J. Biochem. 2012;151(4):383–390 doi:10.1093/jb/mvr141

JB THE JOURNAL OF BIOCHEMISTRY

Varied effects of *Pyrococcus furiosus* prefoldin and *P. furiosus* chaperonin on the refolding reactions of substrate proteins

Received September 29, 2011; accepted December 14, 2011; published online December 30, 2011

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Prefoldin is a molecular chaperone found in the archaeal and eukaryotic cytosol. Prefoldin can stabilize tentatively nascent polypeptide chains or non-native forms of mainly cytoskeletal proteins, which are subsequently delivered to group II chaperonin to accomplish their precise folding. However, the detailed mechanism is not well known, especially with regard to endogenous substrate proteins. Here, we report the effects of *Pyrococcus furiosus* prefoldin (PfuPFD) on the refolding reactions of *Pyrococcus furiosus* citrate synthase (PfuCS) and *Aequorea* enhanced green fluorescence protein (GFPuv) in the presence or absence of *Pyrococcus furiosus* chaperonin (PfuCPN). We confirmed that both PfuPFD and PfuCPN interacted with PfuCS and GFPuv refolding intermediates. However, the interactions between chaperone and substrate were different for each case, as was the final effect on the refolding reaction. Effects on the refolding reaction varied from passive effects such as ATP-dependent binding and release (PfuCPN towards GFPuv) and binding which leads to folding arrest (PfuPFD towards GFPuv), to active effects such as net increase in thermal stability (PfuCPN towards PfuCS) to an active improvement in refolding yield (PfuPFD towards PfuCS). We postulate that differences in molecular interactions between substrate and chaperone lead to these differences in chaperoning effects.

Keywords: chaperonin/citrate synthase/prefoldin/protein folding/*Pyrococcus furiosus*.

Abbreviations: CCT, chaperonin containing t-complex polypeptide; GFP, green fluorescent protein; PfuCPN, *Pyrococcus furiosus* chaperonin; PfuCS, *Pyrococcus furiosus* citrate synthase; PfuPFD, *Pyrococcus furiosus* prefoldin.

Molecular chaperones have been defined as a class of cellular proteins whose function is to assist in the correct folding, successful oligomeric assembly, translocation and degradation of proteins (*1–3*). The functional characteristics of cytosolic chaperones can be divided roughly according to two distinct mechanisms. Molecular chaperones such as trigger factor or HSP70 perform by holding nascent polypeptides and non-native proteins temporarily. In contrast, toroidal chaperonin complexes, such as HSP60, provide a physically secluded space to prevent protein misfolding and aggregation in the crowded environment of the cell (*4*).

Chaperonins are ubiquitous large protein complexes consisting of two 7- to 8-fold symmetrical rings. The complexes assist folding of protein to its native state by binding to and encapsulating non-native proteins within their central cavity, and releasing these proteins in an adenosine 5′-triphosphate (ATP) dependent, repetitive manner (*5–7*). Chaperonins are classified into two subfamilies: one is group I chaperonins found in mitochondria, chloroplasts and the bacterial cytosol, which cooperate with co-chaperonin HSP10 during the substrate encapsulation reaction. The other is group II chaperonins in archaea and the eukaryotic cytosol, which have a helical protrusion motif in their apical domain as a built-in lid (*8*).

Prefoldin, also known as the GimC/Gim proteins, is a hexameric molecular chaperone complex consisting of members from two related classes of subunits. Prefoldin is present in all eukaryotes and archaea, but absent in bacteria (*9*). Eukaryotic prefoldins assemble into a unique hetero hexameric structure containing two related prefoldin α-subunits [PFDN3 (von Hippel–Lindau binding protein 1) and PFDN5] and four related prefoldin β-subunits [PFDN1, PFDN2, PFDN4 and PFDN6 (human leukocyte antigen (HLA) class II region expressed from gene KE2)] (*9–11*). In contrast, archaeal organisms possess only one or two members of each subunit class (*12–14*). Prefoldins were originally found in yeast and characterized by their ability to facilitate the formation of functional tubulins (*15*). Eukaryotic prefoldin interacts with nascent polypeptide chains to stabilize the folding of cytoskeletal proteins (*16*) and transfers these proteins selectively to cytosolic chaperonin (*9*, *11*, *12*). This transfer of target protein from prefoldin to chaperonin occurs rapidly and is independent of nucleotide (*10*). The crystal structure of the archeal prefoldin from *Methanobacterium thermoautotrophicum* has been revealed to be a jellyfish-like structure consisting of two α- and four β-subunits, forming a double β-barrel assembly with six long tentacle-like coiled coils protruding from it. The distal regions of the coiled coils display hydrophobic patches and are responsible for multivalent binding of non-native proteins (*17*).

Although chaperonin and prefoldin are found to exist in archaea, no HSP70 homologues have been

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found so far (*18*–*20*), and cytoskeletal proteins such as actin and tubulin are also not found. Archaeal prefoldins have been demonstrated to protect the aggregation of some heterogeneous proteins such as hen lysozyme, bovine mitochondrial rhodanese, glucose dehydrogenase from *Thermoplasma acidophilum*, and porcine citrate synthase (*12*, *14*, *21*, *22*). Recently, Zako *et al*. (*23*) reported that *P. horikoshii* prefoldin facilitated the refolding of denatured lysozyme, at a temperature (25°C) relatively lower than the physiologically active temperature of *P. horikoshii* (98°C). Importantly, it has been reported that GimC from *M. thermoautotrophicum* could form heterogeneous complexes with yeast homologues, and the Gimβ from *M. thermoautotrophicum* was also able to partially complement yeast mutants lacking *GIM1* or *GIM4* (*12*).

We describe here our experiments on the effects of two *Pyrococcus furiosus* molecular chaperones, the chaperonin PfuCPN and the prefoldin PfuPFD, on the refolding reactions of an endogenous substrate protein citrate synthase (PfuCS) and an exogenous substrate green fluorescent protein (GFPuv). We found that both molecular chaperones could interact with each substrate protein in a specific manner during refolding, but the consequences of each interaction were different. Effects ranged from a passive interaction that led to folding arrest to an active assistance of refolding that resulted in an improved refolding yield. Interestingly, in our experiments we found that PfuPFD, and not PfuCPN, was able to elicit a net increase in refolding yield during the refolding reaction of PfuCS. The ability of PfuPFD to actively improve the refolding of endogenous substrate proteins at high temperatures (70°C) is a novel characteristic, not seen for other prefoldins reported to date.

Materials and Methods

Proteins and reagents

Chaperonin (PfuCPN) from *P. furiosu*s was purified from *Escherichia coli* BLR(DE3)/pET23a(+)PfuCPN (*24*). The harvested cells were disrupted in sonication buffer [50 mM HEPES–KOH pH 8.0, containing 0.5 mg/ml Pefabloc SC (Roche Diagnostics K.K.)] on ice. After the centrifugation, the supernatant was heated at 90°C for 15 min and immediately cooled on ice. Heat-aggregated proteins were removed by centrifugation and filtration. The soluble proteins were applied to a Resource-Q anion exchange column (Amersham Biosciences), which had been equilibrated with 50 mM Tris–HCl buffer, pH 8.5 and eluted with a 0–1 M KCl linear gradient using the ÄKTA$_{FPLC}$ system (Amersham Biosciences) at 4°C. Fractions containing PfuCPN were pooled and concentrated using Amicon ultra-15 centrifugal filter units with ultracel-50 membrane (Millipore). The concentrated PfuCPN (~20 mg/ml) was loaded onto Superose 6 10/300 GL column (GE Healthcare) equilibrated with size-exclusion chromatography buffer (50 mM HEPES–KOH, pH 7.0, containing 150 mM KCl). The purity of PfuCPN was evaluated by SDS–PAGE and dynamic light scattering measurements (Otsuka Electronics Co. Ltd. FDLS-3000). The fractions containing PfuCPN were collected carefully and concentrated to ~5 mg/ml with Amicon ultra-15 centrifugal filter units. The purified protein was stored at −80°C until use.

Prefoldin α and β genes were cloned by PCR amplification from *P. furiosus* genomic DNA (*25*) using gene-specific primers. The *P. furiosus* PfuPFDα gene corresponds to gene locus PF0375 of the *P. furiosus* genomic sequence and the entire locus was cloned. The PfuPFDβ gene was obtained from gene locus PF0382, identified as a putative prefoldin β gene. Homology analysis suggested strongly that the actual prefoldin ORF corresponded to a truncated segment of this locus that started from the 57th methionine residue of the open reading frame (see 'Results' section), and so this truncated fragment was cloned and used for expression. Expression of these two genes was accomplished by incorporating the two fragments in tandem into the multicloning site (MCS) of pET23a(+), with PfuPFDα inserted between the NdeI and BamHI sites of the MCS and PfuPFDβ inserted between the BamHI and EcoRI sites of the MCS. In order to assure expression of the downstream PfuPFDβ gene, a synthetic oligonucleotide fragment derived from the T7 promoter and ribosomal binding sites (*taatacgactcactatagggcgaaggagatatatat)* was inserted immediately adjacent and upstream of the PfuPFDβ ATG start codon.

Purification of PfuPFD was performed according to the methods of Iizuka *et al*. (*14*) with some modifications. The cells were sonicated in buffer (50 mM HEPES–KOH pH 8.0, containing 2 mM EDTA) on ice. After the centrifugation, the supernatant was heated to 80°C for 30 min and then immediately cooled on ice. Heat-aggregated proteins were removed by centrifugation and filtration and the soluble proteins were applied to a Resource-Q anion exchange column, which had been equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 2 mM EDTA and eluted with a 0–1 M KCl linear gradient using the ÄKTA$_{FPLC}$ system at 4°C. Fractions containing PfuPFD were pooled and concentrated using Amicon ultra-15 centrifugal filter units with ultracel-10 membrane (Millipore). The concentrated PfuPFD (~20 mg/ml) was loaded onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with size-exclusion chromatography buffer. Next, fractions containing PfuPFD were applied to a HiPrep 16/10 Heparin FF affinity column equilibrated previously with 50 mM HEPES–KOH, pH 6.5 and eluted with a 0–1 M KCl linear gradient. The purity of PfuPFD was analysed by SDS–PAGE. The PfuPFD fractions were collected and concentrated to the protein concentration of ~5 mg/ml with Amicon ultra-15 centrifugal filter units.

Citrate synthase (PfuCS) gene from *P. furiosus* gene locus PF0203 was cloned from *P. furiosus* genomic DNA by PCR amplification using gene-specific primers (*25*). The amplified PfuCS gene was confirmed by DNA sequence analysis, and the amplified gene was ligated into the MCS of pET23a(+) using the NdeI and HindIII sites. The protein was expressed in *E. coli* BLR(DE3) . Purification of PfuCS was performed according to the method of Muir *et al*. (*26*) with some modifications. The harvested cells were sonicated in buffer (50 mM HEPES–KOH pH 8.0, containing 2 mM EDTA, 20 mM KCl) on ice. After the centrifugation, the supernatant was heated at 85°C for 15 min and immediately cooled on ice. Heat-aggregated proteins were removed by centrifugation and filtration and the soluble proteins were applied to a Toyopearl AF-Red-650 affinity column (Tosoh Corporation), equilibrated previously in wash buffer (50 mM Tris–HCl, pH 7.0, containing 150 mM KCl). After washing the column (~6 column volumes), PfuCS was eluted with elution buffer of 50 mM Tris–HCl, pH 7.0, containing 150 mM KCl, 5 mM oxaloacetate and 1 mM CoA. Fractions containing PfuCS were pooled and concentrated using Amicon ultra-15 centrifugal filter units with ultracel-30 membrane (Millipore). The concentrated PfuCS (~10 mg/ml) was loaded onto Sephacryl S-300 column (GE Healthcare), which had been equilibrated with size-exclusion chromatography buffer. The purity of the protein was analysed by SDS–PAGE. The PfuCS fractions were collected and concentrated to ~5 mg/ml with Amicon ultra-15 centrifugal filter units.

GroE was expressed in *E. coli* JM109/pUCESL and purified according to the published protocols (*27*).

GFPuv was purified from *E. coli* BL21(DE3)/pGFPuv, as described previously (*28*).

Refolding of GFPuv and PfuCS

GFPuv was unfolded by acid as follows: 10 μM GFPuv was incubated in 12.5 mM HCl containing 5 mM DTT at room temperature for 30 min. Refolding reaction was started by 200-fold dilution of the acid-denatured GFPuv into refolding buffer (50 mM HEPES–KOH, pH 7.1, containing 1 M KCl, 3 mM $MgCl_2$ and 1 mM ATP) containing 0.2 μM PfuCPN/0.025–0.2 μM PfuPFD at 60°C. Refolded GFPuv fluorescence was continuously measured using a JASCO FP-6300 fluorescence spectrometer at 509 nm upon excitation at 395 nm with continuous stirring (*28*). The fluorescence intensity of 0.05 μM native GFPuv was set as 100%.

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PfuCS was unfolded as follows: 19 μM PfuCS was incubated in 6 M guanidine hydrochloride at 70°C for 10 min. Refolding reaction was started by 100-fold dilution of the denatured PfuCS into refolding buffer (50 mM HEPES–KOH, pH 7.0, containing 1 M KCl, 3 mM $MgCl_2$ and 2 mM ATP) containing 0.38–0.57 μM PfuCPN/0.095–0.86 μM PfuPFD at 70°C or 0.38 μM GroEL/GroES at 25°C. At the indicated time points, 50 μl of the reaction mixture were withdrawn and added to 950 μl enzyme activity assay buffer (50 mM HEPES–KOH, pH 8.0, containing 150 mM KCl, 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 47 μM acetyl-CoA, 0.2 mM oxaloacetic acid and 2 mM EDTA), which had been pre-incubated at 50°C. The enzymatic activity of PfuCS was assayed by measuring the increase in absorbance of 412 nm (*26*, *29*) using a Hitachi U-2000 or U-2000A spectrophotometer at 50°C. Average values from three independent native PfuCS (0.19 μM) activity measurements was set as 100%.

Protection of PfuCS from thermal inactivation

The ability of PfuCPN to protect protein from heat inactivation was examined using PfuCS at 70°C. The experiments were started by 100-fold dilution (final protein concentration, 0.19 μM) of PfuCS into reaction buffer (50 mM HEPES–KOH, pH7.0, containing 1 M KCl and 3 mM $MgCl_2$) with or without 0.57 μM PfuCPN.

ATP hydrolysis activity assay

ATP hydrolysis activity of reaction mixtures containing 0.2 μM PfuCPN was measured at 60°C in ATPase assay buffer (50 mM HEPES–KOH, pH 7.1, containing 1 M KCl and 3 mM $MgCl_2$), according to the published protocols (*28*). We confirmed that addition of 1 M KCl to the assay was critical for the maximum ATPase activity of PfuCPN (*30*). The amounts of inorganic phosphate were measured colorimetrically with the malachite green reagent (*31*) and the amounts of inorganic phosphate yielded by spontaneous hydrolysis of ATP under the conditions were subtracted.

Results

Preparation of highly purified PfuCPN

In a previous study concerning the PfuCPN protein, we reported that the ATPase activity of recombinant PfuCPN samples showed a strong ATPase activity in the presence of Mn^{2+} or Co^{2+} ions, but not in the presence of Mg^{2+} ions (*24*). Subsequently, it was pointed out that recombinant PfuCPN may be purified in both monomeric and oligomeric forms (*30*), and that differences between these two chaperonin forms may be the cause of our previous results (*30*), suggesting that some functional aspects of this protein may be sensitive to the purity of its preparations. In the present study, we used additional steps to ensure that we were observing the characteristics of the oligomeric form of PfuCPN, mainly by purifying samples using size-exclusion chromatography and monitoring protein preparations using dynamic light scattering. Our present findings reflect the characteristics of the oligomeric form of PfuCPN (Supplementary Fig. S1).

Refolding of denatured GFPuv in the presence of PfuCPN and ATP

First, in order to evaluate the chaperonin function of PfuCPN (16-mer), we investigated its ATPase activities in the presence of Mg^{2+}, as well as its ability to assist the refolding of acid-denatured GFPuv at 60°C (*32*, *33*). As shown in Fig. 1A, when the acid-denatured GFPuv was diluted into the refolding buffer without PfuCPN, GFPuv refolded spontaneously, and its fluorescence intensity was recovered to ~60% of that of native GFPuv within 600 s (Fig. 1A, open circles).

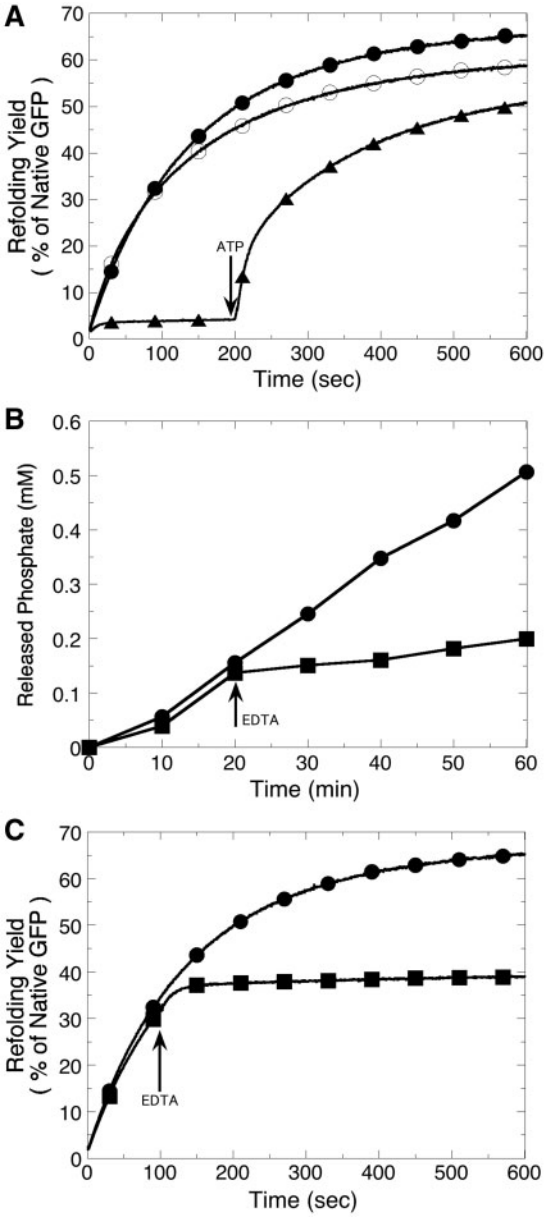

Fig. 1 PfuCPN assisted refolding of GFPuv in the presence of Mg-ATP. (A) GFPuv was unfolded in 12.5 mM HCl containing 5 mM DTT and allowed to refold in refolding mixtures at 60°C. Closed circles indicate the mixture in the presence of 0.2 μM PfuCPN with Mg-ATP. Closed triangles indicate the mixture in the presence of 0.2 μM PfuCPN without ATP, ATP (final concentration, 1 mM) was added to the refolding mixture at 200 s as indicated by an arrow. Opened circles indicate refolding in buffer only. (B) ATP hydrolysis activity in the presence of PfuCPN with or without EDTA. The experimental temperature was 60°C. Closed circles indicate the mixture in the presence of 0.2 μM PfuCPN, 3 mM $MgCl_2$ and 2 mM ATP. Closed squares indicate the mixture in the presence of 0.2 μM PfuCPN, 3 mM $MgCl_2$ and 2 mM ATP, EDTA (final concentration, 10 mM) was added to the mixture at 20 min as indicated by an arrow. The amount of inorganic phosphate was measured by colorimetric reagent assay. (C) The refolding of GFPuv in the presence of PfuCPN with or without EDTA. The acid-denatured GFPuv was diluted into the refolding buffer at 60°C. Closed circles indicate the mixture in the presence of 0.2 μM PfuCPN and Mg-ATP. Closed squares indicate the mixture in the presence of 0.2 μM PfuCPN and Mg-ATP, EDTA (final concentration, 5 mM) was added to the refolding mixture at 100 s as indicated by an arrow.

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In the presence of 0.2 μM PfuCPN and 1 mM ATP, this refolding yield was improved to 65%, slightly better than the spontaneous reaction (Fig. 1A, closed circles). However, in the absence of ATP, an increase in GFPuv fluorescence was not observed, and when ATP was added at 200 s to this mixture, a sharp increase in fluorescence was observed and the fluorescence intensity recovered to ~50% of native values within the next 400 s (Fig. 1A, closed triangles). The result showed that the highly purified PfuCPN has the ability to bind and release folding intermediates of GFPuv in an ATP-dependent manner.

Next, in order to clarify the role of ATP during the refolding reaction, we examined a relationship between ATP hydrolysis activity and protein folding activity of PfuCPN at 60°C, as shown in Fig. 1B and C. Magnesium ion was required for the chaperonin to exert ATPase activity (*5–7*). As shown in Fig. 1B, when EDTA, a chelating reagent of magnesium ion, was added to the mixture in the presence of PfuCPN with Mg-ATP, an immediate halt of the ATPase activity was observed (Fig. 1B, closed squares). In refolding assays of GFPuv, when we added EDTA to the refolding mixture after a 100 s refolding interval, we found that the refolding of GFPuv proceeded for a short interval immediately after EDTA addition, followed by the suppression of further fluorescence recovery after ~50 s (Fig. 1C, closed squares). Adding non-hydrolysable ATP analogues such as ATP-γ-S or ADP also failed to recover GFP fluorescence (data not shown). These results showed that PfuCPN mediated the refolding of GFPuv in a manner associated with ATP-hydrolysis driven release and rebinding. The fact that we could suppress GFPuv recovery by the delayed addition of EDTA after a ~100 s refolding interval suggests that interactions between PfuCPN and GFPuv molecules persist throughout this time frame, and when compared to the ATPase rate, suggests strongly that the refolding of GFPuv in the presence of PfuCPN is a multi-cyclic molecular event.

Suppression of the GFPuv refolding by PfuPFD

Initially, the gene fragment cloned from the locus originally annotated as PfuPFD β-subunit (PF0382) was not expressed in *E. coli* cells under the conditions we tested. Therefore, we compared this gene with PfuPFD β genes from other archaeal prefoldin β proteins, and found that the annotated PfuPFD β contained an extra peptide segment in the amino-terminal region (Supplementary Fig. S2). We decided to modify the isolated gene fragment to truncate this peptide region. The resultant modified gene was expressed well in *E. coli*. PfuPFD (α2 and β4 complexes) were purified from *E. coli* BLR(DE3) cells that harboured an expression vector that contained PfuPFD α and β genes in tandem. The purified products were analysed by gel-filtration and SDS–PAGE to confirm the subunit composition (Supplementary Fig. S3). The composition of PFD α:PFD β in purified oligomers was found to vary between 2:3 and 2:4.

Using this purified product, we next examined its effects on the refolding of acid-denatured GFPuv in

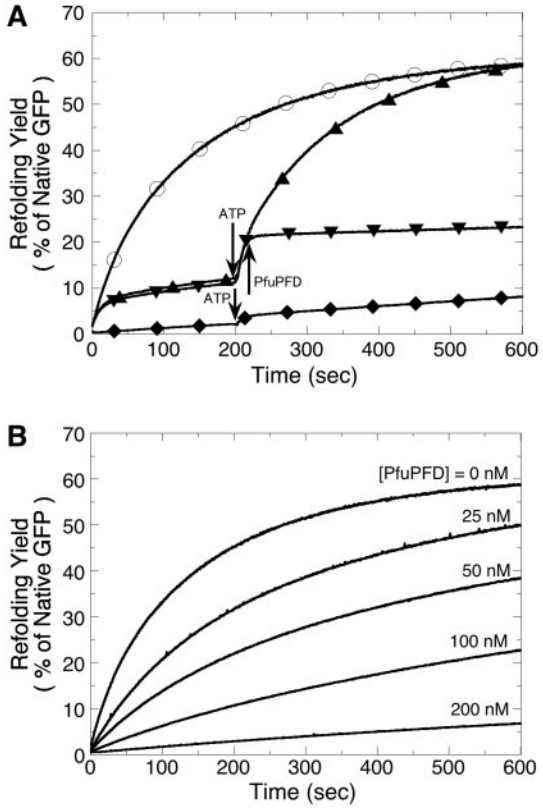

Fig. 2 PfuPFD inhibited the GFPuv refolding by interacting with intermediates. (A) The refolding of GFPuv in the presence of PfuPFD with PfuCPN. The acid-denatured GFPuv was diluted into the refolding buffer at 60°C. Closed diamonds indicate the mixture in the presence of 0.3 μM PfuPFD/0.2 μM PfuCPN without ATP, ATP (final concentration, 1 mM) was added to the refolding mixture at 200 s as indicated by an arrow. Inverted closed triangles indicate the mixture in the presence of 0.2 μM PfuCPN without PfuPFD/ATP, ATP (final concentration, 1 mM) was added to the refolding mixture at 200 s (indicated by a down-pointing arrow), and PfuPFD (final concentration, 0.3 μM) was added to the mixture at 220 s (indicated by an up-pointing arrow). Closed triangles indicate the mixture in the presence of 0.2 μM PfuCPN without ATP, ATP (final concentration, 1 mM) was added to the refolding mixture at 200 s. Opened circles indicate refolding in buffer only. (B) Relative decreases in fluorescence of refolded GFPuv containing various amounts of PfuPFD. The acid-denatured GFPuv was allowed to refold in the refolding mixtures which contained various concentrations of PfuPFD (0–200 nM) at 60°C.

the presence or absence of PfuCPN, as shown in Fig. 2. When denatured GFPuv was diluted into refolding buffer that contained 0.2 μM PfuCPN with or without 0.3 μM PfuPFD, the fluorescence increase of GFPuv was completely suppressed in samples that contained PfuPFD (Fig. 2A, diamonds) and was suppressed to a slightly lesser degree in samples without PfuPFD (Fig. 2A, inverted triangles). This result suggested that the combination of PfuCPN and PfuPFD captured GFPuv refolding intermediates more efficiently than PfuCPN only. Upon addition of ATP at 200 s, GFP fluorescence was observed in samples containing PfuCPN only (Fig. 2A, triangles) as was also shown in Fig.1A. However, samples containing both PfuCPN and PfuPFD remained devoid of GFPuv fluorescence (Fig. 2A, diamonds). In addition, when

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0.3 μM PfuPFD was added to samples containing only PfuCPN after a 220 s refolding interval, the recovery of GFPuv fluorescence was abruptly arrested (Fig. 2A, inverted closed triangles). These results suggested that GFPuv refolding intermediates preferentially bound to PfuPFD over PfuCPN, resulting in a stable protein complex that suppressed GFP refolding regardless of the presence or absence of ATP or PfuCPN. In order to confirm the direct interaction between PfuPFD and GFPuv, the refolding of GFPuv was measured in the presence of varied concentrations of PfuPFD (0–200 nM; Fig. 2B). The refolding rates and final yields of the refolded GFPuv decreased in proportion to the amount of PfuPFD added, indicating clearly that PfuPFD interacts with GFPuv refolding intermediates and traps them tightly.

PfuCPN cannot assist the refolding of PfuCS

As shown above, PfuCPN had the ability to bind GFPuv refolding intermediates and release them in an ATP-dependent manner. In order to probe the ability of PfuCPN to assist the refolding of an endogenous substrate protein, we performed refolding experiments on PfuCS, as shown in Fig. 3. When denatured PfuCS was diluted into the refolding buffer without chaperonin at 70°C, a slight recovery of PfuCS activity (5% of native values) was seen immediately, which then gradually decreased, probably due to the relative instability of PfuCS under the conditions we used (Fig. 3A, opened circles). In the presence of 0.57 μM PfuCPN with or without ATP, PfuCS recovery was not improved, but this activity did not decrease over the next 30 min (Fig. 3A, closed circles and diamonds), suggesting that PfuCPN may contribute to maintaining the active form of PfuCS.

To test that the refolding of PfuCS can be mediated by chaperonin intrinsically, we examined PfuCS refolding in the presence of the *E. coli* GroEL chaperonin at 25°C (Fig. 3A, inset). In the presence of 0.38 μM GroEL and 0.38 μM GroES, the refolding yields of PfuCS were greatly improved upon addition of ATP at 5 min (Fig. 3A, inset, closed circles). The result suggested that PfuCPN may be intrinsically unable to facilitate the refolding of PfuCS at 70°C.

Next, we tested whether PfuCPN can protect PfuCS from heat inactivation by measuring the enzyme activity of PfuCS after incubation at 70°C in the presence or absence of PfuCPN. As shown in Fig. 3B, in the absence of PfuCPN, the activity of PfuCS decreased gradually to ~10% in 60 min (Fig. 3B, opened circles). On the other hand, in the presence of PfuCPN, the activity was maintained significantly (~80%) (Fig. 3B, closed circles). From these results, it was suggested that although PfuCPN could not actively assist the refolding of PfuCS at 70°C, it was able to maintain its active conformation and protect it from heat inactivation.

PfuPFD facilitates the refolding of PfuCS

Finally, we examined the refolding of PfuCS in the presence of PfuPFD. Interestingly, as shown in Fig. 4, the refolding reaction of PfuCS at 70°C was greatly improved in the presence of PfuPFD

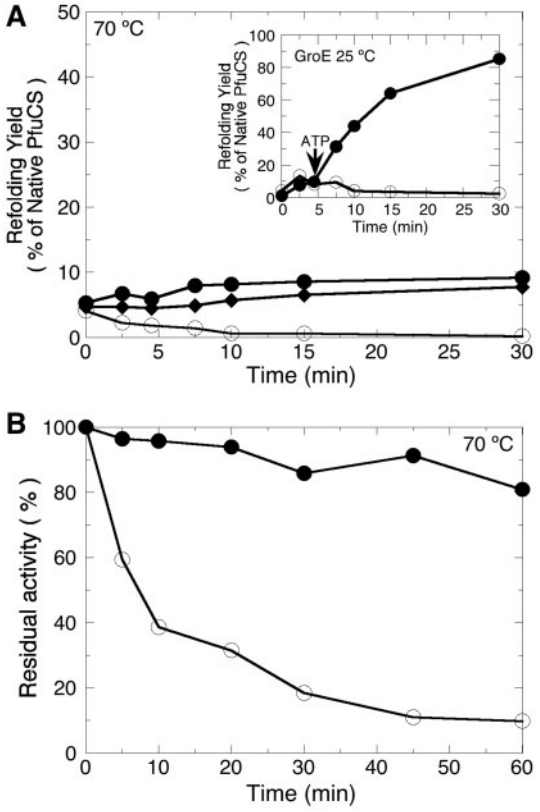

Fig. 3 Effect of PfuCPN on the folding of endogenous substrate protein PfuCS. (A) The refolding of PfuCS in the presence of group II chaperonin PfuCPN. The denatured PfuCS was diluted into the refolding buffer at 70°C. Closed circles indicate the mixture in the presence of 0.57 μM PfuCPN with ATP. Closed diamonds indicate the mixture in the presence of 0.57 μM PfuCPN without ATP. Opened circles indicate refolding in buffer only. PfuCS activity then gradually decreased, because PfuCS was unstable under these conditions. The inset indicates the refolding of PfuCS in the presence of group I chaperonin GroE (GroEL/GroES). The denatured PfuCS was allowed to refold in the refolding buffer at 25°C. The enzymatic activity of PfuCS was measured at 50°C. Closed circles indicate the mixture in the presence of 0.38 μM GroEL and 0.38 μM GroES without ATP, ATP (final concentration, 2 mM) was added to the refolding mixture at 5 min (indicated by an arrow). Opened circles indicate refolding in buffer only. (B) The stability of PfuCS in the presence of PfuCPN without ATP. The experiments was started by 100-fold dilution (final protein concentration, 0.19 μM) of PfuCS into reaction buffer with (closed circles) or without (opened circles) 0.57 μM PfuCPN at 70°C. The enzymatic activity of PfuCS was measured at 50°C and the initial PfuCS activity was set as 100%.

(Fig. 4A, inverted opened triangles), compared with that of the spontaneous reaction (Fig. 4A, opened circles). Moreover, the presence of PfuCPN in addition to PfuPFD resulted in a decrease in the final refolding yield of PfuCS from 55% to 35% (Fig. 4A, closed diamonds), which indicated the presence of a competitive binding reaction. In order to confirm the effects of PfuPFD on PfuCS refolding, refolding of PfuCS was examined in the presence of various concentrations of PfuPFD (Fig. 4B). We found that the refolding yields of PfuCS increased with increasing amounts of PfuPFD. These results suggested that PfuPFD is capable of assisting PfuCS folding, but does not necessarily cooperate with the chaperonin, PfuCPN.

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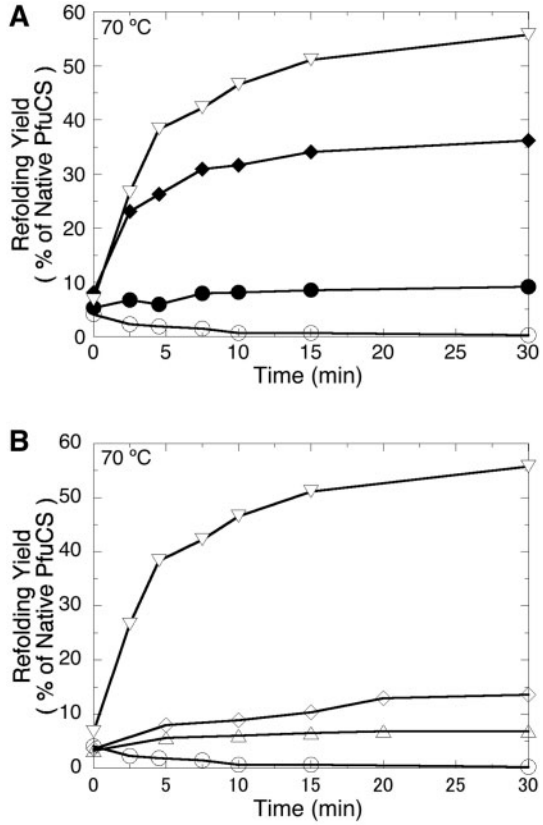

Fig. 4 Assisted refolding of PfuCS by PfuPFD. (A) The refolding of PfuCS in the presence of PfuPFD and/or PfuCPN. The denatured PfuCS was diluted into the refolding buffer at 70°C. Inverted opened triangles indicate the mixture in the presence of 0.86 μM PfuPFD. Closed diamonds indicate the mixture in the presence of 0.57 μM PfuCPN and 0.86 μM PfuPFD with ATP. Closed circles indicate the mixture in the presence of 0.57 μM PfuCPN with ATP. Opened circles indicate refolding in buffer only. (B) Refolding of PfuCS in the presence of various amounts of PfuPFD. The denatured PfuCS was allowed to refold in the refolding mixtures which contained various concentrations of PfuPFD at 70°C. The ratio of PfuPFD to PfuCS monomer were as follows: opened triangles, 0.5:l; opened diamonds, 2:l; inverted opened triangles, 4.5: l. Opened circles indicate refolding in buffer only.

Additionally, we examined the effects of these two chaperones on the refolding of another citrate synthase from a different species, *T. acidophilum*, for comparison. The sequence homology between this enzyme and PfuCS is 66%. Interestingly, PfuPFD and/or PfuCPN were unable to facilitate the refolding of this enzyme at 60°C (Supplementary Fig. S4). This result suggested that differences in the amino acid sequence of various substrate proteins affect the interactions between PfuPFD/PfuCPN and substrate proteins.

Discussion

In mammals, the expression and the functional cooperativity of the various molecular chaperone machineries are extremely complicated; it is reported that the expression levels of HSP70s and HSP40s differ in every tissues and organelles (*34*), and cytoplasmic chaperonin classified in HSP60 group is consist of hetero-oligomeric protein (*35*). In contrast, the molecular chaperone system of hyperthermophilic archaea including *P. furiosus* is relatively simple compared to the eukaryotic system, *i.e.* only small heat shock proteins, group II chaperonin and prefoldin have been annotated in each genome (*18–20*). This fact makes archeal systems an attractive target in studies regarding the molecular function of individual chaperones and cooperative relationships between them.

In the present study, we showed clearly for the first time that the chaperonin from *P. furiosus* was sufficiently capable of binding of substrate protein intermediates and interacting with the folding proteins in an ATP-dependent manner (Fig. 1). Previous studies only addressed the ATPase activity (*24*, *36*) and the interactions with non-native lysozyme (*30*, *36*, *37*). Therefore, we were interested in further probing the functional chaperonin cycle of PfuCPN and tried to characterize it. Through comparisons between our previous experimental results and the results of our present study, we found that the degree of purity of PfuCPN 16-mer was very important in obtaining consistent results, as pointed out by others (*30*). In this study, we took care to isolate only oligomeric forms of PfuCPN by including a size-exclusion chromatography step and also monitoring the fractionation profile by using dynamic light scattering, because PfuCPN samples purified according to our previous procedure may include a monomeric form of the chaperonin (*24*). Although we have limited information on the dissociation characteristics of PfuCPN, our preliminary results suggested that PfuCPN oligomer was unstable under the conditions of the absence of Mg^{2+} ion, or in a lower concentration of KCl at high temperatures. By using our modified procedure, we could obtain highly purified PfuCPN in the active oligomeric form under the conditions applied in the present study, as judged by the refolding characteristics of GFPuv (Figs 1 and 2A).

The characteristics of the binding and Mg-ATP-dependent release of GFPuv refolding intermediates by PfuCPN was similar to that reported in *P. horikoshii* chaperonin (*38*). However, the refolding of PfuCS was not actively assisted by PfuCPN, even though PfuCS is an endogenous substrate protein from *P. furiosus* cells (Fig. 3A). Formation of a binary complex of PfuCPN–PfuCS in the absence of ATP and a partial release of PfuCS in the presence of ATP were confirmed by size-exclusion chromatography experiments (data not shown). Therefore, it is safe to say that PfuCPN has an intrinsic ability to bind PfuCS folding intermediates. However, it is incapable of further assisting PfuCS folding, in sharp contrast to group I GroEL chaperonin. Instead, PfuCPN contributes in maintaining the stability of PfuCS from thermal denaturation at the high temperature (Fig. 3B).

Very interestingly, PfuPFD significantly assisted the refolding of PfuCS at 70°C (Fig. 4). The presence of PfuCPN in addition to PfuPFD did not enhance the refolding of PfuCS; on the contrary, the refolding yield was decreased upon addition of PfuCPN. This finding suggested that PfuPFD competes with PfuCPN to bind

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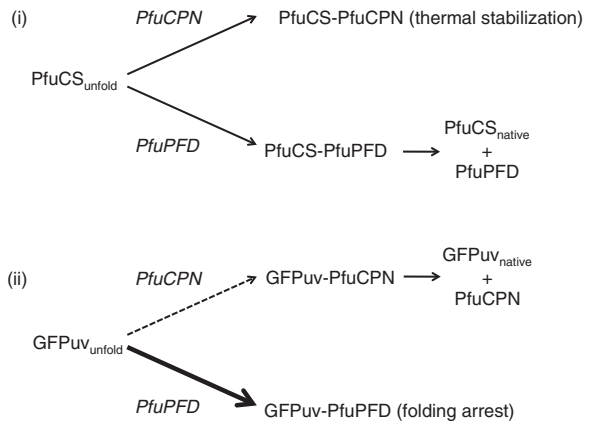

Fig. 5 A schematic model of the folding for PfuCS and GFPuv in the presence of PfuPFD and PfuCPN. In the refolding reaction of PfuCS (i), both PfuCPN and PfuPFD are able to bind folding intermediates of PfuCS. In the case of PfuCPN, this interaction did not result in an improvement in refolding yield of PfuCS from the completely unfolded state, but conferred thermal stability to PfuCS under conditions that would otherwise cause denaturation. In contrast, interaction with PfuPFD results in a net increase in refolding yield relative to the spontaneous reaction. In the refolding reaction of GFPuv (ii), there is a significant preference for GFPuv molecules to bind to PfuPFD, rather than PfuCPN. Interaction with PfuPFD resulted in the immediate arrest of GFPuv refolding, reflecting the relative stability of this interaction. In contrast, binding of GFPuv to PfuCPN displays the more conventional characteristics of chaperonins such as *E. coli* GroEL.

unfolding intermediates of PfuCS, which was similar to the case of *P. horikoshii* prefoldin (*38*) but different from *T.* KS-1 prefoldin (*14*). Moreover, it is interesting to note that the fact that PfuPFD and PfuCPN were unable to assist the refolding of citrate synthase from *T. acidophilum*, a homologue of PfuCS.

A final interesting insight was obtained through comparing the behaviour of the two molecular chaperones in the refolding reactions of GFPuv (Figs 1 and 2) and PfuCS (Fig. 4). From the delayed addition of EDTA in Fig. 1, we saw that the interactions between GFPuv and PfuCPN persisted for a time frame of ~100 s, and this in turn indicated that GFPuv refolding intermediates were binding to and being released by PfuCPN repeatedly during this time. Addition of PfuPFD to this reaction (Fig. 2A) resulted in an immediate arrest of GFPuv refolding caused by PfuPFD binding to GFPuv. This result indicated that GFPuv preferentially bound to PfuPFD rather than PfuCPN under these conditions. In contrast, during the refolding reaction of PfuCS, the addition of PfuCPN to refolding reactions containing PfuPFD resulted in competitive binding between these two chaperones for the folding intermediates of PfuCS (Fig 4A). These two experimental results point towards a variety in the binding affinities that govern the interaction of various protein folding intermediates to these two chaperones. The differences in binding behaviour may be due to differences in kinetic behaviour of the refolding protein, differences in the molecular characteristics of protein folding intermediate surfaces, or possibly the relative reversibility of the overall protein refolding reaction. Nevertheless, differences in affinity between various protein folding intermediates and the two chaperones examined in our experiments have implications that affect our understanding of how these chaperones assist protein folding in *P. furiosus*. Other experiments using various endogenous and exogenous refolding substrate proteins should provide additional data regarding this important result.

Our results are summarized in Fig. 5, which compares the refolding reactions of GFPuv and PfuCS in the presence of PfuCPN and PfuPFD. The behaviour of the two chaperones in these refolding reactions differs, in both the manner in which they interact with protein folding intermediates and the eventual outcome of these interactions. More detailed experiments should provide us with additional insights regarding the molecular chaperone network in *P. furiosus*.

Supplementary Data

Supplementary Data are available *JB* Online.

Acknowledgements

We thank Junjiro Tsunemi for technical assistance.

Funding

The Ministry of Education, Culture, Sports, Science, and Technology of Japan (grants-in-aid for Scientific Research, partial).

Conflict of Interest

None declared.

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