Selective Contribution of Eukaryotic Prefoldin Subunits to Actin and Tubulin Binding*

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Prefoldin (PFD) is a chaperone that interacts exclusively with type II chaperonins, also called chaperonins, are ubiquitous and essential oligomeric double toroidal ATPases dedicated to facilitated folding. Here we demonstrate that functional PFD can spontaneously assemble from its six constituent subunits (PFD1–PFD6), each expressed as a recombinant protein. Using engineered forms of PFD assembled in vitro, we show that the tips of the PFD tentacles are required to form binary complexes with authentic target proteins. We show that PFD uses the distal ends of different but overlapping sets of six subunits within the hexamer. Our data are consistent with the hypothesis that PFD, like the eukaryotic cytosolic chaperonin, has co-evolved specifically to facilitate the folding of its target proteins.

Chaperonins are divided into two categories: type I and type II. Type I chaperonins are found in prokaryotes (e.g. GroEL) and prokaryotically derived organelles. Type I chaperonins are characterized by a homo-oligomeric structure and act in conjunction with a co-chaperonin (GroES in the case of GroEL) (9–12). Type II chaperonins are hetero-oligomers, lack an obligate co-chaperonin, and are found only in eukaryotes (chaperonin-containing T-complex polypeptide-1 (CCT))1 and archaea (the thermosome) (reviewed in Refs. 13–17). The principal targets of CCT are the actins and tubulins, whose proper folding is completely dependent on interaction with CCT in vitro and in vivo (18–21).

Prefoldin (PFD) is a chaperone that interacts exclusively with type II chaperonins. It was originally discovered both in a Saccharomyces cerevisiae screen for genes involved in microtubule formation (where it was called GIM, for genes involved in microtubule biogenesis) (22) and through the purification of an actin folding complex when denatured actin is diluted into crude cytosolic extracts (23). Eukaryotic PFD is a multisubunit complex containing six polypeptides in the molecular mass range of 14–23 kDa. In archaea, on the other hand, PFD is composed of two types of subunits, two α and four β (24). The six subunits associate to form two back-to-back up-and-down eight-stranded β barrels, from which hang six coiled coils. Each subunit contributes one (β subunits) or two (α subunits) β hairpin turns to the β barrels (25). The coiled coils are formed by the N and C termini of an individual subunit. Overall, this unique arrangement resembles a jellyfish. The eukaryotic PFD hexamer is composed of six different subunits; however, these can be grouped into two α-like (PFD3 and -5) and four β-like (PFD1, -2, -4, and -6) subunits based on amino acid sequence similarity with their archaeal counterparts (23, 24). Modeling programs suggest that the subunits will adopt the same β-hairpin turns and coiled coils seen in the archaeal homologues (24, 25). Indeed, recent electron-microscopic three-dimensional reconstructions have demonstrated that eukaryotic PFD has a six-legged structure similar to that seen in the archaeal homologue (26).

Eukaryotic PFD has been shown to bind both actin and tubulin co-translationally (27). The chaperone then delivers the target protein to CCT, interacting with the chaperonin through the tips of the coiled coils (26). Unlike chaperonins, whose function is essential to life, ablation of PFD in the yeast

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1 The abbreviations used are: CCT, chaperonin-containing T-complex polypeptide-1; PFD, hexameric prefoldin; PFD1 to -6, prefoldin subunits 1–6, respectively; Mes, 4-morpholinooethanesulfonic acid; DTT, dithiothreitol; PFDΔx, truncated PFD subunit x; EDC, 1-ethyl-3[3-dimethylaminopropyl]carbodiimide-HCl; AEDP, 3/[2-(aminoethyl)di-thio]propionic acid-HClSmS, millimoles; bvPFD, bovine PFD; PBS, phosphate-buffered saline.
S. cerevisiae results in viable cells. However, these are growth-compromised and have characteristic cytoskeletal phenotypes (22, 23). No authentic target proteins of any archaeal PFD have been identified.

Because eukaryotic PFD is heterohexameric, it has been hypothesized that the individual subunits are not redundant. An important question therefore concerns the contribution of each subunit to the binding of specific target proteins and the organization of the subunits within eukaryotic PFD. Here we present data that demonstrate the ability of the subunits to spontaneously self-assemble into the functional PFD complex. We also show that the distal region of different but overlapping sets of subunits are critical for complex formation with actin versus α- and β-tubulin and suggest a model for the order of the six subunits within the heterohexamer.

EXPERIMENTAL PROCEDURES
Cloning of PFD Subunits
Full-length subunit-encoding fragments were generated by PCR using a human testis cDNA library (Clontech Inc.) as template. The PCR products were cloned into pET23a (pFDT1–4, NdeI/BamHI), pET23b (pFD5, NdeI/XhoI), and pET23d (pFD6, NcoI/EcoRI) expression vectors at the sites indicated.

Expression and Purification of Recombinant PFD Subunits
All recombinant proteins were expressed in Escherichia coli BL21DE3 (28, 29). Cells were pelleted and resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA in the presence of a protease inhibitor mixture (Roche Applied Science). Resuspended bacteria were lysed using a French press, and the lysis products were centrifuged at 50,000 x g for 20 min at 4 °C. Purification protocols for each subunit from either inclusion bodies (pFDT1, pFD3) or soluble supernatants (pFDT2, -4, -5, and -6) are described below. Buffers used in purification procedures are listed in Table I.

PFD1—Inclusion bodies were washed by resuspension in buffer I, recovered by centrifugation at 13,000 x g for 10 min at 4 °C, and dissolved in buffer II. Dissolved inclusion bodies were diluted into 10 volumes of buffer III and dialyzed stepwise at 4 °C against buffer IV containing progressively lower concentrations of urea. Each step lowered the urea concentration by 1 x until all denaturant had been removed. The final dialysate was cleared of particulate matter by centrifugation, and 0.5 ml was loaded onto a 10/30 Superdex 200 FPLC column, equilibrated with buffer V. PFD1 eluted as a single, symmetrical peak with a predicted molecular mass of 20 kDa.

PFD2—The supernatant prepared from lysed bacteria was brought to pH 8.0 by the addition of Tris base, dialyzed with an equal volume of water, and applied to a Q15 anion exchange column equilibrated with buffer VI. The column was developed with a linear gradient containing 1 M NaCl. The majority of the recombinant protein eluted at a conductivity of 6–11 mS/cm as judged by SDS-PAGE. Pooled fractions containing PFD2 were further purified by cation exchange chromatography on a MonoS ion exchange column equilibrated with buffer VII and developed with a linear gradient of the same buffer containing 1 M NaCl. PFD2 eluted from this column as a major peak at a conductivity of 10–20 mS/cm.

PFD3—Inclusion bodies containing PFD3 were twice washed by suspension in buffer VIII and once by resuspension in buffer IX, in each case using a Dounce homogenizer. Material recovered by centrifugation (50,000 x g, 20 min, 4 °C) was dissolved in a minimal volume of buffer X and centrifuged at 60,000 x g for 12 min at 4 °C. Four volumes of buffer XI were added to the recovered supernatant, which was then dialyzed against buffer XII.

PFD4—The bacterial supernatant was diluted with an equal volume of buffer XIV and applied to a Q15 anion exchange column that was washed and developed with a linear gradient of the same buffer containing 1 M NaCl. PFD4 eluted from the column in the range 20–28 mS/cm.

PFD5—The bacterial supernatant was exchanged into buffer XIV using a column of Sephadex G25 and applied to a Q15 anion exchange column, which was washed and developed with a linear gradient similar to that used to purify PFD4. PFD5 eluted at a conductivity of 4–12 mS/cm. Fractions containing PFD5 were pooled, concentrated, and applied to a Superdex 200 gel filtration column equilibrated in buffer XV. PFD5 emerged as a single peak with an apparent molecular mass of 40 kDa.

PFD6—The bacterial supernatant was exchanged into buffer XVI using a column of Sephadex G25 and applied to a Superase column equilibrated and eluted as described for PFD4. PFD6 eluted in the range of 11–20 mS/cm.

Cloning, Expression, and Purification of Truncated PFD Subunits
Truncated subunits (designated Δ) were generated by PCR and cloned into the pET23a (PFD1Δ, -2, -3, -5, and -6) or pET23b (PFD4Δ) expression vectors at the NdeI/BamHI sites. The extent of the different truncations is shown in Table II. With the exception of PFD5Δ (where the truncation starts at M62), the recombinant truncated subunits contained an additional methionine at their N terminus.

Cells expressing the truncated proteins were lysed, and pellets and supernatants were prepared as described above for the corresponding wild type proteins. All proteins were moderately to well expressed, and all but PFD5Δ were to some extent soluble. Lysis products were centrifuged at 50,000 x g for 20 min at 4 °C. Purification protocols for each subunit from either insoluble inclusion bodies (PFD1Δ, -2, -3, -5, -6) or soluble supernatants (PFD4Δ) were as follows.

PFD1Δ, PFD2Δ, and PFD6Δ—Inclusion bodies were twice washed by resuspension in buffer VIII and once by resuspension in buffer IX. Material recovered by centrifugation (50,000 x g, 20 min, 4 °C) was dissolved in a minimal volume of buffer X and centrifuged at 60,000 x g for 12 min at 4 °C. The recovered supernatant was diluted into 10 volumes of buffer XI and dialyzed stepwise at 4 °C against buffer XII and then buffer XIII. The final dialysate was concentrated to 0.5 ml using Centricon YM3 units (Amicon) and applied to a Superdex 75 FPLC column equilibrated in buffer XIV. The recombinant proteins emerged from the column as symmetrical peaks with apparent molecular masses of ~10 kDa.

PFD3Δ—Inclusion bodies containing PFD3Δ were treated as described above through dialysis, except that they were washed only with buffer IX.

PFD4Δ—The supernatant prepared following bacterial lysis was
desalted into buffer XVI using a column of Sephadex G25 and applied to anion exchange column, which was washed and developed with a linear gradient of the same buffer containing 0.5 mM NaCl. PFD4 eluted in the conductivity range 20–22 mS/cm. Appropriate fractions were pooled, concentrated, and fractionated on a Superdex 75 FPLC column equilibrated in PBS. PFD4 eluted with an apparent molecular mass of ~10 kDa.

PFD3—Inclusion bodies containing PFD3 were treated as described above for PFD1, except that the dialysis step was terminated at buffer XII.

Assembly of PFD Hexamers and Subcomplexes

To generate wild type recombinant PFD, equimolar amounts (2–8 nmol) of all six subunits were combined in a total volume of 100–300 μl, brought to a final concentration of 1.5 M urea, and dialyzed against PBS for 1.5 h. The final dialysate was brought to 0.2 mM NaCl, concentrated to 60 μl using a Centricon YM-10 membrane (Amicon), and applied to a Superdex 200 PC 3.2/30 Smart system (Amersham Biosciences) column equilibrated with PBS. Various PFDs containing different truncated subunits were assembled in a similar manner. The PFD2/PFD3 dimer was prepared by combining equimolar amounts (5–10 nmol) of PFD2 and PFD3 in a final volume of 200–400 μl. Reaction products were dialyzed against PBS for 1.5 h, concentrated, and fractionated in the same manner as for wild type PFD. The PFD5/PFD6 complex was generated by co-incubating equimolar amounts (5–10 nmol) of PFD5 and PFD6 in a volume of 15–30 μl for 10 min at 30 °C. The reaction products were brought to a final volume of 60 μl with PBS and applied to a Superdex 200 PC 3.2/30 column.

**PFD Functional Assay**

CCT purification from rabbit reticulocyte lysate, in vitro translation of actin, and generation of denatured, radiolabeled target protein were performed as previously described (18, 30–32). Generation of PFD/actin binary complexes, their transfer to CCT, and analysis of the reaction products on non-denaturing gels was done as described in Ref. 23.

**PFD-Target Protein Binary Complex Formation**

35S-Radiolabeled, denatured actin or α- or β-tubulin were diluted 100-fold to a final concentration of ~0.1 μM into PBS containing various PFDs (1 μM) and ~0.1 μM purified rabbit hemoglobin. The reaction was incubated at 30 °C for 10 min. The reaction products were separated by non-denaturing electrophoresis as described in Ref. 23.

**Cross-linking of PFD2-PFD3, PFD5-PFD6, Trimeric Complexes, and PFD2-PFD1**

Cross-linking reactions were done using 0.5 μM purified PFD2-PFD3 dimer in the presence of 20 mM EDC and various concentrations of AEDP (both from Pierce), in the range 0–20 μM, in PBS for 40 min at room temperature. The reaction was quenched by the addition of Tris-HCl, pH 8.0, to 40 μM, and the products were analyzed by SDS-PAGE. The purified PFD5/PFD6 complex was covalently cross-linked at a final concentration of 0.9 μM in PBS for 20 min at room temperature using glutaraldehyde in the range 0.0015–0.5%. The reaction was quenched by the addition of Tris, pH 8.0, to 40 μM, and the products were analyzed by SDS-PAGE.

To generate trimers containing PFD2-PFD3-PFD1 and PFD2-PFD3-PFD4, subunits were added to the cross-linking reaction containing PFD2-PFD3 at the following concentrations: PFD1, 1.5 μM; PFD4, 3.5 μM; PFD5, 0.8 μM; PFD6, 0.7 μM; and PFD5-PFD6, 0.5 μM. Each reaction was done in PBS containing 20 mM EDC and 5 mM AEDP for 40 min at room temperature and quenched and analyzed as described above.

To generate dimers of PFD1/2, subunits were incubated together in the presence of 20 mM disulfosuccinimidyl tartrate in PBS. The subunits were all used at a concentration of about 3 μM. The reaction was allowed to proceed for 30 min at room temperature and was then quenched with 80 mM Tris, pH 8.0. The products were analyzed by SDS-PAGE.

**RESULTS**

**Purification of Recombinant PFD Subunits and Their Assembly into Functional Hexamer**—We cloned all six subunits of human PFD into pET vectors and expressed them separately in *E. coli*. All six were to some extent soluble and were purified to minimally 80% homogeneity as judged by Coomassie staining following SDS-PAGE (Fig. 1A). The purified products behaved in a manner consistent with the size expected on the basis of the predicted open reading frame, with the exception of PFD3; this appeared smaller than the expected polypeptide. Analysis by N-terminal peptide sequencing revealed that PFD3, when expressed as a recombinant protein in *E. coli*, initiates at Val31 rather than at Met1 (data not shown). To circumvent this unwanted result, we reduced the GC content wherever possible in the region encompassing the first nine amino acids, consistent with maintaining the *bona fide* PFD3 amino acid sequence. This resulted in a construct that initiated at M1 in *E. coli*.

Because PFD is an oligomer, we sought to determine whether its assembly from its constituent subunits could occur in the absence of other factors. To do this, we co-incubated approximately stoichiometrically equal amounts of each subunit and analyzed the resulting products by gel filtration. In this experiment, a major symmetrical peak emerged from the column with an apparent molecular mass of about 120 kDa (PFD runs larger than its true molecular mass due to the extended nature of the molecule) (Fig. 1B). The contents of this peak migrated as a single species when run on a nondenaturing gel and contained all six subunits upon analysis by SDS-PAGE (Fig. 1C). We conclude that the PFD hexameric complex can spontaneously assemble from its individual subunits.

Individually, none of the subunits exhibited chaperone abil-
ity (data not shown). To determine whether the spontaneously assembled hexameric complex was functional in terms of its ability to transfer target protein to CCT, radiolabeled, unfolded actin was diluted into buffer containing hemoglobin (as a negative control), buffer containing purified bovine PFD (bvPFD) (as a positive control for actin-PFD binary complex formation), or buffer containing bvPFD followed by the addition of CCT (as a positive control for the transfer of radiolabeled actin from PFD to CCT). In each case, the products were analyzed by non-denaturing gel electrophoresis (Fig. 1D, lanes 1–3). In parallel experiments using recombinant PFD, a complex formed with a mobility slightly less than that formed using the bovine homologue. We found that recombinant PFD-bound, labeled actin was as efficiently transferred to CCT as in the control experiment done using bvPFD purified from testes (Fig. 1D, compare lanes 2 and 3 with lanes 4 and 5). We conclude that recombinant PFD is functional in terms of its ability to deliver its bound target protein to CCT.

**Binding of Actin and Tubulin Target Proteins to PFD—** Electron microscopy studies suggest a similar jellyfish-like structure for archaeal and eukaryotic PFD (26), and algorithms also predict coiled-coil structures for the eukaryotic PFD tentacles similar to those seen in the archaeal homologue. To investigate the possibility that the tips of the eukaryotic PFD tentacles are also involved in target protein recognition, we generated truncated forms of each of the six subunits in which at least three distal turns in both the N- and C-terminal α-helices were removed, resulting in subunits with trimmed coiled-coils. In all cases, four or more turns of each α-helix were maintained, making coiled-coil formation possible (Table II). Each construct engineered for the production of truncated subunits was expressed in *E. coli*, and the recombinant protein was purified. Various PFD hexamers were assembled in the same manner as the wild type molecule, each containing from one to six truncated subunits. All combinations assembled spontaneously and migrated as symmetrical peaks when assayed by gel filtration or as a single band when analyzed by native gel electrophoresis. An example of such a truncated, assembled PFD (in this case, PFD truncated in all six subunits, migrating with an apparent molecular mass of about 100 kDa) is shown in Fig. 2A; the content of this species (Fig. 2A, inset) was confirmed by spontaneous assembly and analysis of various PFD(Δ1–6) preparations in which one or more subunits had been individually radiolabeled (data not shown). As expected, the PFDs containing truncated subunits all migrated with molecular masses slightly less than that of wild type PFD, in proportion to their content of truncated subunits.

Actin and tubulins are known targets of PFD (22, 23). When presented with radiolabeled, denatured actin or α- or β-tubulin, bvPFD and wild type PFD formed a stable binary complex. However, PFD in which all six subunits had been truncated completely failed to form a stable complex with either actin or α- or β-tubulin (Fig. 2B). We conclude that at least some of the tentacle’s tips play an essential role in the binding of target proteins to eukaryotic PFD.

Archaeal PFD is assembled from only two different types of subunit, α and β (24). The tips of the tentacles are largely hydrophobic, as is the cavity that they form. In contrast, eukaryotic PFD contains six different subunits, all of which have charged residues at the distal end of the coiled-coils (26). This suggests the possibility that eukaryotic PFD may have evolved to interact with its target proteins differentially, using specific combinations of subunits to bind each target protein. To explore this idea, we used the variously truncated, assembled PFDs to investigate the role played by different subunits in binding to either actin or α- or β-tubulin. When presented with radiolabeled, denatured actin, PFD containing N and C truncations in either PFD1, 2, 5, or 6 formed a binary complex in a manner similar to that seen in experiments using wild type PFD (Fig. 2C, compare lanes 1 and lanes 2 and 3 and lanes 6 and 7). In contrast, PFD containing a truncated form of PFD3 or PFD4 (PFDΔ3 and PFDΔ4, respectively; see Table II) greatly diminished the ability of PFD to stably bind actin (Fig. 2C, compare lanes 1 and lanes 4 and 5). The same double truncation in PFD4 and a similar truncation in PFD5 strongly diminished stable binary complex formation of α- and β-tubulin with PFD (Fig. 2D, compare lanes 1 and lanes 5 and 6). However, PFDΔ3 and corresponding truncations in PFD1 and PFD2 had little if any impact on the ability of PFD to form a stable binary complex with either tubulin compared with wild type PFD (Fig. 2D, compare lanes 1 and lanes 2–4). These data suggest that eukaryotic PFD utilizes a different although overlapping set of subunits to form binary complexes with actin and tubulin target proteins. Alternatively, PFD subunits may contribute differentially to the binding of actins and tubulins.

**PFD2 and -3 and PFD5 and -6 Form Stable Subcomplexes—** The PFD hexamer spontaneously self-assembles by means of the subunits’ intrinsic mutual affinity. We decided to investigate the possibility that some of these interactions might be sufficiently strong to generate stable subcomplexes containing...
PFD2-PFD3 was subjected to chemical cross-linking. The products of this reaction, detected by Coomassie staining after separation by SDS-PAGE, included monomeric PFD2 and PFD3 and a species that behaved with the precise expected molecular mass of a dimer of PFD2 and PFD3 (Fig. 3B). No higher order species were detected in this experiment. The yield of dimeric product increased as a function of increasing concentration of cross-linker. We conclude that the PFD2-PFD3 complex exists as a stable dimer.

In similar experiments, we found that purified PFD5 (which migrates as a symmetrical peak upon gel filtration and appears as a single species on a native gel) and PFD6 (which also migrates as a symmetrical peak upon gel filtration and has an isoelectric point of 9.67 that results in its migration toward the cathode under conditions where PFD5 enters the native gel) also spontaneously associated to form a co-complex (Fig. 3C). This material cannot be a co-migrating admixture of PFD5 and PFD6 both because of its migration properties on gel filtration and its distinctive behavior upon native gel electrophoresis. The PFD5-PFD6 complex emerged from the gel filtration column at an earlier position upon gel filtration compared with the PFD2-PFD3 dimer, although it migrated more slowly than the fully assembled hexamer. The apparent size of this complex (about 80 kDa) suggested that it might be a tetramer. To investigate this possibility, we covalently cross-linked the complex and analyzed the reaction products by SDS-PAGE. Whereas other higher order species were seen at higher cross-linking concentrations, the first two species to appear behaved as a dimer of PFD5 and -6 and an apparently tetrameric complex, whose yield dramatically increased with increasing concentrations of cross-linker (Fig. 3D). These data suggest that the PFD5-PFD6 complex assembled from its constituent polypeptides most probably exists as a stable tetramer.

As spontaneously assembling complexes, the PFD2-PFD3 dimer and the PFD5-PFD6 complex are potential intermediates in the assembly of the functional PFD hexamer. To investigate this possibility, purified PFD2-PFD3 was incubated with equimolar amounts of the remaining four subunits, and the products of this reaction were resolved by gel filtration. The most abundant species emerging from the gel filtration column migrated on native gels in a manner identical to functional, recombinant PFD assembled from individual subunits (Fig. 4A, compare lanes 1 and 2). In parallel experiments, PFD2-PFD3, PFD5-PFD6, PFD6, and PFD4 were co-incubated, as were PFD5-PFD6 and the other four individual subunits. In each case, these combinations also produced species that ran indistinguishably from recombinant PFD on native gel electrophoresis (Fig. 4A, lanes 3 and 4). We also found that covalently cross-linked PFD2-PFD3 assembled efficiently into hexameric PFD upon co-incubation with the remaining subunits (data not shown). We conclude that the PFD2-PFD3 dimer and the PFD5-PFD6 complex are potential building blocks in the assembly of the functional PFD hexamer, either contributing directly to the assembly of the PFD molecule (in the case of PFD2-PFD3) or donating subunits from PFD5-PFD6 subcomplexes (possibly PFD5-PFD6 dimers) for the assembly reaction.

Arrangement of Subunits within the PFD Hexamer—The archaeal PFD has two α and four β subunits. The two α subunits exist opposite one another, two of their four β hairpin turns forming the shared side of the two β barrels of the body of the PFD jellyfish. The α subunits are therefore separated from each other at the level of the tentacles by two β subunits on each side. Whereas the archaeal α subunits do interact with one another across the body of the jellyfish, the interactions of the β subunits in the body are exclusively with their nearest neighbors (25). PFD3 and PFD5, like the archaeal α subunits, a subset of the six subunits. To do this, all possible combinations of two, three, or four purified subunits were co-incubated, and the reaction products were analyzed by gel filtration. We found that PFD2 (which appears as a single species on a native gel and migrates as a symmetrical peak upon gel filtration) and PFD3 (which aggregates in the absence of minimally 2 M urea, appearing in the void volume of gel filtration columns) assembled spontaneously, resulting in the generation of a major peak that migrated with an apparent molecular mass of about 50 kDa (Fig. 3A). This material migrated as a single species upon native gel electrophoresis (Fig. 3A, inset) and contained only PFD2 and PFD3 upon analysis by SDS-PAGE (data not shown). To investigate the nature of this complex, purified

![Fig. 3. Purification and analysis of Subcomplexes PFD2-PFD3 and PFD5-PFD6.](http://www.jbc.org/)

A, absorbance profile of reaction products of co-incubated PFD2 and PFD3 were fractionated on a Superdex 200 gel filtration column. Gel filtration markers are indicated by the arrowheads above the profile. β-LG, β-lactoglobulin. The asterisk designates the migration position of an apparent PFD2-PFD3 dimer. The arrowheads below the x axis indicate the elution positions of the two constituent subunits, PFD2 and PFD3, when fractionated in isolation. Note that the appearance of PFD3 in the void volume is a result of aggregation in the absence of urea and that PFD2 reproducibly eluted as a symmetrical peak (not shown). Inset, assembled PFD2-PFD3 behaves as a unique species upon analysis by native gel electrophoresis compared with PFD2 and PFD3. B, the PFD2-PFD3 complex exists as a dimer. The PFD2-PFD3 complex was cross-linked with increasing amounts of AEDP, and the products of the reaction were analyzed by SDS-PAGE. The locations of molecular size markers (in kDa) are shown on the left; the locations of the PFD2-PFD3 dimer, PFD3, and PFD2 are indicated on the right. C, PFD5 and PFD6 spontaneously assemble into an oligomer. Reaction products of co-incubated PFD5 and PFD6 were fractionated on a Superdex 200 gel filtration column. Gel filtration markers are indicated by the arrowheads above the profile. The arrowheads below the x axis indicate the elution positions of the two constituent subunits, PFD5 and PFD6, when fractionated in isolation; each migrates as a symmetrical peak (not shown). Inset, assembled PFD5-PFD6 migrates differently from PFD5 and PFD6 upon native gel electrophoresis. D, the PFD5-PFD6 complex exists as an apparent tetramer. The PFD5-PFD6 complex shown in C was cross-linked with increasing levels of glutaraldehyde, and the reaction products were analyzed by SDS-PAGE. Molecular size markers (in kDa) are shown on the left. Expected migration positions of the PFD5-PFD6 tetramer, PFD5-PFD6 dimer, PFD5, and PFD6 are indicated on the right. In the experiments shown in A and C, similar amounts of protein were used in the two assembly reactions; the difference in the amount of absorbance reflects the relative absorbance of the constituent subunits. In the experiments shown in B and D, essentially identical cross-linked products were observed using several different cross-linking reagents.
Contribution of Prefoldin Subunits to Target Protein Binding

FIG. 4. The complexes of PFD2-PFD3 and PFD5-PFD6 are assembly-competent, and the PFD2-PFD3 complex is flanked by PFD1 and PFD4. A, the PFD2-PFD3 dimer and the PFD5-PFD6 complex can contribute to the assembly of the PFD hexamer. Various combinations of PFD2-PFD3, PFD5-PFD6, and other PFD subunits were co-incubated, and the reaction products were fractionated on a gel filtration column. Material present in the peak corresponding to the apparent molecular mass of functional, recombinant PFD was analyzed by native gel electrophoresis. The arrowhead shows the migration position of the PFD hexamer. B, PFD2-PFD3 forms trimers with PFD1 and PFD4. Products of cross-linking reactions containing PFD2-PFD3 with or without other subunits or control reactions containing other subunits alone were analyzed by SDS-PAGE. The arrowhead and asterisk show the positions of species migrating at a size expected for trimers not present in either cross-linked PFD2-PFD3, PFD1, or PFD4 alone. In a parallel experiment, PFD5-PFD6 was cross-linked alone or in combination with PFD2-PFD3. Molecular size markers (in kDa) are shown. C, cross-linking of PFD2 and PFD1. Products of cross-linking reactions containing PFD2 and either PFD1 or PFD4 or control reactions containing each of the three individual subunits alone were analyzed by SDS-PAGE. The arrowhead shows the position of a species migrating at the size expected of a PFD2-PFD1 dimer, not present in either cross-linked PFD2 or PFD1 alone. In a parallel experiment, PFD2 and PFD4 were cross-linked together or individually. Molecular size markers (in kDa) are shown on the left.

have two β hairpin turns and are therefore α-like (23). Given the close sequence and structural homologies that exist between archaeal and eukaryotic PFD, it is very likely that PFD3 and PFD5 occupy positions opposite one another in the overall structure of the eukaryotic PFD hexamer. However, the location of the remaining subunits within eukaryotic PFD cannot be assigned on the basis of the structure of archaeal PFD.

To address this issue, we attempted various kinds of cross-linking experiments on intact PFD molecules. This seemed a potentially ideal approach, given the stability of the heterohexamer. However, we found that all such cross-linking experiments led to the generation of multiple species, possibly as the result of covalent bridging within the β-barrels that form the body of the molecule. We therefore took an alternative approach, in which we used the same conditions that successfully covalently cross-linked the PFD2-PFD3 complex to cross-link each of the remaining four subunits either individually (as controls) or in combination with the prepurified PFD2-PFD3 complex. When the reaction products were analyzed by SDS-PAGE, a new band not seen in negative controls of cross-linked PFD2-PFD3 or PFD1 alone appeared in the products of the reaction containing all three of these subunits (Fig. 4B, lanes 1–9). This band migrated at about 55 kDa, the size expected of a trimeric complex between the three subunits. Similarly, when PFD2-PFD3 and PFD4 were co-incubated and cross-linked, the products of this reaction also included a new band that migrated with the expected size for a trimer of the three subunits (also around 55 kDa) not seen in the negative controls (Fig. 4B, lanes 4 and 5). In contrast, experiments done with PFD5 and PFD6, each cross-linked with PFD2-PFD3, failed to produce any bands not seen in the negative controls (cross-linked PFD5 or PFD6, respectively, and PFD2-PFD3) (Fig. 4B, lanes 6–9). Furthermore, in a parallel experiment, cross-linking of a reaction containing prepurified PFD5-PFD6 and PFD2-PFD3 failed to produce any bands suggestive of an interaction between these two complexes (Fig. 4B, lanes 10 and 11). We cannot rule out the possibility that the cross-linked species observed in reactions containing PFD2-PFD3 and either PFD1 or PFD4 might be nonspecific, especially given the modest propensity of PFD4 (and indeed of PFD5 and PFD6) to form homo-oligomeric species under identical conditions. Nevertheless, given the complete absence of any detectable interaction between PFD2-PFD3 and PFD5 or PFD6, these data suggest an arrangement within the PFD complex in which PFD2-PFD3 is flanked by PFD1 and PFD4 rather than PFD5 or PFD6.

Because the α subunits are separated from one another by two β subunits on each side, and assuming that the overall arrangement of the α- and β-like subunits is conserved between archaeal and eukaryotic PFDs, if PFD2-PFD3 is indeed flanked by PFD1 and PFD4, then the PFD5-PFD6 pair must also be nested between PFD1 and -4. The positions of PFD3 and PFD5 and the evidence presented in Fig. 4B point to two possible models for the overall arrangement of subunits within the hexamer (i.e. PFD3-PFD2-PFD1-PFD5-PFD6-PFD4 or PFD3-PFD2-PFD4-PFD5-PFD6-PFD1). We therefore did further cross-linking experiments to assess with which subunit (i.e. either PFD1 or PFD4) PFD2 might interact. We expected that any potential interaction between PFD2 and its neighboring subunit might be relatively weak, given the highly cooperative assembly of this chaperone and our observation that PFD2 preferentially forms a stable dimer with PFD3. Nevertheless, when the products of a cross-linking reaction containing PFD1 and PFD2 were analyzed by SDS-PAGE, a new band appeared that was not seen in either of the negative controls of PFD2 or PFD1 cross-linked alone (Fig. 4C, compare lanes 1 and 2 with lane 3). This band migrates at the predicted size of a dimer of PFD2 and PFD1 at about 35 kDa, exactly halfway between the homodimeric species of each subunit. No new bands were seen in a corresponding cross-linking reaction containing PFD2 and PFD4 when the reaction products were compared with negative controls done with these subunits cross-linked alone (Fig. 4C, compare lanes 4–6). Notwithstanding the caveats delineated above concerning the data shown in Fig. 4B, these data are consistent with PFD2 existing adjacent to PFD1 and are compatible with only one of the candidate models, presented in simple form in Fig. 5A.

DISCUSSION

Eukaryotic PFD is a hetero-oligomer of six different subunits in the molecular mass range 14–23 kDa. The chaperone binds to nascent, unfolded actins and tubulins cotranslationally and delivers them to CCT, with which it interacts (23, 27, 33). No
counterpart of PFD exists in prokarya; however, in archaea, PFD from *Methanobacterium thermoautotrophicum* exists as a hexamer assembled from two α-subunits and four β-subunits to form a jellyfish-like structure in which each tentacle is formed from the two α-helices of a subunit. The subunits can assemble spontaneously to form the hexameric particle (24, 34). To assess whether spontaneous assembly of eukaryotic PFD can also occur, and as a starting point for studies using engineered subunits to investigate the CCT and target protein binding properties of PFD, we cloned, expressed, and purified all six subunits. We found that when PFD1 to -6 are co-incubated, they assemble spontaneously into hexameric PFD that is functional in terms of its ability to donate its target protein to CCT (Fig. 1). The ability of PFDs from both classes of organisms to assemble spontaneously is consistent with the high degree of homology between the α-archaeal subunits and the α-like eukaryotic subunits (PFD3 and -5) as well as the archaeal β-subunits and the β-like eukaryotic subunits (PFD1, -2, -4, and -6). We note, however, that assembly reactions lacking one or more subunits failed to yield biochemically stable complexes in good yield, with the exception of PFD2-PFD3 and PFD5-PFD6. These observations suggest that the interactions leading to complete hexamer formation are strongly cooperative.

Truncations of the tentacle tips of *M. thermoautotrophicum* PFD α- and β-subunits result in a loss of the ability to form a binary complex with denatured rhodanese and firefly luciferase. These substrates were used since no physiological target proteins of archaeal PFD are known. In contrast, there is incontrovertible genetic and biochemical evidence that actin and tubulin are bona fide targets of eukaryotic PFD (22, 23). Electron microscopy studies have shown that eukaryotic PFD when occupied with actin forms a volume consistent with PFD (35–37). We therefore sought evidence that a similar difference in target protein binding properties might exist in the case of eukaryotic PFD. Our analysis depended on assaying the ability of actin or α- or β-tubulin to form stable binary complexes with engineered forms of PFD containing one or more truncated tentacle tips. We observed a dramatically reduced yield of actin-PFD in reactions done with PFD containing PFD3 or PFD4 (Fig. 2C), whereas the yield of binary complex as judged by this assay was unaffected by corresponding deletions in the other subunits. Our proposed model of the PFD hexamer places PFD3 and PFD4 adjacent to one another (Fig. 5). In that event, these two subunits may both contribute to an actin binding site in PFD.

Deletion analysis, alanine scanning substitution analysis, and peptide competition experiments have defined two PFD binding sites in actin and α-, β-, and γ-tubulins (38). The ability of a polypeptide containing the two binding sites of actin or tubulin to prevent binding of either full-length protein to PFD suggested that the chaperone uses at least some of the same subunits to bind these target proteins, although the PFD subunits might contribute differently to the binding of these proteins. These experiments and the data we present here are consistent with the notion that PFD uses different but overlapping combinations of subunits to bind actin and tubulin and that both CCT and PFD have coevolved with their target proteins.

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