[Frontiers in Bioscience 15, 708-717, January 1, 2010]

Structure and function of archaeal prefoldin, a co-chaperone of group II chaperonin

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1. ABSTRACT

Molecular chaperones are key cellular components involved in the maintenance of protein homeostasis and other unrelated functions. Prefoldin is a chaperone that acts as a co-factor of group II chaperonins in eukaryotes and archaea. It assists proper folding of protein by capturing nonnative proteins and delivering it to the group II chaperonin. Eukaryotic prefoldin is a multiple subunit complex composed of six different polypeptide chains. Archaeal prefoldin, on the other hand, is a heterohexameric complex composed of two α and four β subunits, and forms a double β barrel assembly with six long coiled coils protruding from it like a jellyfish with six tentacles. Based on the structural information of the archaeal prefoldin, substrate recognition and prefoldin-chaperonin binding mechanisms have been investigated. In this paper, we review a series of studies on the molecular mechanisms of archaeal PFD function. Particular emphasis will be placed on the molecular structures revealed by X-ray crystallography and molecular dynamics induced by binding to nonnative protein substrates.

2. INTRODUCTION

Molecular chaperones are ubiquitous proteins that are required for the correct folding, transport, and degradation of proteins within the cell (1-3). Typically, molecular chaperones can protect nonnative polypeptides from forming aggregates and can provide them with an appropriate environment in which they can fold properly. In addition, recent research has unveiled many other functions for molecular chaperones more or less unrelated to protein folding but pertinent, for example, to immunity and to cancer (4-7).

The molecular chaperone system of archaea varies among genera and species but in general is quite complex, including representative of virtually all components present in higher organisms (8). However, in hyperthermophilic archaea, the system is much simpler than that of other organisms. Within hyperthermophilic archaea, only five kinds of chaperones have so far been identified: group II chaperonin, prefoldin (PFD), small heat shock protein, peptidyl-prolyl *cis-trans* isomerase and AAA

proteins (ATPases associated with various cellular activities), and NAC (8, 9). These archaea lack the Hsp70 chaperone system as well as Hsp90 and Hsp100, whereas these are thought to be indispensable chaperones in all other organisms (10-12). In lieu of these other systems, it is believed then that group II chaperonin and its cofactor, prefoldin, play the major role in the folding of proteins in hyperthermophilic archaea.

PFD was first identified in yeast as Gim (genes involved in microtubule biogenesis) in a genetic screen for mutants that are synthetically lethal in combination with a mutated yeast gamma-tubulin (tub4-1) (13). It was later named Prefoldin after it was characterized as a co-chaperone of group II chaperonin in eukaryotes (14). PFD, which occurs ubiquitously in archaea and eukaryotes (9), stabilizes nonnative proteins and subsequently delivers them to a group II chaperonin to facilitate proper folding (14-17). Eukaryotic PFD is a multiple subunit complex composed of six different polypeptides (two α-type and four β-type subunits) that range from 14 kDa to 23 kDa in molecular mass (15). Archaeal PFD, on the other hand, is composed of two kinds of subunits: two α subunits and four β subunits. The substrates of eukaryotic prefoldins are mainly nascent cytoskeletal proteins (15, 16, 18). Biochemical studies have revealed that the specific subunits of PFD proteins contribute to the binding of substrate proteins (19). Archaeal prefoldins have been shown to prevent the aggregation of various proteins (20-23).

The focus of this review is on archaeal prefoldins. Here, we review a series of studies and investigate our understanding of the molecular mechanisms of archaeal PFD function. Particular emphasis will be placed on the molecular dynamics induced by binding to nonnative protein substrates. The data described ought to help in the understanding of prefoldin biology and functions not only in archaea but also in eukaryotes and, thus, provide basic information applicable to the study of important problems in health and disease, such as the human chaperonopathies (24) and their pathophysiology at the molecular level.

3. STRUCTURE OF ARCHAEAL PREFOLDIN

3.1. 3D structure of Archaea Prefoldin

The crystal structures of a PFD from *Methanobacterium thermoautotrophicum* (MtPFD) (25) and from *Pyrococcus horikoshii* OT3 (PhPFD) (26) have been reported. These two PFDs share a common quaternary fold composed of two α subunits and four β subunits (Figure 1A). The structure of PFD, a double β barrel assembly with six long coiled coils protruding from it, resembles a jellyfish with six tentacles. A large central cavity is formed by the six coiled coils. According to these crystal structures, each coiled coil tentacle is fully solvated and its polar and charged side chains are almost exposed to the solvent. The average B-factor of the coiled coil region of each subunit is much higher than that of the β barrel assembly region and the coiled coil tentacles have few inter-subunit interactions within the hexameric complex, indicating that each individual coiled coil is highly flexible. This flexibility should be favorable for widening the central cavity and capturing a variety of nonnative proteins.

The α and β subunits share a similar architecture. In the β subunit, the N- and C-terminal regions form a coiled coil structure, which is connected by a β hairpin linker consisting of two short β strands. The α subunit has a similar architecture, although the α helical regions are connected by two β hairpins (Figure 1B). The extra β hairpin is involved in the dimerization of α subunits in the $\alpha_2\beta_4$ hexamer.

3.2. Structure of the hydrophobic groove at the distal region of each subunit

Chaperones interact with the hydrophobic surface of unfolded proteins of various sequences, sizes and shapes. Hydrophobic grooves exist in the coiled coils of both the α and β subunits of PFD (Figure 1C). Since the hydrophobic grooves are facing the central cavity, they are thought to be involved in binding nonnative substrates. The hydrophobic residues in the hydrophobic groove of the α subunit do not face one another in an orderly way, as compared to those of the β subunit. Thus, the hydrophobicity of the groove of the β subunit is higher than that of the α subunit, indicating that the β subunit is essential for binding nonnative proteins.

3.3. Molecular dynamics in nonnative substrate protein binding

Molecular dynamics (MD) simulation studies of substrate nonnative protein binding by PFD under high temperature conditions have been reported (26). MD simulation has indicated that at high temperatures, the residues of the coiled coil regions on both α and β subunits show greater flexibility when compared to those of the β assembly region. Thus, the β assembly contributes to the maintenance of the width of the central cavity and the overall unique structure of PFD. In addition, short segments in the connecting linkers between the α helical and β assembly regions exhibit even higher flexibility, suggesting that these regions play a role of a "hinge" and allow flexible movements by the coiled coils of all subunits (Figure 2).

To investigate the precise substrate binding site and the motion of PFD during substrate binding, a docking simulation of PFD with a denatured protein was carried out. The docking simulations demonstrated that the β subunit makes a noticeable positional change and the hydrophobic groove at the distal region stabilizes hydrophobic interactions with a denatured protein (Figure 3). The simulations also showed that in order to stabilize the PFD-substrate complex, the two α subunits of the hexameric complex changed their conformation and position depending on the size and binding mode of the unfolded protein. Lundin *et al.* have analyzed the substrate binding properties of *Methanothermobater thermoautotrophicus* by substituting mutant coiled coil tips in PFDs (23). They have also proposed that while substrate binding depends on the spatial arrangement of residues generally intrinsic to coiled coils, PFDs have additionally evolved features specific to their chaperone activities.

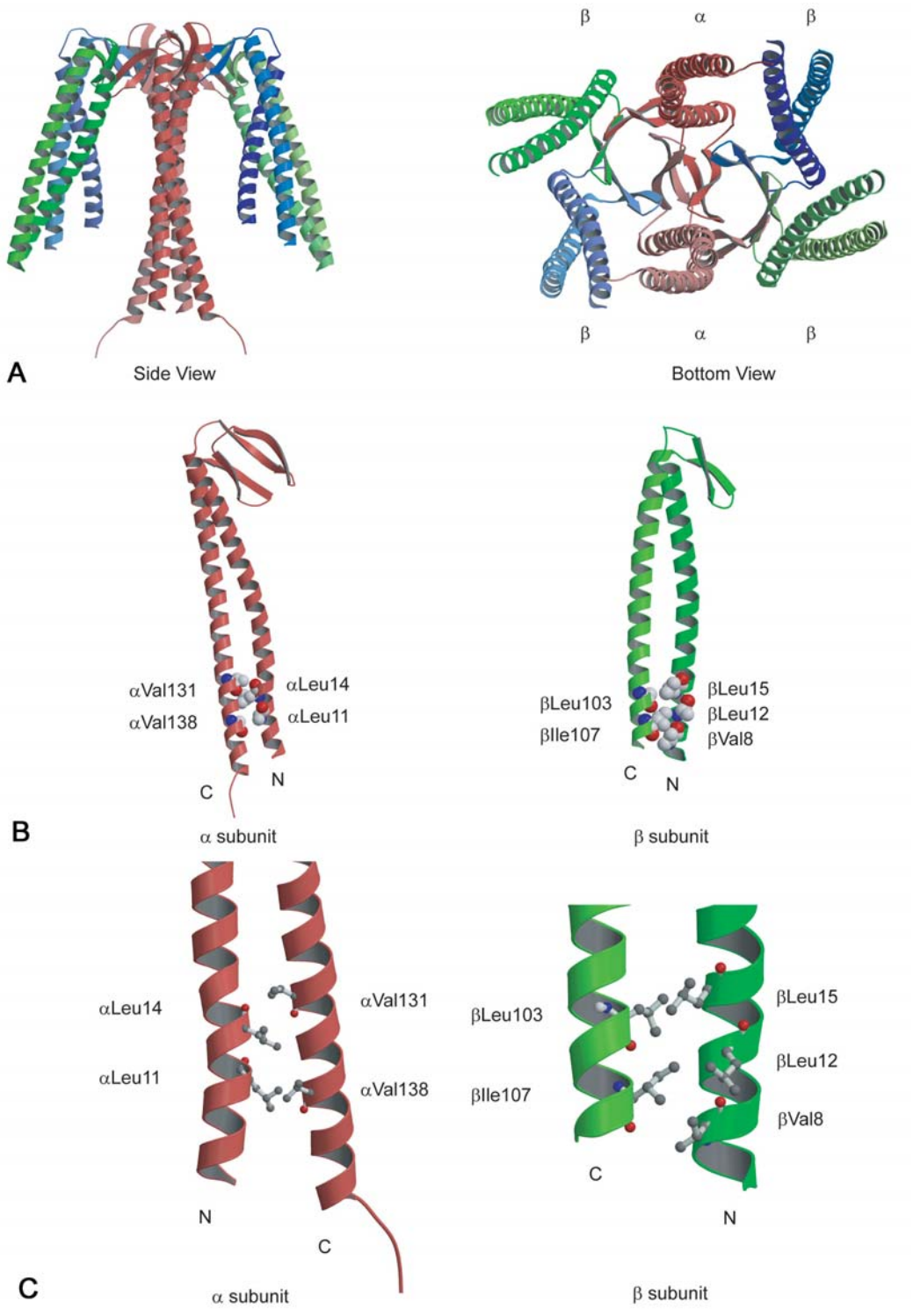

Figure 1. Crystal structure of *Pyrococcus horikoshii* OT3 prefoldine. (A) Overall structure of the $\alpha_2\beta_4$ hexameric complex of PhPFD showing side and bottom views. An α subunit and two β subunits are shown in red (α subunit), green and blue (β subunits), respectively. (B) The overall structure of α and β subunits. The hydrophobic residues (αLeu11, αLeu14, αVal131, αVal13, βLeu8, βLeu12, βLeu15, βLeu103 and βIle107) at the distal region of each subunit are in CPK representation. (C) The α and β subunits hydrophobic groove structure at the distal region of the coiled coil. The hydrophobic residues are shown by ball-and-sticks models. These figures are reproduced with permission from 26.

3.4. Substrate binding site in archaeal PFDs

Chaperones need to bind unfolded polypeptide substrates of various sequences, sizes and shapes, and interact with exposed hydrophobic surface on those substrates. In PFDs, hydrophobic grooves are located at the distal region of each subunit, as mentioned above. Martin-Benito *et al.* have analyzed the substrate binding properties of *Pyrococcus horikoshii* PFD by cryo-EM(27). PhPFD selectively uses an increasing number of subunits to interact with substrate proteins of increasingly larger sizes. For example, lysozyme (14 kDa) interacts with a pair of β subunits and green fluorescent protein (GFP) (27 kDa) interacts with a pair of the β subunits and one of the α subunits. In the case of a much larger substrate protein, conalbumin (75 kDa), all six PhPFD subunits are used for the interaction. These results suggest that the β subunits are more important than the α subunits for substrate protein interaction.

The deletion of the hydrophobic groove of the β subunit impaired chaperone activities of *Methanothermobater thermoautotrophicus* and *Pyrococcus horikoshii* PFDs (22, 25). These results also showed that the β subunits are more important than the α subunits for substrate binding. In PhPFD, the hydrophobic groove at the distal region of the β subunit is formed by βVal8, βLeu12, βLeu15, βLeu103, βIle107, and βLeu111. These residues are all essential for binding with a nonnative substrate. βI107 serves not in substrate binding itself but rather as a central core around which the hydrophobic region is formed (26). Conversely, truncation of the 6 C-terminal amino acids of the β subunit (residues 112-117) showed no observable effect on PhPFD's chaperone activities (22). These results provide convincing evidence for the important role of the hydrophobic groove in interaction with unfolded proteins.

4. CHAPERONIN-PREFOLDIN SYSTEM

4.1. CPN-PFD system: Non native protein transfer from PFD to CPN

Chaperonins (CPNs) are ubiquitous molecular chaperones that form double-ring assemblies of 800-900 kDa with a central cavity for binding and folding substrate proteins in an ATP-dependent manner (28-30). Their activity is assisted by PFD; archaeal PFD can transfer denatured protein to archaeal CPN (17, 21, 22, 31). Siegers et al. showed that CCT-mediated productive actin folding is accelerated at least 5-fold in the presence of eukaryotic PFD (16). In contrast, we have mainly studied the archaeal PFD; in particular, the functional cooperation between PhPFD and a group II chaperonin (17, 21, 22, 31-33). The effect of PhPFD on the chaperonin-mediated refolding of acid-denatured (GFP) was examined using a Thermus thermophilus HB8 CPN (group I) and Thermococcus sp. Strain KS-1 (*T.*KS-1) CPN (group II). PhPFD delivers unfolded proteins to both group I and group II chaperonins for refolding (21). In addition, archaeal PFD can also transfer denatured proteins to eukaryotic CCT (20). Vainberg *et al.* showed that PFD transfers its bound, denatured actin to CCT in a nucleotide-independent manner *in vitro* (34). These results suggested that substrate transfer to CPN is a general role for PFD.

Using the CPN-PFD system, a kinetic study of the interaction between PhPFD and non-native substrate proteins, based on single molecule imaging studies, showed that substrate proteins and PhPFD are in a dynamic equilibrium of association and dissociation. Importantly, dissociation of substrate proteins from the PhPFD complex was accelerated by the presence of CPN (31). Another important piece of evidence on substrate transfer was obtained by measurement of the substrate transfer rate constant using GFP as a substrate (17). In this experiment, a lag time observed during the initial period of GFP refolding, which is thought to reflect the transfer of GFP from PhPFD to CPN, was used to evaluate the transfer rate constant. The lag time correlated positively with the transfer rate constant of denatured GFP from PFD to CPN and the binding rate constant between PFD and CPN, indicating that the substrate transfer rate from prefoldin to chaperonin is strongly associated with its affinity for the chaperonin.

4.2. CPN binding site

PFDs have been shown to interact directly with group II chaperonins through their distal regions (22, 27, 35). Using EM images of eukaryotic CPN-PFD complexes, Martin-Benito *et al.* showed that the sites of interaction between PFD and CPN are located at the level of the outer regions of the PFD jellyfish's "tentacles" and the inner surface of the apical domains of CPN (35). It seems that eukaryotic PFD inserts the tips of its tentacles into the cavity of CCT. Martin-Benito *et al.* have also reported EM images of the archaeal CPN-PFD complex (PhCPN-PhPFD) (27), showing that the binding mode of archaea CPN and PFD is similar to the eukaryotic system. However, PhPFD does not penetrate into the archaeal CPN cavity as much as eukaryotic PFD penetrates into the CCT cavity, suggesting that the substrate transfer and binding mechanism between archaeal and eukaryotic systems are slightly different. EM studies have shown that the tentacles of PhPFD use their coiled-coil tips to interact with the CPN apical domains.

To identify the interaction sites of PhPFD and CPN in more detail, interaction studies using various mutants forms of these proteins have been performed (17, 21, 22). Truncation analyses of PhPFD subunits demonstrated that both the N- and C-terminal regions of the outer β subunits are more important for CPN interactions than the two central α subunits. Interestingly, two acidic amino acid residues located in the helical protrusion of CPN, namely the 250^{th} and 256^{th} residues, have been shown to be important for strong binding to PFD. Corresponding basic amino acid residues are also present in the distal region of C-terminus helix of β subunit tentacles, which are conserved in several archaeal PFDs. This observation suggests that electrostatic interactions are important for the interaction between archaeal PFD and CPN. In addition, the measurement of the mobility of Met residues on PhPFD by using ^{13}C NMR spectroscopy showed that Met11(β) of β subunit became immobile upon the binding to CPN, suggesting that N-terminal helix of coiled coil of β subunit is also partially involved in the interaction with CPN (36). These results show that both the distal N- and C-terminal regions of the β subunit are important for interaction with a chaperonin. These dual functions of the prefoldin tips, substrate binding

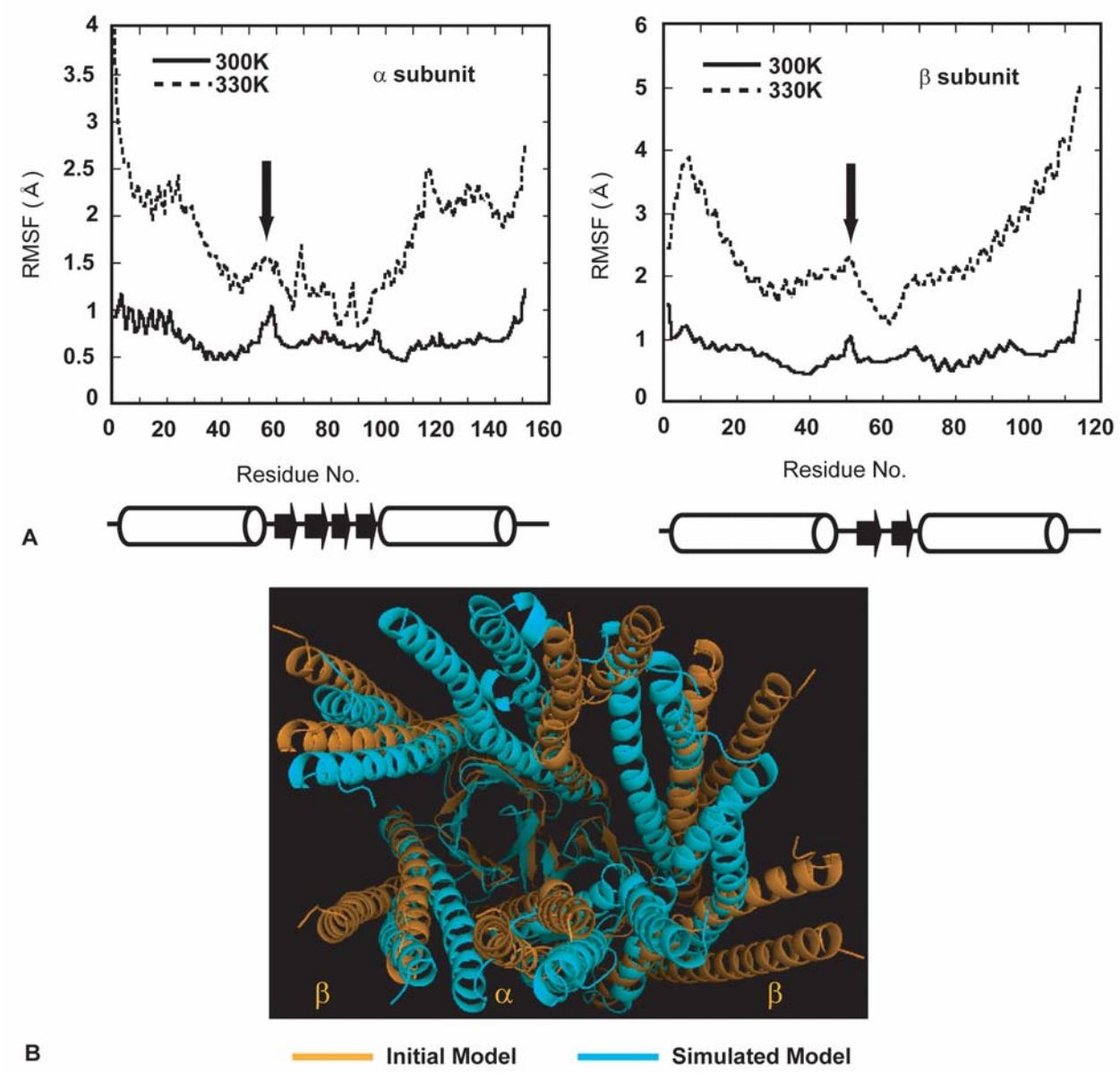

Figure 2. Fluctuation of PhPFD during MD simulation. (A) Fluctuation along the whole trajectory of the Cα atoms PhPFD during MD simulation at 300 K (solid line) and 330 K (dashed line). Arrows indicate the hinge region of each subunit. (B) Superimposed structure of the initial model (orange) and simulated model (blue). The simulated model is the result of the 1 ns of MD calculation at 330 K.

and chaperonin binding, could represent an important property for facilitating the functional cooperation with a chaperonin.

5. OTHER STRUCTURE AND FUNCTIONS

5.1. Combinatorial diversity of PFD subunit

Most archaeal genomes contain one α-type and one β-type PFD subunit, whereas eukaryotic genomes typically contain six unique PFD subunit genes (9). Archaeal PFD is a 90 kDa hetero-hexameric complex ($\alpha_2\beta_4$) composed of two α and four β subunits. Recent genome analysis has revealed that the hyperthermophilic archaea *Thermococcus kodakaraensis* KOD1 has two pairs of prefoldin subunit genes (TK1005 and TK1121 for α subunits, and TK0643 and TK1122 for β subunits), which likely reflects the existence of two different chaperonin subunits. In addition, *Thermococcus* sp. strain KS-1 (*T.* KS-1) has four different prefoldin complexes composed of two pairs of prefoldin subunits (α1, α2, β1 and β2), and they form four different functional prefoldin complexes (α1-β1, α2-β1, α1-β2 and α2-β2). Their properties as molecular chaperones have been characterized. They all exist as $\alpha_2\beta_4$ heterohexamers, and can protect several proteins from

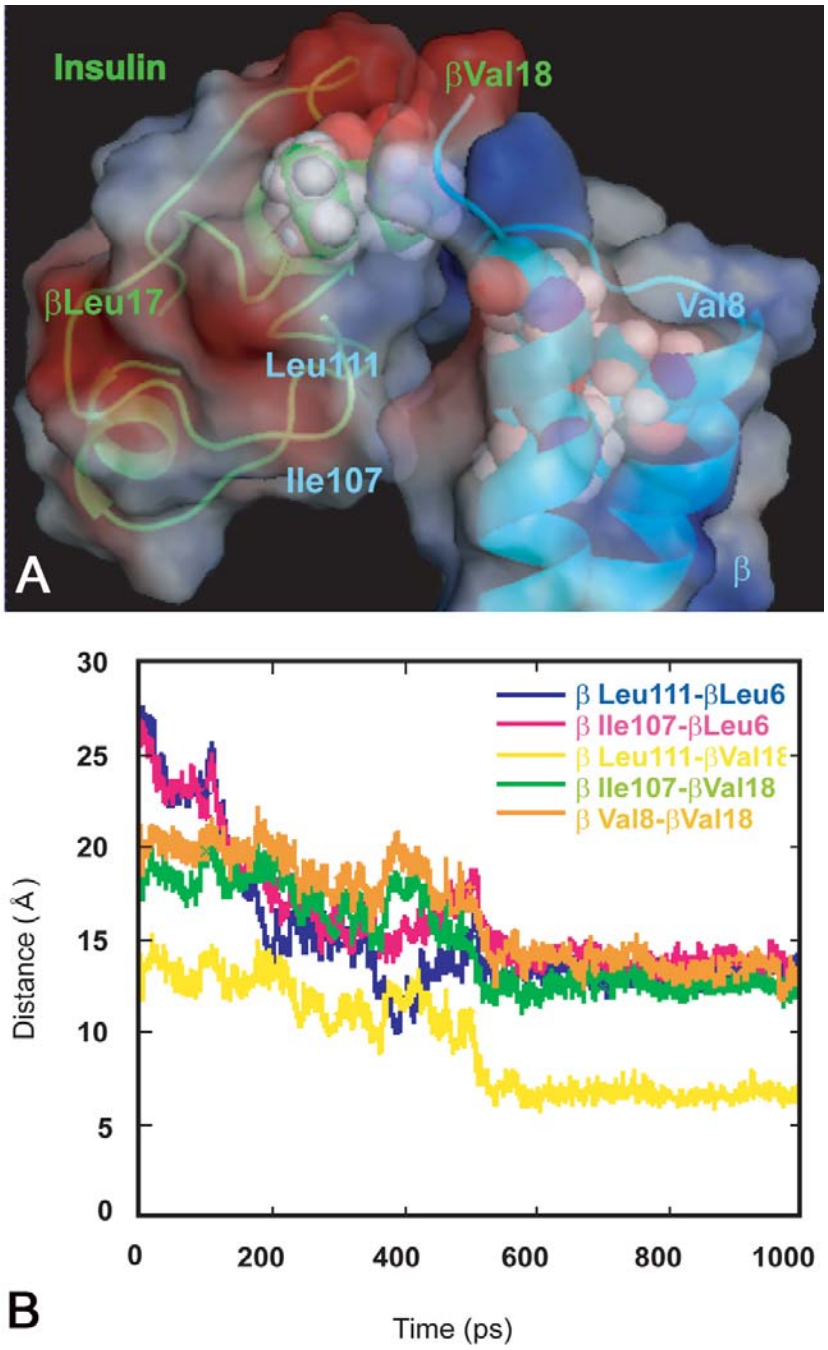

Figure 3. Detail of the interface between the insulin model and at the distal region of PhPFD. (A) The interactions between the denatured insulin model and hydrophobic groove of β subunit after 1 ns MD simulated calculation at 330 K. (B) The distance between the Cα atoms of the interacting hydrophobic residues of the PhPFD β subunits and the insulin model structure. These figures are reproduced with permission from 26.

forming aggregation with different levels of activity (Figure 4). In comparisons of the collaborative activity between the prefoldin complexes and their cognate chaperonins, prefoldin complexes containing β1 subunit interacted with chaperonin strongly, and there is a strong correlation positive between the binding affinity and the substrate transfer rate from prefoldin to chaperonin.

Immunoprecipitation with a subunit specific antibody followed by mass spectrometric analysis of *Thermococcus kodakaraensis* PFDs has shown that α1-β1 (PfdA-PfdB) and α2-β2 (PfdC-PfdD) complexes dominate in the cell (37). Danno *et al.* concluded that the α1-β1 complex plays a crucial role at all growth temperatures and the α2-β2 complex contributes to survival in a high

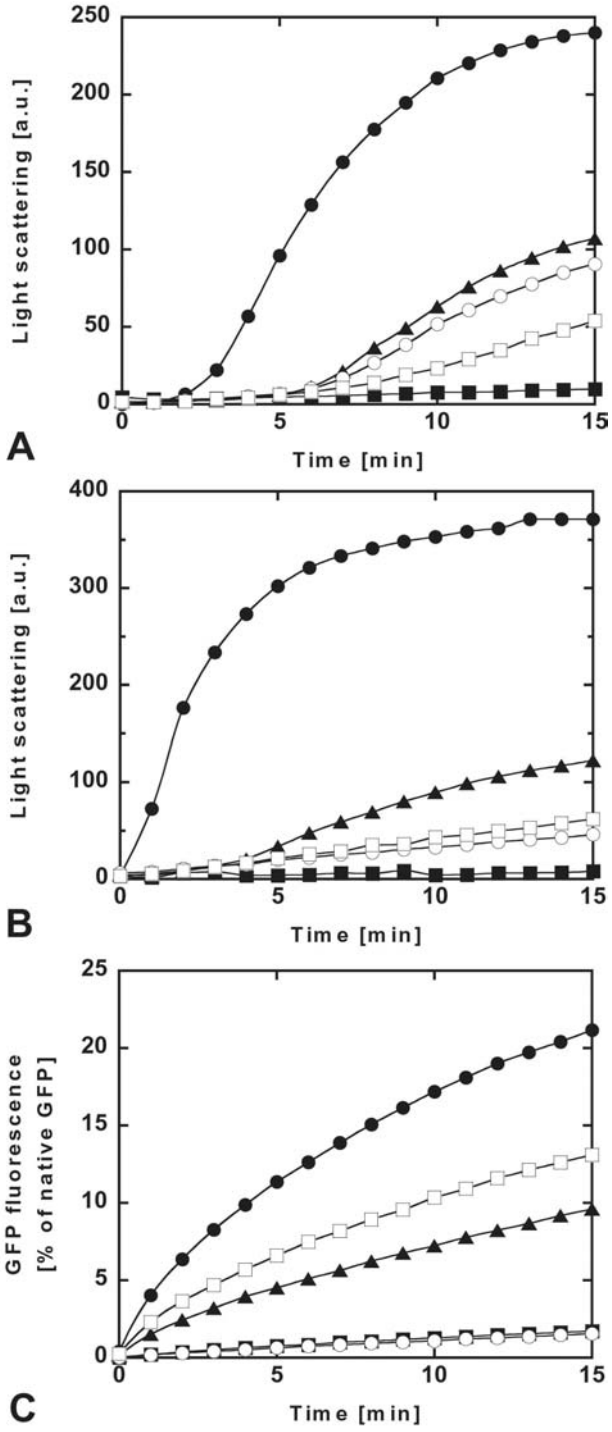

Figure 4. Chaperone activity of *T.* KS-1 prefoldin complexes. (A) Effects of *T.* KS-1 prefoldin complexes on the aggregation of citrate synthase (CS) at 45 °C were monitored. CS was incubated in the absence (*filled circles*) or presence of prefoldins (*filled squares*, α1-β1 complex; *filled triangles*, α2-β1 complex; *open circles*, α1-β2 complex; *open squares*, α2-β2 complex). (B) Effects of *T.* KS-1 prefoldin complexes on the aggregation of catalase at 60 °C were monitored. Catalase was incubated in the absence (*filled circles*) or presence of prefoldins (*filled squares*, α1-β1 complex; *filled triangles*, α2-β1 complex; *open circles*, α1-β2 complex; *open squares*, α2-β2 complex). (C) Effects of *T.* KS-1 prefoldin complexes on the refolding of GFP at 60 °C were monitored. Acid-denatured GFP was diluted in the folding buffer with (*filled squares*, α1-β1 complex; *filled triangles*, α2-β1 complex; *open circles*, α1-β2 complex; *open squares*, α2-β2 complex) or without prefoldins (*filled circles*). The amount recovered is expressed as a percentage of the fluorescence intensity of native GFP. These figures are reproduced with permission from 32.

Table 1. K_D values for the interaction between the truncated mutant PhPFDs and PhCPN. K_D values were calculated by the resonance unit at the equilibrium. Relative K_D values were calculated by dividing the K_D values by that of the wild-type PhPFD

PhPFD		K_D (10^{-8} M)	Relative K_D
α subunit	β subunit		
Wtα	Wtβ	2.63	1
Wtα	tc5β	4.59	1.74
Wtα	tc6β	11.0	4.18
Wtα	tc7β	28.8	11.0
Wtα	tc8β	30.8	11.7
Wtα	tn10β	44.7	17.0
tc8α	Wtβ	2.13	0.81
tc8α	tc5β	35.5	13.5
tc8α	tc6β	26.0	9.89
tc8α	tc7β	57.7	21.9
tc8α	tc8β	69.3	26.3
Wtα	tn10β	44.7	17.0
tn17α	Wtβ	2.03	0.77

The truncated mutants were designed according to the terminus, number of residues and subunit type that was mutagenized, as exemplified by tc8α, a mutant α subunit with an 8-amino acid truncation from the C terminus, and tn10β, a mutant β subunit with a 10-amino acid truncation from the N terminus.

temperature environment. Although other complexes were not observed, this does not exclude the possibility that they may form in relatively low numbers. It seems that the *Thermococcus* strains have gained an improved chaperone system that could provide an advantage for growth in a wider range of temperatures.

5.2. Homomeric complex of PFD subunit

As described above, prefoldin is known to exist and function as hexamer with 6 coiled-coil tentacles. Recently, we found that both β1 and β2 can form stable homo-tetramers with four coiled coil tentacles resembling the jellyfish-like structure of heterohexameric PFD (38). The tetrameric β1 subunits can protect against aggregation of relatively small proteins, implying that PFD β1 subunit acts as a molecular chaperone in some archaea. The size of the entire β1 homo-tetramer complex is smaller than that of the hexameric PFD. This size difference might be responsible for the different chaperone activities and substrate specificities of various homo- or hetero-oligomeric PFDs.

Recently, a filamentous PFD (γ PFD) was discovered in the hyperthermophilic archaeon *Methanocaldococcus jannaschii* (39). The γ PFD, which is up-regulated over 20-fold in response to heat shock, is homologous to the α PFD of the *M. jannaschii*. This homologue did not assemble with either the α or β subunit but it formed a long filamentous structure that was shown to have chaperone activity *in vitro*. Transmission electron microscopy (TEM) measurements revealed that the lengths of γ PFD filaments vary from 200 nm to over 2 μm, whereas the width of the filaments, as deposited on mica or carbon support grids, are uniform at 8.4 ± 0.4 nm. Based on the TEM observation and the homology model from the α subunit in the crystal structure of the $\alpha_2\beta_4$ PFD complex from *M. thermoautrophicum*, a potential model of the filament assembly was postulated. In this model, assemblies composed of two γ PFD units, homologous to β barrels, are stacked in the same manner as in the $\alpha_2\beta_4$ complex, along the elongation direction of the filament with their coiled coils parallel to the support grid surface. In addition, it was also reported that truncating the coiled coil length could serve to redirect the orientation of γ PFD on the surface (40).

6. CONCLUSION

Over the past five years, we have increased our understanding of the details of the molecular mechanisms of archaeal PFD. The *Pyrococcus horikoshii* OT3 PFD is one of the best studied, and we now understand how PFDs interact with nonnative proteins and CPNs, and also how PFD makes and controls motion in its unique tertiary complex. Recently, additional prefoldin subunits, α2, β2 and γ, were found in archaea, suggesting that archaeal species have the unique ability to adapt to changes in various environmental conditions. We have already characterized various prefoldin complexes from *Thermococcus* KS-1, suggesting that each PFD complex differs in its ability to recognize substrate proteins and chaperonins. However, since the structural studies were restricted to the ternary complex PFD-nonnative protein-CPN, it is difficult to provide substantial and precise information concerning the substrate and CPN recognition of each PFD complexes during substrate transfer from PFD to CPN. The structure of PFDs, complexed with substrate protein or CPN, needs to be determined in order to clearly establish how PFD changes its conformation and transfers the substrate protein to CPN.

Moreover, structural investigations and improved functional characterizations are needed to better understand the basis of the structure-function relationship that directs the regulatory function of the *Thermococcus* KS-1 prefoldins. Such investigations will lead to a more full understanding of the distribution of PFD subunit assembly. In addition, elucidation of the basic molecular mechanisms pertaining to PFD functions and interactions with substrate and CPN will pave the way for studies aiming at understanding pathologies involving defective chaperones.

7. ACKNOWLEDGEMENT

This work was supported by grants-in-aids for scientific research (17028013, 19370038 and 20059013) and a grant from the National Project on Protein Structural and Functional Analyses from the Ministry of Education, Science, Sports, and Culture of Japan.

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Abbreviations: PFD: prefoldin; CPN: chaperonin; PhPFD: *Pyrococcus horikoshii* OT3 prefoldin; MtPFD: *Methanobacterium thermoautotrophicum* prefoldin; MD: molecular dynamics; CS: citrate synthase; GFP: green fluorescent protein;

Key Words: Archaea, Molecular Chaperone, Prefoldin, Chaperonin, Molecular Dynamics, Review

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