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Biochemical and Biophysical Research Communications 317 (2004) 176–180
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First characterization of co-chaperonin protein 10 from hyper-thermophilic *Aquifex aeolicus*☆

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Received 20 February 2004

Abstract

All known co-chaperonin protein 10 (cpn10) molecules are heptamers of seven identical subunits that are linked together by β-strand interactions. Here, we report the first characterization of a cpn10 protein from a *thermophilic* organism: *Aquifex aeolicus*. Primary-structure alignment of *A. aeolicus* cpn10 (*Aae*cpn10) shows high homology with mesophilic cpn10 sequences, except for a unique 25-residue C-terminal extension not found in any other cpn10. Recombinant *Aae*cpn10 adopts a heptameric structure in solution at pH values above 4 (20 °C). Both monomers and heptamers are folded at 20 °C, although the thermal stability of the monomers (pH 3; $T_m \sim 58$ °C) is lower than that of the heptamers (pH 7; $T_m \sim 115$ °C). *Aae*cpn10 functions in a GroEL-dependent in vitro activity assay. Taken together, *Aae*cpn10 appears similar in secondary, tertiary, and quaternary structure, as well as in many biophysical features, to its mesophilic counterparts despite a functional temperature of 90 °C.
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Keywords: *Aquifex aeolicus*; Co-chaperonin protein 10; Circular dichroism; Protein unfolding; Cross-linking; Thermostability

Protein–protein interactions are of fundamental importance in biology because they determine a wide array of protein structures and functions [1]. In addition to heterogeneous protein–protein complexes, many proteins are oligomeric due to the association of identical subunits. In fact, the majority, 70–80%, of all enzymes are oligomeric [2]. The function of quaternary structure, i.e., the arrangement of multiple subunits into an oligomer, may be to allow for cooperative effects, formation of novel active sites, provide additional stability, increase solubility or decrease osmotic pressure [3]. The heptameric co-chaperonin protein 10 (cpn10) is an attractive model for studies of the interplay between polypeptide folding and protein–protein assembly. The primary function of the cpn10 heptamer is to assist cpn60 in folding of nonnative proteins. Upon binding to cpn60, cpn10 forms a cap covering the central cavity of cpn60, and folding of nonnative proteins is achieved through cycles of ATP-dependent binding and dissociation [4–7].

Both structure and function of cpn10 appear conserved throughout nature [4–7]. Crystal structures for *E. coli* cpn10 (GroES), *Mycobacterium tuberculosis*, and *Mycobacterium leprae* cpn10 and bacteriophage T4 Gp31 proteins have been reported [8–11]. Human mitochondrial cpn10 is 37% identical to GroES in terms of primary structure: X-ray and NMR data revealed that its overall fold is identical to that of GroES [12]. In all known cases, each cpn10 subunit adopts an irregular β-barrel topology in the native heptamer. The dominant interaction between the subunits is an anti-parallel pairing of the first β-strand in one subunit and the final β-strand in the other subunit [8]. Our recent biophysical work on human mitochondrial cpn10 [13–15] has shown that isolated cpn10 monomers are folded, but that they have only marginal stability (20 °C, pH 7). From a thermodynamic cycle, we could conclude that more than 85% of the overall heptamer stability is governed by the inter-protein interactions.

Proteins from thermophilic organisms are often similar in sequence and structure to their mesophilic

☆ *Abbreviations:* Cpn10, co-chaperonin protein 10; GuHCl, guanidine hydrochloride; CD, circular dichroism.

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.025

homologs, although they are much more resistant to thermal perturbation [16–18]. Efforts to determine the origin of thermostability in *monomeric* proteins have led to several hypotheses, such as stabilization by an increased number of ionic interactions, an increased extent of hydrophobic-surface burial, an increased number of prolines, and smaller surface loops [18]. Little is known about the mechanisms, i.e., interplay between polypeptide folding and protein–protein assembly, governing thermostability of *oligomeric* proteins. To begin to address this issue, we have cloned, expressed, and performed an initial biophysical characterization of a thermostable cpn10 molecule: *Aquifex aeolicus* cpn10 (*Aae*cpn10). *A. aeolicus* is a hyper-thermophilic bacterium that grows at 89 °C for which the complete genome has been sequenced [19]. We find that *Aae*cpn10 is heptameric and exhibits high sequence homology with mesophilic cpn10 variants, except for an extra C-terminal tail. The similarity in terms of overall tertiary and quaternary structure is evident from biophysical comparisons and the ability of *Aae*cpn10 to function in an in vitro activity assay. *Aae*cpn10 monomers exhibit lower thermal stability than the heptamers, which unfold in a reversible process at temperatures above 100 °C (pH 7). Notably, unfolded monomers can only be refolded at conditions favoring assembly.

Materials and methods

Cloning, expression, and purification. The *A. aeolicus* genomic DNA was obtained from Dr. J. Meyer (CEA-Grenoble, France). The *Aae*cpn10 gene (mopB or Aq2199 [19]; Accession No. NP_214511; 369 bp) was downloaded from NCBI and used to design PCR primers. The primers were 5′-GCT TTT GCA TAT GAA ATT AAG ACC CCT TTA CG-3′ (Forward) and 5′-CAG GAT CCT TAG TTT TGC CCT TGC-3′ (Reverse) which included a *Nae*I and *Bam*HI cleavage site, respectively (underlined). The amplicon was ligated into a pET24c vector. Insertion of the *Aaecpn10* gene was confirmed by restriction-endonuclease digestion on 1% agarose gels. The *Aae*cpn10-containing vector was transformed into *E. coli* BL21(DE3) cells and grown overnight on LB plates (37 °C, 30 μg/ml kanamycin). Mini-expression of the vector allowed for DNA sequencing, which confirmed the presence of the insert and the correct DNA sequence.

To isolate the *Aae*cpn10 protein, transformed BL21(DE3) cells were grown overnight in LB media (30 μg/ml kanamycin). After 4 h of incubation of 1-L cultures, protein expression was induced by the addition of 2 mM IPTG. Cells were harvested and re-suspended in 100 mM Tris, pH 7.5. Cells were lysed using a French press. Precipitates, 50% and 75% saturated with ammonium sulfate, contained over-expressed *Aae*cpn10 according to gel-electrophoresis. These pellets were re-suspended in 100 mM Tris, pH 7.5, and placed in a water bath of 85 °C for 5 min (destroying most *E. coli* proteins but not *Aae*cpn10), followed by 3 min on ice, before centrifugation. The supernatant, containing *Aae*cpn10, was injected to a Q-Sepharose column (Amersham–Pharmacia) connected to an FPLC chromatography system (Amersham–Pharmacia). Protein fractions were pooled, concentrated, and dialyzed into 5 mM phosphate, pH 7. 5 mM $MgCl_2$ had to be added to allow for high *Aae*cpn10 stock concentrations. Gel-electrophoresis of purified *Aae*cpn10 showed a single band of the predicted molecular weight (13.6 kDa, 122 amino acids) indicating >98% purity. Protein yield was approximately 25–30 mg protein per 1-L culture. Protein concentration was determined using ε_{280} of 9800 M^{-1} cm^{-1} (one Trp and three Tyr). Amino-acid sequence alignments were made using CLUSTALW multiple alignment [20] and secondary-structure predictions were obtained as described in [21]. Human mitochondrial cpn10 was prepared as previously described [13,15].

Biophysical studies. Absorption spectra were measured on a Cary 50 spectrophotometer. For far-UV circular dichroism (CD) measurements, an OLIS spectropolarimeter was used with a digitally controlled water bath. Thermal unfolding of *Aae*cpn10 at pH 7, monitored by CD at 220 nm, occurred in a single reversible transition (midpoint corresponding to T_m). Different equilibration times (5–10 min) at each temperature did not change the profiles, and re-scans of original samples gave identical results. Thermal unfolding of *Aae*cpn10 was monitored for different protein concentrations (3, 30, 100 μM; pH 7) and guanidine hydrochloride (GuHCl) concentrations (1.5, 2, 2.5, and 3 M; pH 7) and at pH 3.

Gel filtration was performed on a 16/60 Superdex 75 column (Pharmacia). Calibration was performed with Pharmacia Low Molecular Weight Calibration Kit (pH 7, 20 °C). The solution pH and temperature were varied as indicated. Protein samples (*Aae*cpn10 and human cpn10) for cross-linking were incubated for ~10 min at a specific temperature (10, 30, 50, 70, and 90 °C) before addition of gluteraldehyde (1% w/v final). After 2 min incubation at the specific temperature, the reactions were quenched by addition of 50 mM $NaBH_4$. Following 20 min further incubation, the cross-linked cpn10 solutions were precipitated with 10% w/v trichloroacetic acid. The resulting pellets were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a pre-cast 4–12% Novex gel.

The full procedure for the citrate synthase activity assay has been reported [13]. In short, 40 μM pig-heart citrate synthase was denatured in GuHCl (20 min, 20 °C, 3 mM DTT, and 2 mM EDTA). While vortexing, denatured citrate synthase was added to reaction mixtures of cpn10 and *E. coli* GroEL. ATP was added to a final concentration of 2 mM to initiate chaperonin-assisted refolding. After 1 h, an aliquot of the reaction was added to oxaloacetate and acetyl-CoA. Citrate synthase activity (catalyzing the condensation of oxaloacetate and acetyl-CoA to citrate and CoA) was measured by the decrease in absorption at 233 nm, which corresponds to acetyl-CoA disappearance. The percent recovery of citrate synthase activity was normalized to the activity of the native protein.

Results and discussion

Primary and secondary structure of Aaecpn10

Aquifex aeolicus cpn10 (*Aae*cpn10) was cloned and over-expressed in *E. coli*. The purified protein has a molecular weight of 13.6 kDa per monomer in agreement with the prediction based on its primary sequence (122 residues). The amino-acid sequence of *Aae*cpn10 is aligned with some other mesophilic cpn10 sequences in Fig. 1 and color-coded according to secondary-structure prediction. It is clear that the thermophilic protein is highly homologous to the others in terms of primary structure: most striking, the *Aae*cpn10 sequence is 56% identical to that of GroES. This observation implies that the common tertiary (β-barrels) and quaternary (heptamers) structures of mesophilic cpn10 proteins (Inset, Fig. 2A) are also retained in the thermophilic *Aae*cpn10. Surprisingly, the *Aae*cpn10 is 22–25 residues longer than the mesophilic proteins: it has a C-terminal extension,

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                    interface
A.aeolicus      MKLRPLYDKIVVERLEEKEEKTPSGIIIPDTAKEKPQLGKVVAVGPGKLLDNGE
M.tuber         VNIKPLEDKILVQ-ANEAETTTASGLVIPDTAKEKPQEGTVVAVGPGRWDEDGE
E.coli          MNIRPLHDRVIVK-RKEVETKSAGGIVLTGSAAAKSTRGEVLAVGNGRILENGE
Human     MAGQAF RKFLPLFDRVLVE-RSAAETVTKGGIMLPEKSQGKVLQATVVAVGSGSKGKGGE
Yeast   MSTLLKSAKSIVPLMDRVLVQ-RIKAQAKTASGLYLPEKNVEKLNQAEVVAVGPGFTDANGN

                                                    interface
A.aeolicus      -LKPLSVKEGDVVLFNK-YAGNEVEIE-GKIYLVMSEDEVLAVVEDYSSLIGGEVRWQQRQLSTTRKQGQN
M.tuber         KRIPLDVAEGDTVIYSK-YGGTEIKYN-GEEYLILSARDVLAVVSK
E.coli          -VKPLDVKVGDIVIFNDGYGVKSEKID-NEEVLIMSESDILAIVEA
Human           -IQPVSVKVGDKVLLPE-YGGTKVVLD-DKDYFLFRDGDILGKYVD
Yeast           -KVVPQVKVGDQVLIPQ-FGGSTIKLGNDDEVILFRDAEILAKIAKD
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Fig. 1. Alignment of *Aae*cpn10, GroES, human cpn10, *M. tuberculosis* cpn10, and yeast cpn10 amino-acid sequences. Residues are color-coded according to secondary-structure predictions (blue, β-sheet; green, α-helix; black, random coil). Subunit–subunit interface areas are indicated.

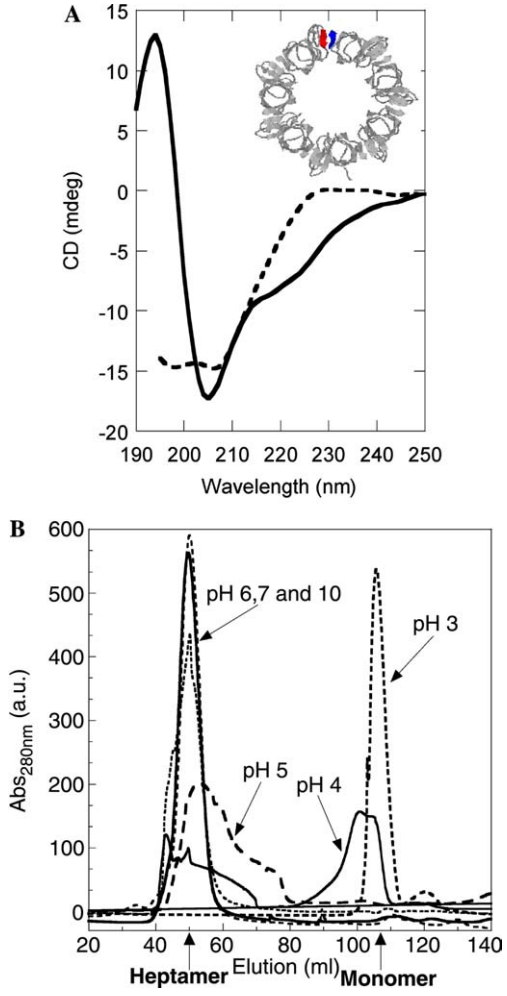

Fig. 2. (A) Far UV CD spectra of *Aae*cpn10 (solid line) and human cpn10 (dotted line); pH 7, 20 °C. Inset: Ribbon diagram of human cpn10 heptamer [12]. Strands involved in one interface are highlighted. (B) Gel-filtration elution profiles for *Aae*cpn10 (20 °C) as a function of solution pH (3–10). *Aae*cpn10 monomer (13.6 kDa) and heptamer (94 kDa) elution volumes (105 and 50 ml, respectively) are indicated. On this column at pH 7, heptameric cpn10 (70 kDa) elutes at 56 ml, molecular weight standards give 45 ml (70 kDa), 48 ml (67 kDa), 64 ml (44 kDa), 74 ml (24 kDa), and 84 ml (13 kDa).

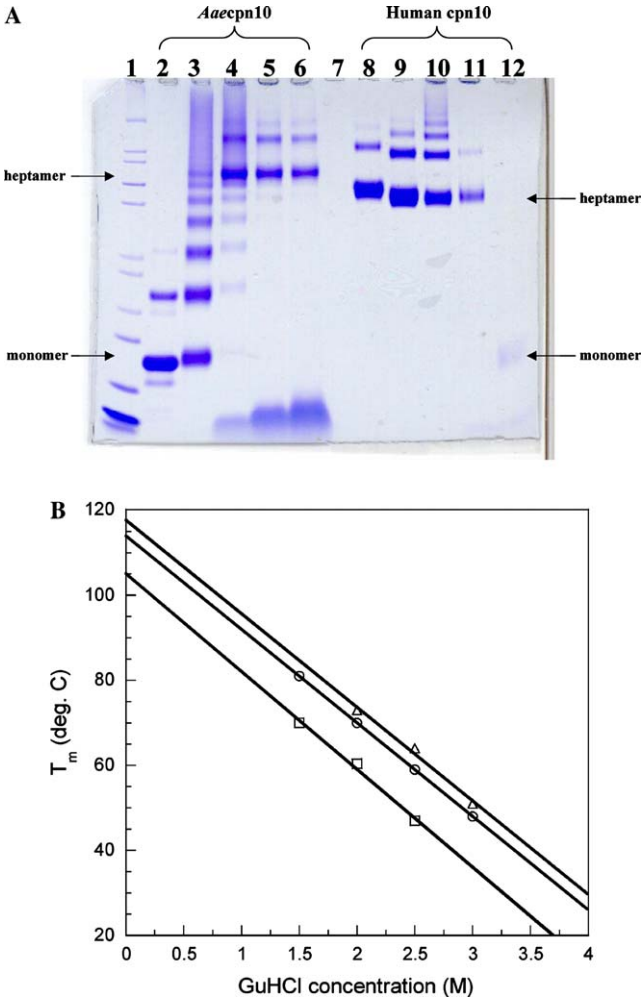

Fig. 3. (A) SDS–PAGE of *Aae*cpn10 and human cpn10 cross-linked with gluteraldehyde at various temperatures (10–90 °C). Lane 1, standard; lanes 2–6, *Aae*cpn10; and lanes 8–12, human cpn10. Lanes 2 and 8, 10 °C; lanes 3 and 9, 30 °C; lanes 4 and 10, 50 °C; lanes 5 and 11, 70 °C; and lanes 6 and 12, 90 °C. (B) Thermal midpoints (T_m), determined from $CD_{220\,nm}$-monitored unfolding curves, for *Aae*cpn10 as a function of GuHCl concentration, for 3 (squares), 30 (circles), and 100 (triangles) μM *Aae*cpn10 (pH 7). Linear extrapolation gives T_m in the absence of denaturant in each case.

predicted to have partly helical structure. Using SWISS-PROT and TREMBL Protein Knowledge data-bases, all available cpn10-like sequences were aligned (84 full-length sequences). Of those, only *Aae*cpn10 has the C-terminal extension. There was no match found for the C-terminal sequence in a BLAST search.

In support of similar secondary structure, purified *Aae*cpn10 exhibits a far-UV CD signal (20 °C, pH 7)

that matches those reported for GroES, *M. tuberculosis* cpn10, and yeast cpn10 [22–24], in all cases including a negative minimum at ~205 nm and a shoulder at 220 nm (Fig. 2A). When compared to the CD signal of human cpn10, which has a positive peak at 230 nm due to restricted tyrosine environment [15], *Aae*cpn10 appears to have more α-helical character (typically, negative minima at 222 and 208 nm), suggesting that the C-terminal extension may form a helix in solution (work on a C-terminal deletion variant is in progress).

Overall structure of Aaecpn10

Gel-filtration and gluteraldehyde cross-linking have been previous methods to accurately determine the quaternary structure of cpn10 proteins [15,22,24]. In Fig. 2B, we show gel-filtration traces of *Aae*cpn10 as a function of pH (20 °C). At neutral pH, *Aae*cpn10 elutes as a heptamer, as is also the case for the studied mesophilic cpn10 proteins. The *Aae*cpn10 heptamer elutes at a volume corresponding to a slightly lower molecular weight (according to the globular molecular-weight standards) than that of the heptamer; since this is also the case for the human cpn10 heptamer, we attribute it to the non-spherical shapes of these molecules. We note that in order to avoid precipitation during concentration of purified *Aae*cpn10, excess Mg^{2+} ions had to be added. Although gel-filtration of *Aae*cpn10 without added Mg^{2+} reports on heptamers, tightly bound Mg^{2+} ions may still be present in the protein. Assembly of human cpn10 and GroES is not dependent on metal ions [15,23], whereas it has been shown for *M. tuberculosis* cpn10 that Mg^{2+} is required for heptamer assembly [24]. The existence of metal-binding sites in cpn10 proteins (and perhaps also in *Aae*cpn10) is consistent with the diffuse binding of heavy metals observed during GroES crystallization [8].

*Aae*cpn10 is heptameric at pH conditions between 6 and 10, but dissociates to monomers at pH values below 5 (Fig. 2B). (When compared to the molecular weight standards, the elution volume attributed to the monomer corresponds to a somewhat lower molecular weight than that of *Aae*cpn10 monomers. However, gel electrophoresis of this fraction confirmed that no proteolytic degradation had occurred.) The pH dependence for *Aae*cpn10 contrasts the behaviors of human cpn10 and GroES, which are heptameric also at pH 3 [24], but is similar to what has been found with yeast cpn10 [22]. *Aae*cpn10 monomers and heptamers have identical secondary structure according to far-UV CD (data not shown).

Gluteraldehyde cross-linking of *Aae*cpn10 (20 °C, pH 7, 40–1200 μM protein) resulted in a set of, roughly equal in terms of intensity, bands corresponding to monomers, dimers, trimers, tetramers, pentamers, hexamers, and heptamers. This result confirms that heptamers form, however, it implies that the lysine side-chains, which are involved in cross-linking, are not fully available for cross-linking in the heptameric structure at this temperature. With human cpn10 at the same conditions, only heptamers are observed upon cross-linking [15]. As expected, cross-linking (20 °C) of *Aae*cpn10 at pH 4 resulted in mostly monomers (data not shown).

Cross-linking of *Aae*cpn10 and human cpn10 (pH 7) was performed as a function of temperature (10–90 °C). Human cpn10 cross-links as a heptamer up to 70 °C, after which only a faint band corresponding to monomers is seen (Fig. 3A). This is in accord with the reported thermal transition for human cpn10 occurring at ~70 °C [15] and shows that the cross-linking assay works efficiently in the tested temperature range. Notably, cross-linking of *Aae*cpn10 becomes dominated by heptamers when the temperature is increased: at 50–90 °C, no smaller oligomers than heptamers are observed (Fig. 3A). This suggests that the lysines in the *Aae*cpn10 heptamer become more available for the cross-linker at higher temperatures. Moreover, it reveals that the native *Aae*cpn10 heptamer is present in solution even at 90 °C (pH 7). In agreement, gel-filtration experiments on *Aae*cpn10 (pH 7) at 50 °C resulted in protein-elution volumes corresponding to heptamers (data not shown).

In vitro function of Aaecpn10

To test the similarity of *Aae*cpn10 to the mesophilic cpn10 proteins in terms of function, an in vitro activity assay was performed (20 °C) [15]. GroEL-dependent refolding of citrate synthase was measured in the presence of human, *E. coli*, and *A. aeolicus* cpn10 proteins (data not shown). The three proteins gave similar results in terms of promoting citrate synthase refolding: human cpn10 induced 50% refolding, GroES 55%, and *Aae*cpn10 65% refolding of citrate synthase at our conditions. In contrast, a mutant of human cpn10, shown not to assemble into heptamers [13], gave negative results (less than 3% refolding of citrate synthase) at the same conditions.

Aaecpn10 thermal stability

Human cpn10 and GroES, in accord with being mesophilic, have thermal midpoints around 70 °C (pH 7) [15,23]. The transitions are protein-concentration dependent since unfolding and disassembly are coupled in the reaction. In Fig. 3B, we show thermal midpoints (probed by far-UV CD) for the *Aae*cpn10 heptamer collected, in the presence of low GuHCl concentrations to lower the transitions to below 100 °C. Extrapolation of the midpoints, for each protein concentration, yielded the thermal midpoint in the absence of denaturant in each case. Like human cpn10 and GroES [15,23], the thermal process is reversible and protein-concentration dependent, suggesting a coupled unfolding/dissociation

reaction: the more protein present, the higher is the midpoint. The thermal transitions (buffer, pH 7) occur at 105, 114, and 118 °C for 3, 30, and 100 μM *Aae*cpn10, respectively, in accord with the *A. aeolicus* bacterium growing at 89 °C.

Unfolding of the *Aae*cpn10 monomers was tested at low pH. Here, the thermal transition occurred already at 58 °C, revealing that much of the *Aae*cpn10 heptamer stability comes from the interface interactions. Interestingly, unfolded *Aae*cpn10 monomers could not be refolded at low pH. The unfolded monomers (20 °C) had to be brought to pH 7 (conditions favoring assembly), where assembly and refolding readily occurred. It thus appears that assembly in the unfolded state is required as a template for polypeptide folding in the case of *Aae*cpn10. In some analogy, refolding of yeast cpn10 required a template in the form of negatively charged membranes [22]. Differences in solution behavior among various cpn10 proteins (such as pH dependence and refolding conditions) may explain non-chaperonin activities, such as antigenicity [24].

Concluding remarks

Here, we report an initial biophysical characterization of a thermostable co-chaperonin protein (*Aae*cpn10). Many hallmark characteristics of mesophilic cpn10 molecules, including the heptameric structure, appear retained in the thermostable variant although the (functional or structural) role of *Aae*cpn10's unique C-terminal tail is unknown. With the recombinant protein at hand, future biophysical and crystallographic work may define the molecular details that allow *Aae*cpn10 to function at high temperatures despite its overall similarity to the mesophilic counterparts.

Acknowledgments

We thank J. Meyer, CEA-Grenoble, for providing the *A. aeolicus* genome. The National Institutes of Health (GM 059663) are acknowledged for financial support.

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