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Molecular characterization of the group II chaperonin from the hyperthermophilic archaeum *Pyrococcus horikoshii* OT3

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Abstract The group II chaperonin from the hyperthermophilic archaeum *Pyrococcus horikoshii* OT3 (PhCPN) and its functional cooperation with the cognate prefoldin were investigated. PhCPN existed as a homo-oligomer in a double-ring structure, which protected the citrate synthase of a porcine heart from thermal aggregation at 45°C, and did the same on the isopropylmalate dehydrogenase (IPMDH) of a thermophilic bacterium, *Thermus thermophilus* HB8, at 90°C. PhCPN also enhanced the refolding of green fluorescent protein (GFP), which had been unfolded by low pH, in an ATP-dependent manner. Unexpectedly, functional cooperation between PhCPN and *Pyrococcus* prefoldin (PhPFD) in the refolding of GFP was not observed. Instead, cooperation between PhCPN and PhPFD was observed in the refolding of IPMDH unfolded with guanidine hydrochloride. Although PhCPN alone was not effective in the refolding of IPMDH, the refolding efficiency was enhanced by the cooperation of PhCPN with PhPFD.

Keywords Chaperonin · Folding · Hyperthermophilic archaea · Molecular chaperone · Prefoldin

Introduction

Molecular chaperones are ubiquitous proteins that are required for the correct folding, assembly, transport, and degradation of proteins within the cell (Bukau et al. 2000; Ellis 1996; Gething and Sambrook 1992; Laksanalamai et al. 2004; Mogk et al. 2001; Hartl and Hayer-Hartl 2002; Valpuesta et al. 2002). The chaperonins are seven- to nine-membered double-ring complexes of 800–1,000 kDa, and capture non-native proteins in a central cavity to promote correct folding in an ATP-dependent manner (Ellis and Hartl 1996; Bukau and Horwich 1998; Ranson et al. 1998; Yoshida and Kawaguchi et al. 2002). They are classified into two groups: group I chaperonins are found in bacteria and organelles of eukaryotes, and group II in archaea and in the cytoplasm of eukaryotes (Kim et al. 1994; Kubota et al. 1995). The group I chaperonin is a complex of a tetradecamer that is capped by the heptameric co-chaperone, GroES (Hartl 1996; Fenton and Horwich 1997; Sigler et al. 1998). In contrast, group II chaperonins exist as an eight- or nine-membered rotationally symmetrical double-ring in a toroidal structure composed of homologous subunits of about 60 kDa and lack co-chaperonins corresponding to GroES (Archibald et al. 1999; Gutsche et al. 1999). The crystal structure of the group II chaperonin from an thermoacidophilic archaeum *Thermoplasma acidophilum* and the hyperthermophilic archaeum *Thermococcus* sp. strain KS-1 suggest that the long, helical protrusions in their apical domain play the equivalent role of GroES as a built-in lid of the cavity (Ditzel et al. 1998; Klumpp and Baumeister 1998; Shomura et al. 2004). Contrary to studies with group I chaperonins, there is not much information on the protein folding mechanisms in group II chaperonins, especially concerning archaeal chaperonins. Protein folding activity in vitro has only been investigated in a few species of archaeal group II chaperonins, such as a native *Sulfolobus* chaperonin (Guagliardi et al. 1994; Guagliardi et al. 1995), a recombinant *Methanococcus* chaperonin (Furutani et al. 1998), a recombinant chaperonin from *Methanococcus maripaludis* (Kusmierczyk and Martin 2003), and recombinant and native chaperonins from *Thermococcus* sp. strain KS-1 (Yoshida et al. 1997, 2000, 2001).

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The group II chaperonin was found to cooperate with a novel chaperone, prefoldin (Geissler et al. 1998; Vainberg et al. 1998; Hansen et al. 1999; Martin-Benito et al. 2002). Prefoldin/GimC has been shown to participate in the maturation of actin and members of the tubulin family by transferring them in the unfolded state to a cytosolic chaperonin, CCT. Although there are no actin or tubulin proteins in archaea, prefoldin homologues have been identified as well as group II chaperonins (Vainberg et al. 1998; Leroux et al. 1999). Archaeal prefoldin consists of only two species of subunits, α and β, whereas eukaryotic prefoldins are composed of two different but related α-class subunits and four related β-class subunits. The crystal structure of the archaeal prefoldin from *Methanobacterium thermoautotrophicum* has been determined at a resolution of 2.3 Å (Siegert et al. 2000). It resembles a jellyfish in that its body consists of a double-β-barrel assembly with six long, tentacle-like coiled coils protruding from it. The distal regions of the coiled coils expose hydrophobic patches and are required for multivalent binding of non-native proteins (Siegert et al. 2000). The conformation of eukaryotic prefoldin was also shown by electron microscopy and was similar to that of archaeal prefoldin (Martin-Benito et al. 2002). However, the interaction and functional cooperation between chaperonin and prefoldin are not well understood. We have already reported that prefoldin from *Pyrococcus horikoshii* OT3 (PhPFD) cooperates functionally with both groups I and II chaperonins in the refolding of the green fluorescent protein (GFP) in vitro (Okochi et al. 2002).

In the present study, we have expressed, purified, and characterized the group II chaperonin from the hyperthermophilic archaeum, *P. horikoshii* OT3. The *Pyrococcus* chaperonin (PhCPN) existed as a homo-oligomer in a double-ring-shaped structure, and its activity as a molecular chaperone was characterized using citrate synthase (CS), isopropylmalate dehydrogenase (IPMDH), and GFP as substrates. The functional cooperation of PhCPN and PhPFD during ATP-dependent protein folding was also investigated.

Materials and methods

Plasmids, bacterial strains, and reagents

A plasmid for expressing IPMDH from *Thermus thermophilus* was obtained from Dr. Yamagishi of Tokyo University of Pharmacy and Life Science. IPMDH expressed in *Escherichia coli* was purified using a Butyl Toyopearl 650 column (Tosoh, Tokyo, Japan), and then by anion-exchange chromatography on a UnoQ6 (Bio-Rad, Calif., USA). The plasmid for expression of a mutant GFP (histidine-tagged GFP mutant with an extra alanine in the N-terminal region and substitutions of F99S, M153T, V163S, and L165F, which exhibits relatively high thermal stability), pET21C-GFP, was a gift from Dr. Taguchi of Tokyo Institute of Technology. It was purified as described previously (Iizuka et al. 2001). PhPFD was expressed and purified as described previously, except for the change of the gel-filtration column to a HiLoad 26/60 Superdex 200 (Amersham Biosciences, N.J., USA) (Okochi et al. 2002). *E. coli* strains used in this study were DH5α for the preparation of plasmids and BL21(DE3) for expression. Restriction enzymes, *ExTaq* DNA polymerase, and other reagents for gene manipulation were purchased from TaKaRa Shuzo (Kyoto, Japan). CS and ATP were obtained from Sigma–Aldrich (Mo., USA) and Wako Pure Chemicals (Osaka, Japan), respectively.

Polymerase chain reaction, cloning, and sequencing

A shotgun clone of *Pyrococcus horikoshii* OT3 containing a chaperonin ORF, PH0017, was used as a template for PCR amplification of the chaperonin gene (Kawarabayasi et al. 1998a, b). Oligonucleotide primers (5′-CAT-ATG-GCA-CAG-TTA-GCA-GGT-CA-3′ and 5′GGA-TCC-TCA-GTC-TAG-GTC-GCT-ACT-AC-3′) were designed to add *Nde*I and *Bam*HI restriction digestion sites at both ends of the chaperonin ORF. The amplified DNA fragment was subcloned in a pT7Blue T vector (Novagen, Wis., USA) by TA cloning. After sequence confirmation, the ORF was digested by *Nde*I and *Bam*HI, and then ligated into pET23b (Novagen) to obtain the plasmid, pPH0017E.

Expression and purification of *P. horikoshii* chaperonin

E. coli BL21(DE3), transformed with pPH0017E, was cultured at 37°C in 2×YT medium (1.6% tryptone, 1% yeast extract, and 0.5% NaCl) containing 100 μg/ml ampicillin without isopropyl-1-thio-β-D-galactoside induction. Cells were harvested by centrifugation and stored at −80°C. The harvested cells were resuspended in 50 mM TrisHCl (pH 7.5) and disrupted by sonication. The suspension of disrupted cells was centrifuged at 25,000 *g* for 45 min at 4°C, and $MgCl_2$, glycerol, and dithiothreitol were added to the supernatant to a final concentration of 25 mM, 5% (v/v) and 1 mM, respectively. The supernatant was heated at 80°C for 30 min, and denatured protein was removed by centrifugation (25,000 *g*, 45 min, 4°C). Then, it was applied to a DEAE Toyopearl column equilibrated with buffer A [50 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, and 1 mM dithiothreitol] containing 5% (v/v) glycerol. Proteins were eluted with a linear gradient of NaCl from 0–500 mM in the same buffer. Fractions containing PhCPN were collected and concentrated by ultra filtration (Centriprep YM-10, Millipore, Mass., USA). The concentrate was applied to a gel-filtration column (HiLoad 26/60 Superdex 200) equilibrated with buffer A containing 150 mM NaCl. Fractions containing PhCPN oligomers were then applied to an anion-exchange column (SOURCE 15Q PE 4.6/100, Amersham Biosciences)

equilibrated with buffer A and eluted with a linear gradient of NaCl. The eluted PhCPN oligomer was then loaded onto a gel-filtration column (Bio-Prep SE 1000/17, Bio-Rad) equilibrated with buffer A containing 100 mM NaCl. The eluted PhCPN oligomer was desalted and concentrated by ultra filtration (Centriprep YM-10). The purified PhCPN oligomer was analyzed using a HPLC gel-filtration column, G3000SW$_{XL}$ (Tosoh) with 50 mM Tris-HCl buffer (pH 7.3) containing 1 mM dithiothreitol, and 150 mM NaCl at a flow rate of 1.0 ml/min.

Electron microscopy

The purified PhCPN (1 mg/ml) was diluted tenfold in 50 mM Tris-HCl buffer (pH 7.3) containing 1 mM dithiothreitol and 150 mM NaCl. An aliquot of the PhCPN solution was mounted on carbon-coated grids, and then it was negatively stained with 1% uranyl acetate for 30 s. The excess of the solution was absorbed from the grids using pre-water-soaked filter paper. Transmission electron microscopy was performed with a PHILIPS Tecnai 20F at an anode voltage of 120 kV. Images were recorded at a magnification of ×50,000 onto Kodak electron image films (SO-163).

ATPase activity measurements

ATPase activity was assayed at temperatures between 65 and 95°C in 200 μl reaction buffer [12.5 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 150 mM KCl] containing 150 ng PhCPN. After 5 min preincubation at the assay temperature, the reaction was started by adding ATP to a final concentration of 1 mM. After incubation for 0, 5, 10, and 15 min, 20 μl reaction mixture was sampled and the reaction terminated by the addition of 780 μl 2% (w/v) perchloric acid at 4°C. The amount of phosphate released was measured by the malachite green assay (Baykov et al. 1988; Geladopoulos et al. 1991). The amount of ATP hydrolyzed without addition of PhCPN at each temperature was subtracted for the calculation of ATPase activity.

Thermal aggregation measurements of CS and IPMDH

Thermal aggregation of CS from porcine heart was monitored by measuring the light scattering at 500 nm with a spectrofluorophotometer (RF-5300PC, Shimazu, Kyoto, Japan) at 45°C with continuous stirring. Monitoring started with the addition of CS (150 nM as a monomer) to 50 mM Tris-HCl buffer (pH 8.0) preincubated at 45°C, with or without PhCPN.

Thermal aggregation of IPMDH from the thermophilic bacterium *T. thermophilus* was monitored at 90°C by measuring the absorbance at 360 nm with an UV-spectrophotometer (UV-1600PC, Shimadzu). PhCPN was added to 50 mM Tris-HCl buffer (pH 8.0) preincubated for 5 min at 90°C prior to the measurement; then, the monitoring started with the addition of IPMDH (final concentration, 600 nM). The mixture was continuously stirred during the measurement.

Fluorometric monitoring of GFP refolding

GFP (25 μM) was unfolded in 12.5 mM HCl. The unfolded GFP was diluted 150-fold and preincubated at 60°C in 50 mM Tris-HCl buffer (pH 7.5) containing 25 mM $MgCl_2$, 100 mM KCl, and 5 mM dithiothreitol. The fluorescence of GFP was continuously monitored with a spectrofluorophotometer (RF-5300PC) at an emission wavelength of 510 nm with excitation at 396 nm. To confirm the chaperone activity, PhCPN was added in the dilution buffer at a molar ratio of 1:3 (unfolded GFP:PhCPN) and the folding reaction started with the addition of ATP. To see the effect of PhPFD, PhPFD, and PhCPN were added in the dilution buffer at a molar ratio of 1:3:3 (unfolded GFP: PhCPN: PhPFD). ATP was added to a final concentration of 1 mM after 5 min incubation. The fluorescence of native GFP was also monitored and its intensity was taken as 100%.

IPMDH refolding measurements

IPMDH (6.0 μM) was denatured with 6 M guanidine hydrochloride in 1.0 M potassium phosphate buffer (pH 7.8) and incubated at room temperature for 30 min. Native IPMDH solution not containing guanidine hydrochloride was also prepared for the control experiment. The denatured IPMDH was diluted 60 times (final concentration, 0.1 μM) into 0.1 M potassium phosphate buffer (pH 7.8) containing 1.0 mM $MgCl_2$, 1.0 M KCl, and PhCPN (0.2 or 0.4 μM), and incubated at 68°C for 10 min. The IPMDH reaction was initiated with the addition of 0.9 mM NAD^+, 0.4 mM (2R*3S*)-3-isopropylmalic acid, and 1.0 mM ATP. Recovery of IPMDH activity was measured as a rate of increase in absorbance at 340 nm with continuous stirring. For the spontaneous refolding assay, PhCPN was omitted from the dilution buffer. To see the effect of PhPFD on the IPMDH refolding, 0.2 μM PhPFD was added in the dilution buffer instead of PhCPN. The cooperation of PhPFD and PhCPN was examined by the addition of 0.2 μM PhCPN to the above prepared PhPFD–IPMDH mixture 5 min prior to the start of assay.

Other methods

Proteins were analyzed by polyacrylamide gel electrophoresis on 10% polyacrylamide gels containing SDS (SDS-PAGE) or 6% polyacrylamide gels without SDS (native-PAGE). Gels were stained with Coomassie Brilliant Blue R-250. Protein concentrations were

measured by the method of Bradford with bovine serum albumin as the standard (Bradford 1976).

Results

Expression and purification of the recombinant PhCPN

Pyrococcus species contain only one chaperonin gene per whole genome (Archibald and Roger 1999). The chaperonin of *Pyrococcus horikoshii* OT3 (PhCPN) was expressed in *Escherichia coli* BL21(DE3), using an expression vector, pET23b. PhCPN was overexpressed in the soluble fraction and partially purified by heat treatment at 80°C for 30 min. PhCPN was further purified by ion-exchange and gel-filtration chromatography. Figure 1 shows the SDS and native-PAGE of purified PhCPN. Although not homogeneous, the preparation is sufficiently pure for studying ATPase activity and protein refolding. HPLC gel-filtration chromatography of the purified chaperonin showed that it existed as a homo-oligomer. However, a significant proportion of oligomers dissociated in native gel electrophoresis (Fig. 1). Electron microscopy observation of the negatively stained PhCPN revealed that it existed as a double-ring-shaped structure with a diameter of about 16 nm (Fig. 2). Recently, we determined the crystal structure of group II chaperonin from *Thermococcus* sp. strain KS-1 (Shomura 2004). It existed as a hexadecameric homo-oligomer. Since PhCPN is highly homologous to the strain KS-1 chaperonin, we think that PhCPN also exists as a hexadecameric homo-oligomer.

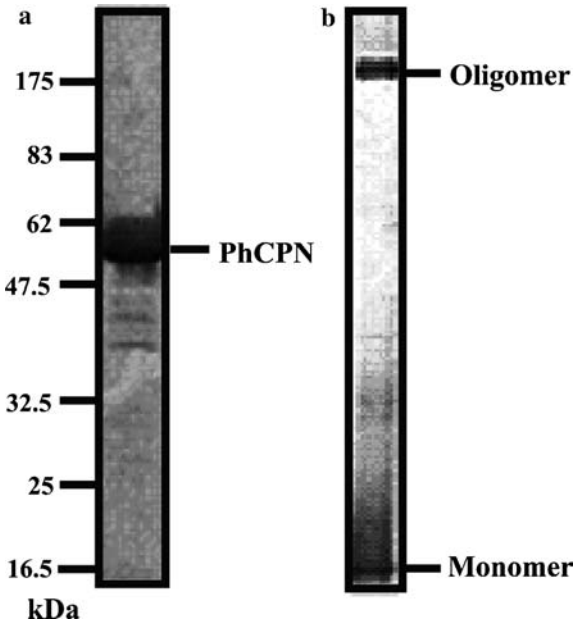

Fig. 1 SDS (**a**) and native-polyacrylamide gel electrophoresis (**b**) of purified group II chaperonin of *Pyrococcus horikoshii* OT3 (*PhCPN*)

ATPase activity

The ATPase activity of the recombinant PhCPN was measured at temperatures between 50 and 95°C (Fig. 3). The PhCPN oligomer showed weak ATPase activity, which intensified with temperature. The highest ATPase activity of PhCPN was obtained at 90°C (0.042 μmol/min/mg), which is close to the optimal growth temperature of *P. horikoshii*. The ATPase activity of PhCPN at 90°C was comparable with the chaperonin of *Thermococcus* sp. strain KS-1 (Iizuka et al. 2001).

Prevention of thermal aggregation of CS and IPMDH by PhCPN

The effects of PhCPN on the thermal aggregation of CS from porcine heart and IPMDH from *Thermus*

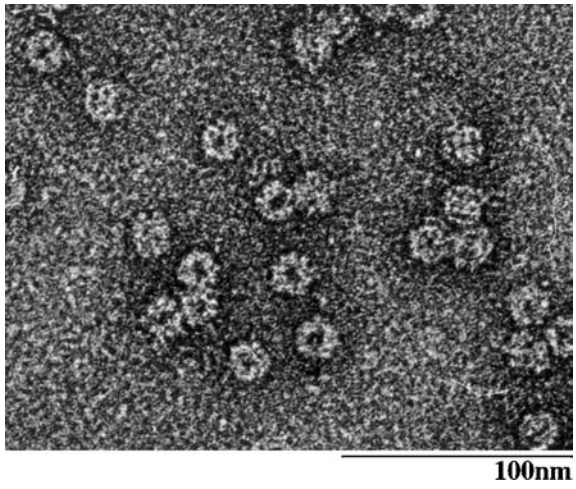

Fig. 2 Electron micrograph of the negatively stained PhCPN

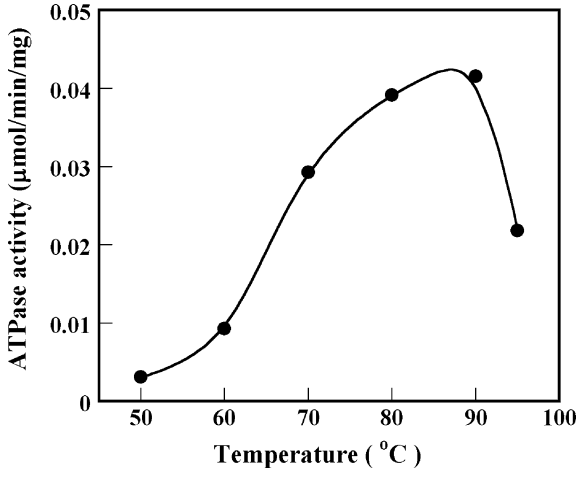

Fig. 3 ATPase activities of PhCPN measured at temperatures between 50–95°C. PhCPN (150 ng) was preincubated in the reaction buffer for 5 min at the indicated temperatures, and the ATPase reaction was started with the addition of ATP and terminated with 2% perchloric acid. The amount of phosphate released was measured by malachite green assay

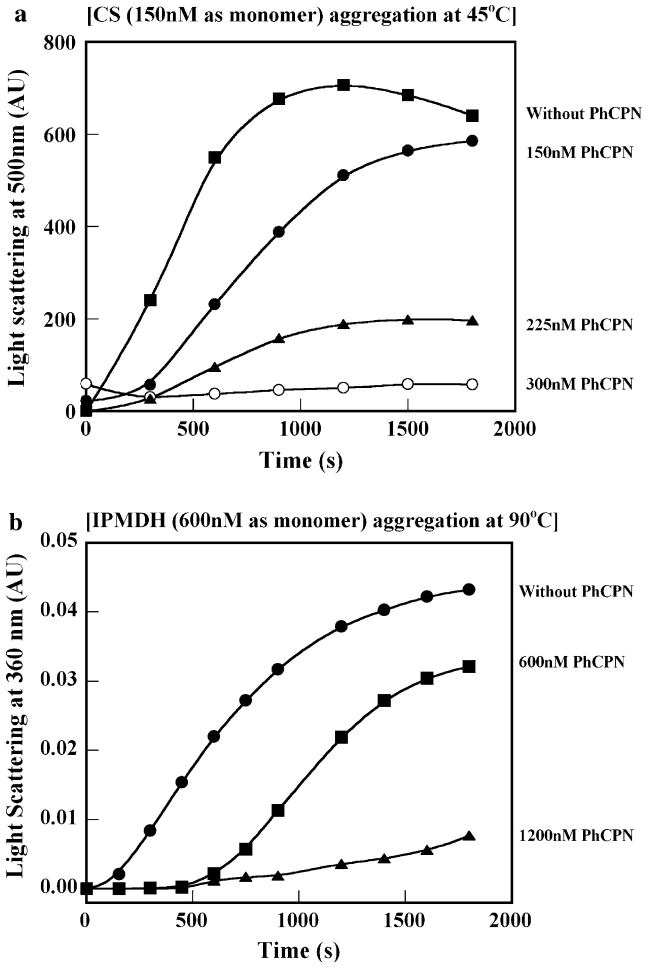

Fig. 4 Prevention of the thermal aggregation of citrate synthase (*CS*) and isopropylmalate dehydrogenase (*IPMDH*) by PhCPN. **a** CS at the concentration of 150 nM as a monomer was incubated in a spectroflurophotometer cell at 45°C in the buffer containing 50 mM Tris-HCl (pH 8.0) plus PhCPN of 0 nM (*closed squares*), 150 nM (*closed circles*), 225 nM (*closed triangles*), and 300 nM (*open circles*). The formation of aggregation was monitored by measuring apparent light scattering at 500 nm with a spectrofluorophotometer. **b** IPMDH at 600 nM as a monomer was incubated in a spectrophotometer cell thermostatted at 90°C in 50 mM Tris-HCl (pH 8.0) buffer in the absence of PhCPN (*closed circles*) or in the presence of 600 nM (*closed squares*), and 1,200 nM (*closed triangles*) PhCPN. The aggregation of IPMDH was monitored by measuring apparent light scattering at 360 nm with a spectrophotometer

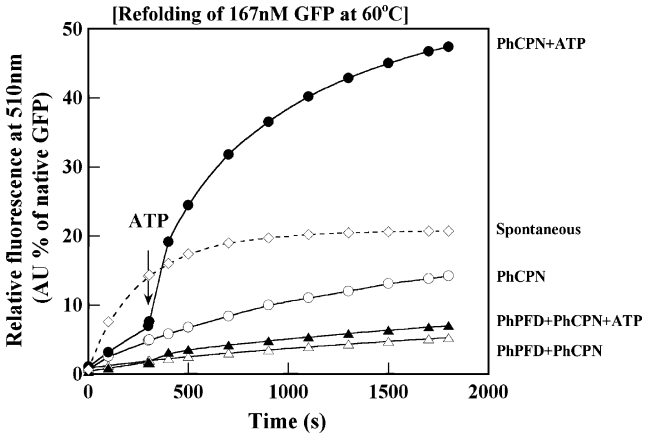

Fig. 5 Refolding of green fluorescent protein (*GFP*) mediated by PhCPN. A reaction mixture containing 167 nM GFP was incubated at 60°C with 500 nM PhCPN (*closed circles*, *open circles*) or without (*open diamonds*). The effect of the addition of 500 nM PhPFD and 500 nM PhCPN (*closed triangles*, *open triangles*) to the folding mixture was also investigated. The recovery of GFP fluorescence was continuously monitored at an emission wavelength of 510 nm with excitation at 396 nm. The fluorescence intensity of native GFP at the same concentration was taken as 100%. The *arrow* indicates the time of addition of ATP (final concentration, 1 mM, *closed circles*, *closed triangles*)

thermophilus HB8 were investigated. When CS was diluted in 50 mM Tris-HCl buffer preincubated at 45°C, it aggregated with increased light scattering. When PhCPN was included in the buffer, the aggregation of CS was inhibited (Fig. 4a). At a 1:1 molar ratio of PhCPN to CS, the heat aggregation reduced to 70–80% of that in the absence of PhCPN. CS was almost completely protected from thermal aggregation when the proportion of PhCPN:CS exceeded 2:1 (300 nM PhCPN).

The molecular chaperone activity of PhCPN was also examined using a thermophilic enzyme, IPMDH. IPMDH is a homodimer with a subunit molecular weight of about 37 kDa (Imada et al. 1991). Although it is much more stable against thermal denaturation than mesophilic enzymes, it denatures to aggregate at 90°C, as detected by light scattering at 360 nm. Aggregation was almost completely prevented when a twofold excess of PhCPN was included (Fig. 4b). It has thus been shown that addition of twofold molar excess of PhCPN could prevent aggregation of both CS and IPMDH at 45 and 90°C, respectively.

GFP refolding by PhCPN

The ATP-dependent protein refolding activity of PhCPN was examined at 60°C, using GFP, a monomeric protein of 27 kDa, as the substrate (Fig. 5). When the acid-denatured GFP, which has no fluorescence, was diluted in the folding buffer in the absence of PhCPN, GFP refolded spontaneously, and its fluorescence recovered up to 21%. In the presence of PhCPN in the folding mixture at a molar ratio of 3:1 (PhCPN:GFP), spontaneous refolding of GFP was inhibited. An addition of ATP induced the enhancement of GFP fluorescence. These results showed that PhCPN was able to capture the unfolded GFP to prevent it from spontaneous refolding and subsequently, to refold it in an ATP-dependent manner.

In the presence of PhPFD and PhCPN in the folding mixture (at molar ratio of 3:3:1 with PhPFD:PhCPN:GFP), the spontaneous refolding of GFP was inhibited more extensively compared with reactions without PhPFD. However, refolding of GFP was only slightly induced by the addition of ATP. Taking into account these results, the unfolded GFP seemed to be tightly

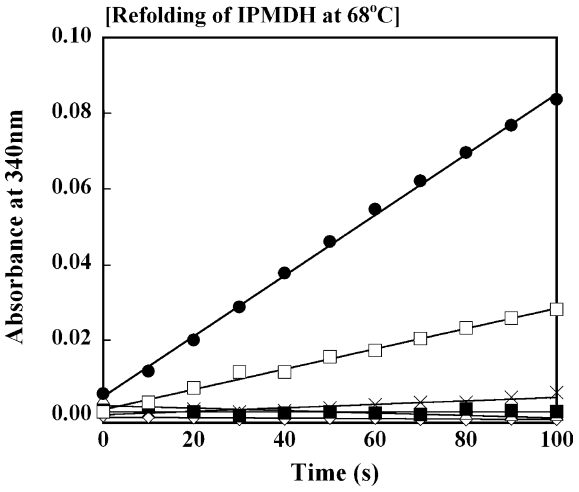

Fig. 6 *Pyrococcus* prefoldin (PhPFD)-assisted refolding of IPMDH by PhCPN. IPMDH from the thermophilic bacterium, *Thermus thermophilus* was denatured in 6 M guanidine HCl, and then diluted by 50-fold (final concentration, 0.2 μM) in dilution buffer containing 0.4 μM PhCPN (*closed squares*); 0.4 μM PhCPN and 1 mM ATP (*cross-hatches*); 0.8 μM PhCPN and 1 mM ATP (*open squares*); 0.4 μM PhPFD (*open triangles*); 0.4 μM PhPFD, 0.4 μM PhCPN, and 1 mM ATP (*closed circles*); or nothing (*open diamonds*)

captured by PhPFD and not effectively transferred to PhCPN for refolding.

Prefoldin-assisted refolding of IPMDH by PhCPN

The effect of PhPFD on the refolding of IPMDH by PhCPN was examined at 68°C (Fig. 6). The native IPMDH is stable at 68°C; thus, the absorbance at 340 nm immediately increases due to the generation of NADH (data not shown). However, spontaneous refolding of the denatured IPMDH (0.1 μM) was not observed at this temperature. When PhCPN at 0.2 μM and ATP were added to the dilution buffer, a little increase in absorbance was observed. In the presence of PhCPN at 0.4 μM, the activity of IPMDH was recovered up to 3% of for the specific activity of native IPMDH. In contrast, when PhPFD was included in the dilution buffer and PhCPN was added 5 min after the incubation, the refolding of IPMDH was further enhanced and the highest yield in IPMDH refolding (8% recovery as compared with the native IPMDH) was obtained. These results showed that refolding of IPMDH was enhanced by cooperative effect of PhPFD and PhCPN. We present evidence that PhPFD plays a role as a trap for denatured IPMDH.

Discussion

The molecular chaperone systems of hyperthermophilic archaea are relatively simple, and only group II chaperonins and small heat shock proteins have been found in their genome sequences. HSP70/HSP40 chaperones, previously thought to be ubiquitous and important in all organisms, are absent (Gribaldo et al. 1999; Laksanalamai et al. 2004). Therefore, the group II chaperonin and its co-factor, prefoldin, might play the major role in the molecular chaperone systems for hyperthermophilic archaea.

The chaperonins of *Pyrococcus* species consist of one subunit and are highly homologous to the β subunits of *Thermococcus* sp. KS-1 chaperonin (Yoshida et al. 1997). *Thermococcus* sp. KS-1 chaperonin is composed of two highly homologous subunits, and both subunits can assemble into homo-oligomers, with full protein folding activity (Yoshida et al. 1997, 2000). Interestingly, subunit composition of *Thermococcus* sp. KS-1 chaperonin changes with growth temperatures, and the thermostability of the natural hetero-oligomeric chaperonin increases as the increase of β-subunit content (Yoshida et al. 2001). The chaperonin homo-oligomer composed of only β subunits is thermodynamically more stable than that of α subunits (Yoshida et al. 2002). The fact that *Pyrococcus* species express homologues of the β subunit of *Thermococcus* sp. KS-1 may reflect extremely high thermotolerance and also their relatively high lower-temperature limit for growth of *Pyrococcus horikoshii*.

In the present study, we have carried out functional characterization of the chaperonin from *P. horikoshii*. PhCPN was highly expressed in *Escherichia coli*, assembled into a hexadecameric homo-oligomer, which exhibited hyperthermophilic ATPase activity, protected porcine heart citrate synthase and thermophilic IPMDH, and from thermal aggregation at 45 and 90°C, respectively. It arrested spontaneous refolding of acid-denatured GFP and assisted refolding of GFP in an ATP-dependent manner. Contrary to the previous observation using PhPFD and chaperonins from *Thermococcus* sp. strain KS-1 and *Thermococcus thermophilus* HB8 (Okochi et al. 2002), PhCPN-dependent GFP refolding was not enhanced by PhPFD (Fig. 5). The captured GFP by PhPFD might not be efficiently transferred from PhPFD to PhCPN. Recently, we have found that the interaction of PhPFD with PhCPN was stronger than that with other chaperonins (Okochi et al. 2004). Thus, the inefficiency is not due to the problem in prefoldin–chaperonin interaction. The plausible interpretation is that the transfer of the unfolded protein from PhPFD to PhCPN might be limited at 60°C. Another interpretation might be that the unfolded GFP captured by PhPFD was transiently transferred to PhCPN; however, refolding of GFP did not occur efficiently. On the other hand, denatured IPMDH was captured by PhPFD and was transferred to PhCPN showing ATP-dependent folding at 68°C (Fig.6). The refolding efficiency was much higher than that without PhPFD. We propose that transfer of substrate occurs as a result of the difference of affinity. Normally, affinity for substrate of hyperthermophilic chaperonin decreases at lowered temperature. Therefore, the difference in refolding efficiency might be caused by the difference in

affinities for substrate of PhCPN and PhPFD at the different temperatures adopted for the measurement. It is also possible that the substrate specificities of PhPFD and PhCPN may be critical.

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