell extracts? One clue comes from the results of Ahern and Klibanov (2), who showed that the irreversible inactivation of enzymes at extreme temperatures can result from the covalent modification of certain amino acid residues. In particular, at temperatures approaching 100°C, the deamidation of asparagine and glutamine residues is greatly accelerated. While there is some evidence that hyperthermophilic proteins contain fewer Asn and Gln residues than their less thermostable counterparts (42), such residues are, nevertheless, typically present in significant amounts. Thus, the 18.8-kDa subunit of PfpI contains five Asn and one Gln residue according to the nucleotide-derived amino acid sequence (18). In addition, Hensel and Jakc (21) argued that high ionic strength tends to protect labile amino acid residues from solvent exposure, thereby slowing the deamidation process. They demonstrated that heat-induced irreversible changes to some intracellular hyperthermophilic proteins are minimized through the stabilizing influence of novel organic phosphates and potassium ions which are present at high intracellular concentrations in hyperthermophilic archaea. Hence, PfpI-C, in a purified form, might be expected to be more thermally labile than when present in a cell extract in the presence of high concentrations of potassium and organic phosphates (38). We show here that high concentrations of potassium phosphate do afford some stabilizing effect on purified PfpI-C, although not to the extent expected for the same activity in cell extracts, suggesting that other factors are involved. Similarly, recombinant PfpI, produced in *E. coli*, would not be exposed to the cytoplasmic factors present in *P. furiosus*. Accordingly, the recombinant protein and purified PfpI-C1 have comparable thermal stabilities.

Because very limited amounts of PfpI-C1 could be purified, it was not possible to perform deamidation experiments along the lines of those described by Ahern and Klibanov (2) and Hensel and Jakc (21). However, that PfpI-H is derived from PfpI-C by deamidation is strongly supported by the difference in their pI values, which are 6.1 and 3.8, respectively. From the amino acid sequence of the 18.8-kDa subunit of PfpI (18), the calculated pI is 6.1, the same as that found experimentally for PfpI-C (Table 2). Assuming that the Asn and Gln residues are deaminated in the thermal conversion of PfpI-C to PfpI-H, the

calculated pI of the 18.8-kDa subunit is 4.8, which approaches the measured pI for PfpI-H of 3.8. Other thermal modifications to amino acids that lead to changes in net charge could be responsible for the remaining pI differential. Clearly, knowledge of the three-dimensional structures of PfpI in its various forms is essential for exploring these issues in a more meaningful way (5, 11). In addition, intracellular chaperones, subunit packing, concentration stabilization, or as yet unidentified factors could play some role in the intracellular stabilization of the protease assembly.

PfpI in cell extracts was able to digest other proteins in *P. furiosus* and has at least two specificities cleaving at the carboxyl side of both hydrophobic and basic residues in the P1 position. It is not clear at present why the trypsin substrate yielded a higher temperature optimum for the protease than the chymotrypsin substrates. Nevertheless, these characteristics distinguish PfpI from single-cut, single-subunit proteases, such as trypsin or chymotrypsin, and it appears to be a processive type of protease with broad specificity, as seen with ClpP (31, 32), the archaeal proteasome (4), and the eukaryotic proteasome (40). The high homology of the gene encoding PfpI to open reading frames of unknown function in *E. coli* and *Staphylococcus aureus* is also intriguing (18). Further kinetic characterization, substrate specificity studies, and immunological analysis of PfpI are currently under way to better understand the structure and function relationships of this activity to the intracellular proteases in all three domains of life.

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