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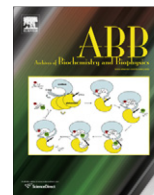

A modulator domain controlling thermal stability in the Group II chaperonins of Archaea

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ABSTRACT

Archaeal Group II chaperonins (Cpns) are strongly conserved, considering that their growth temperatures range from 23 to 122 °C. The C-terminal 15–25 residues are hypervariable, and highly charged in thermophilic species. Our hypothesis is that the C-terminal is a key determinant of stabilization of the Cpn complex. The C-terminus of the Cpn from the hyperthermophile *Pyrococcus furiosus* was mutated to test this hypothesis. C-terminal deletions and replacement of charged residues resulted in destabilization. The stability of ATPase activity declined in proportion to the reduction in charged residues with Ala or Gly. An EK-rich motif (^{528}EKEKEKEGEK5^{37}) proved to be a key domain for stabilization at or near 100 °C. Mutations ''tuned'' the Cpn for optimal protein folding at lower optimal temperatures, and Glu substitution was more potent than Lys replacement. Pf Cpn stability was enhanced by Ca^{2+}, especially in the mutant Cpn lacking C-terminal Lys residues. This suggests that Glu-Glu interactions between C termini might be mediated by Ca^{2+}. The C-terminal of a Cpn from the psychrophilic archaeon *Methanococcoides burtonii* was replaced by a domain from the hyperthermophile, resulting in increased thermostability and thermoactivity. We conclude that localized evolutionary variation in the C-terminus modulates the temperature range of archaeal Cpns.

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Introduction

Molecular chaperones are essential multifunctional cellular systems that assist folding and assembly of newly synthesized proteins, translocation of unfolded proteins across membranes, as well as refolding and degradation of misfolded and aggregated proteins [1–3]. Chaperonins (Cpns)[1] are ubiquitous, hollow complexes that promote correct folding of a wide range of unfolded, misfolded or partially folded proteins [4–6]. Cpns are divided into two groups: Group I Cpns, represented by GroEL in all bacteria, mitochondria and chloroplasts, and Group II Cpns, occurring in eukaryotes and Archaea [5,7,8]. Group I and Group II Cpns share similar quaternary structures consisting of a double toroid cylinder assembled into two rings of subunits stacked back to back. This arrangement generates two adjacent and identical cavities that induce unfolded proteins to fold correctly [6,9]. The two chambers open and shut turn and turn about, activated by ATP hydrolysis at each subunit [2,5]. Group I Cpns are composed of 14 identical subunits arranged in two heptameric rings and require a co-chaperone, GroES, to facilitate protein folding by shutting the chamber orifices as originally shown by Horwich and colleagues [2,10]. Group II Cpns form double rings with an 8- or 9-fold rotary symmetry and have built-in lids, allowing them to function without a co-chaperone [11].

The Archaea are similar to Eukarya in that they have Group II Cpns, however hyperthermophilic Archaea encode minimal Cpn complexes that simplify the study of these chaperones [12]. By contrast, the thermoacidophilic Archaea *Sulfolobus* spp. contain three different Group II subunits while *Methanosarcina acetivorans* encodes five Cpn subunits in both Group I and Group II [8,13]. In species with multiple Cpn homologs, one or two subunits may be up-regulated upon heat shock (e.g. Cpn β-subunit of *Thermococcus* strain KS-1 (*T.* KS-1) [14], and the Cpn α- and β-subunit of *Sulfolobus shibatae* [15]). Heat shock inducible subunits and complexes are typically more stable than non-heat shock regulated subunits from the same organism. The paralogy, heterologous complex formation and variable stability of these Cpns greatly complicates their study. Pf Cpn expression is significant under normal growth conditions and is highly induced upon 105 °C heat shock, probably contributing to the exceptional thermal tolerance of *Pyrococcus* spp. [16]. In contrast to Archaea that encode two, three or more subunits able to form heterologous Cpn complexes, the model archaeon, *Pyrococcus furiosus* (Pf), a hyperthermophile growing optimally at 100 °C, encodes only one Cpn gene [13,17]. Pf Cpn complexes therefore have identical subunits and are minimally complex which renders Pf Cpn an ideal model Cpn for

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[1] *Abbreviations used:* Pf, *Pyrococcus furiosus*; Hsp, heat shock protein; Cpn, chaperonin; GDH, glutamate dehydrogenase; MDH, malate dehydrogenase.

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protein structure analysis and mutational dissection [13,17]. We reported previously that the recombinant Pf Cpn was exceptionally stable *in vitro* [13], resisting 4.5 M guanidine hydrochloride and 105 °C, however the mechanism of its exceptional stability remains unclear.

In all crystal structures of Group II Cpns, the C- and N-terminal regions together form an equatorial domain, but about 20 amino acid residues of the C-terminus remained unresolved in all available structures [18–21]. The C-terminal residues are localized in the equatorial domain close to the inter-ring interface and are thought to project into the inside of the ring and to be involved in intra/inter-ring contacts [20,22]. Current insights into the biological function of the C-terminus stem mainly from structural studies of bacterial Group I Cpn (GroEL) [18]. For example, C-terminally truncated *Escherichia coli* GroEL retains oligomeric structure and protein folding activity, but has reduced thermostability [21,23,24]. The GGM repeat in the GroEL C -terminus is closely involved in modulating the rate of ATP hydrolysis [24,25]. A hydrophilic segment "KNDAAD" in the GroEL C-terminus was found to be critical for substrate protein folding within the central cavity [26]. However, the C-termini of most Group II Cpns lack these characterized domains and are divergent from GroEL. There is limited information of the role of the C-terminus of Group II Cpns. The thermostability difference between α-Cpn and β-Cpn from *T.* KS-1 is thought to arise mainly from the differences in their C termini [21]. By comparing archaeal Group II Cpns from organisms with different growth temperatures we observed that they are conserved and collinear in the internal domains but have highly divergent C-terminal regions, which increase in the proportion of charged residues in proportion with growth temperature. Hyperthermophiles have clustered charged residues whereas the psychrophilic Archaea have few or no charged residues (Table 1). Because ionic interactions have been considered to contribute significantly to the thermostability of hyperthermophilic proteins [27–30], we formed the hypothesis that Cpn thermostability is determined largely by the variable charges of their C-termini. To examine this hypothesis, we carried out targeted mutation studies in order to produce Pf Cpn complexes with the C-terminal charge densities corresponding to Cpns from Archaea with lower growth temperatures. We also replaced the C-terminal domain of a Cpn from a psychrophilic archaeon with the C-terminus from the *P. furiosus* Cpn, producing a chimera with enhanced thermal stability and increased optimal temperature.

Materials and methods

Chemicals, enzymes and reagents

Escherichia coli strains used in this study were DH5α for the initial cloning of the pET expression plasmid and BL21(DE3) for recombinant protein expression. Restriction enzymes, polymerase and ligase were purchased from New England Biolabs (Beverley, MA). All other chemicals were of analytical grade from Sigma–Aldrich (St. Louis, MO). All solutions were made up in ultra-pure water. The native gel protein marker was NativeMark™ unstained molecular weight protein standard from Invitrogen (Carlsbad, CA). Glutamate, ATP, EDTA, DTT, β-NADH and NAD were purchased from Sigma–Aldrich (St. Louis, MO). Bovine glutamate dehydrogenase (GDH) and porcine heart malate dehydrogenase (MDH) were purchased from Amresco (Solon, OH).

Cloning, expression and purification of Pf Cpn wild-type and mutants

Pf Cpn was prepared as previously reported [13]. Genes for Pf Cpn mutants (CD1, CD2, EKD, 0E, 0K, 4E, 3E, 2E, 1E and MA) were amplified by PCR with above sense primer and the corresponding antisense primers listed in Table 2. Genes of Pf Cpn ND was amplified by the antisense primer of Pf Cpn and the following sense primer: 5′-AATTCCATGGGCATGCTCGTTGATAGCC-3′. The mutants were cloned, expressed and lyzed using the protocol for Pf Cpn [13] with modification. The supernatant extracts were heated at 80 °C (WT, CD1, CD2, EKD, 4E, 3E, 2E, 1E and ND) or 65 °C (0E, 0K and MA) for 30 min, then they were purified to homogeneity by two successive rounds of anion exchange: HiTrap™ Q HP cartridge from Biorad (Hercules, CA) and Bio-Scale™ macro-prep high Q cartridge from GE healthcare (Uppsala, Sweden).

Cloning, expression and purification of Mb Cpn and MbPf Cpn

Methanococcoides burtonii (Mb) DSM 6242 Cpn gene (GenBank Accession No. CP000300.1) was synthesized by Integrated Device Technology (Coralville, IA). It was amplified by PCR with following primers with digestion sites (underlined):

sense, 5′-GGCCCATATGATGGCAGGACAGATGTC-3′;
antisense, 5′-GGCCGGATCCTTACATCATTGGAGGCATTC-3′.

The gene for MbPf Cpn was produced by the sense primer and the following antisense primer: 5′-GGCCGGATCCTCAGTCTAGATCACTGCTGAAGTCCTCGCTTCCTCCTCCCTTCTCACCTTCTTTCTCCTTCTCTTTCTCGAGCTTGCTGGCCCTGAGGATCATTACTG-3′. Mb Cpn and MbPf Cpn were cloned using the procedure for Pf Cpn [13] with modification. During their induced expressions, IPTG was added into the culture at OD_{600} of 0.7 to a final concentration of 1 mM. Induction was allowed to proceed at 20 °C for 12 h. Harvested cells were suspended in extraction buffer (25 mM Hepes–KOH, pH 7.2, 100 mM KCl) lyzed by French Press and centrifuged at 25,000*g* for 30 min. The supernatants were purified by Protino Ni-TED 1000 Kit (Macherey–Nagel, Düren).

Table 1
Comparison of the C-termini of Group II Cpns.

Species	C-termini of Group II Cpns	Opt./Max. Temp. (°C)	Negatively charged residues
Pyrodictium occultum	EEKEEKEKEKKEEGEE	105/110	10
Pyrobaculum aerophilum str.IM2β[a]	KREEKGKKKEGEEGEEKKEETKFD	100/104	10
Pyrobaculum islandicum DSM 4184	EKEKEEKKGEEEKKEEKKEFD	100/103	11
Methanopyrus kandleri	SKEEEEEEEEGGSSEF	98/110	9
Pyrococcus furiosus	EKEKEKEGEKGGGSEDFSSDLD	100/103	9
Pyrobaculum arsenaticum DSM 13514	EKEEKGEKKEEKKEEFD	95/103	9
Pyrococcus horikoshii OT3	EKEKEGEKGGGSEEFSGSSDLD	95/100	8
*Archaeoglobus fulgidus*β[a]	EKEKGPEGESGGEEDSEE	83/95	9
Desulfurococcus	EKDKEDKGGSNDFGSDLD	85/95	7
Thermococcus kodakarensis Kod1β[a]	EKDKEGGKGGSEDFGSDLD	65/95	7
Sulfolobus shibatae	GGSEPGGKKEKEEKSSED	75/80	6
Thermoplasma acidophilum DSM 1728	SSSSSSNPPKSGSSSESSED	60/65	3
Methanococcus burtonii DSM 6242 2146[a]	APPMPDGGMGGMPPMM	18/23	1

[a] Indicated Cpn subunit.

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