

Mechanisms of Stability of Robust Chaperones from Hyperthermophiles

Frank Robb

Center of Marine Biotechnology, University of Maryland Biotechnology Institute,
701 E. Pratt St, Baltimore, MD 21202

All of the important catalytic tasks in living cells are carried out by proteins folded into conformations that are essential for the biological function of the cell. In the case of thermally stable proteins, the basis for high temperature stability is still under active study. Activity and stability of enzymes at high temperature is an obvious and critically important adaptation for the survival of thermophiles at the extremes of their temperature ranges. One of the novel aspects of our project is that we proposed to study protein folding at or above 100°C in the hyperthermophilic archaeon *Pyrococcus furiosus* (Pf) which grows optimally at 100°C and encodes single genes for the heat shock regulated Group II chaperonin, Pf Cpn and an α -crystallin homolog, the small Heat Shock Protein (sHsp).

Pf Cpn, a 16-mer oligomer, exhibited optimal ATPase activity at 90°C, pH 7.2 and KCl concentrations from 300 to 500 mM. Pf Cpn is exceptionally stable, retaining 80% of its activity after exposure at 100°C for 3h, and resisting extreme pH, high ionic strength, and up to 4.5M guanidine hydrochloride (Gdn-HCl), a strong denaturant. Pf Cpn bound specifically to denatured lysozyme and ATP addition resulted in protection of lysozyme from thermal inactivation and aggregation at 100°C.

In examining the superior stability and protein folding activity of the small Heat Shock Protein (sHSP) and the thermosome (HSP60), chaperones which are both induced by heat shock at 105°C. We compared the ion pair content in chaperones from *Pyrococcus furiosus*, with optimal growth at 100°C, with homologous proteins from *Methanococcus jannaschii*, an 88°C extreme thermophile. We have previously shown that ion-pair networks contribute significantly to the stability of extremely stable HSPs from hyperthermophiles,

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directing the formation of quaternary nanostructures of very high molecular weight. Current experiments have revealed the structural basis for the dimer formation in sHSP by means of deletion of loops that orient the neighboring subunits head-to-tail.

By applying heat shock conditions (105°C, elevated pressure) to large cultures of *P. furiosus* and other hyperthermophiles in a specialized fermentor we have purified the native versions of chaperones that we have previously characterized by recombinant expression. We are currently comparing the thermostability of the native vs recombinant sHSP and HSP60 chaperones, and will determine whether the native proteins have posttranslational modifications.

Mutations in the C-terminus of the *P. furiosus* CPN60 have revealed that it is the determinant for thermostability (see Fig 1).

Substrate binding also stabilized the 16-mer oligomer of Pf Cpn in 3M Gdn-HCl and activated ATPase hydrolysis in 3-5 M Gdn-HCl. Unfolded lysozyme enhanced the thermostability of Pf Cpn, with an increased optimal temperature for ATPase activity of 100°C. This suggests a molecular basis for adaptive mechanisms to function as a protein foldase at the optimal growth temperature of Pf, 100°C and as a salvage chaperone during heat shock conditions of 105°C. In surface plasmon resonance (Biacore) experiments, Pf Cpn facilitates the release of denatured lysozyme from immobilized sHsp with or without ATP. Pf sHsp transferred denatured lysozyme to Pf Cpn, consistent with cooperative functions of these chaperones during cellular heat shock. This is the first evidence for cooperative transfer of non-native proteins between a group II chaperonin and a cognate sHsp (Luo and Robb, in revision).

From a study of the C-terminal domains of chaperonins from hyperthermophiles, mesophiles and psychrophiles we identified a motif of charged residues, specifically in the hyperthermophiles with multiple, adjacent glu and lys residues. The *P. furiosus* C-terminal contains the motif AASKLEKEKEKEGEGKGGG with five glu/lys repeats. The HSP60 from the psychrophile *Methanococcoides burtonii* contains no glu or lys in the

corresponding domain, although the psychrophile and hyperthermophile chaperonins show general conservation. We constructed mutants of this region including a 25 amino acid deletion. This mutant was functional in folding and ATPase activities, yet was 10⁰C less thermostable than the wild type (Luo and Robb, in preparation). In ongoing mutagenesis studies we have added synthetic subdomains, including one in which all lys residues are replaced by glu in the C terminal deletion mutant and tests of the stability of these mutants is in progress. Preliminary data suggest that the "all-glu" version of the Pf Cpn is functional in folding and ATPase activities, however it is radically destabilized with a T_{1/2} of 29 seconds at 100⁰C compared to 4.5 hours in wild-type Pf Cpn. The C-terminal domains of the *Thermoplasma* and *Thermococcus* CPNs, which are homologous to Pf Cpn, are not resolved in current crystal structures and we hypothesize that this domain is a key determinant of high temperature stability in chaperonins.

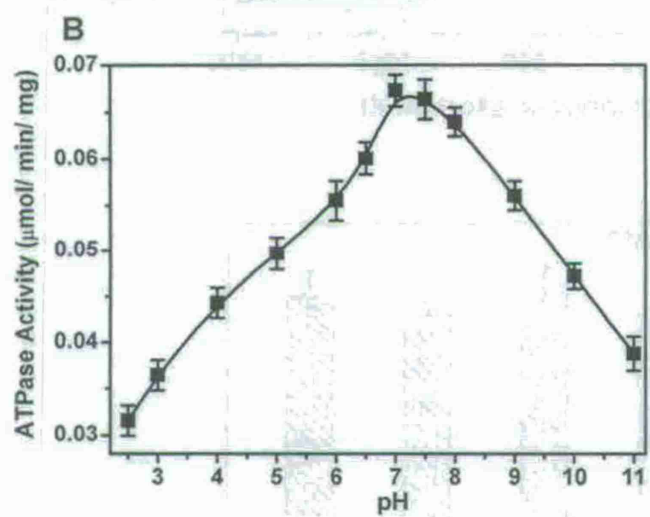
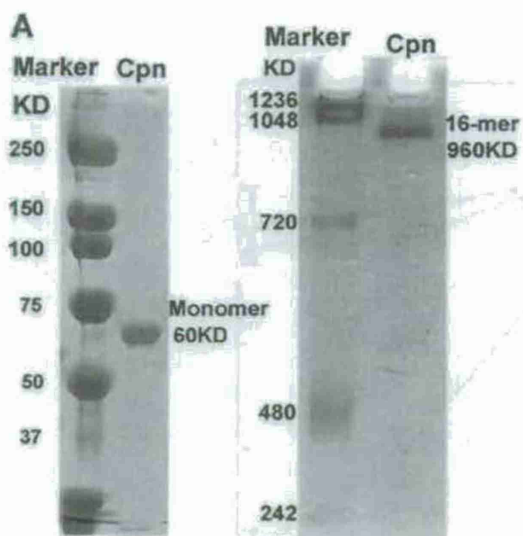
We concluded that Glu residues in the C-terminal are crucial to thermostability of Pf Cpn. We considered whether other negatively charged residues (E⁵⁴², D⁵⁴³, D⁵⁴⁷ and D⁵⁴⁹) in ⁵⁴²EDFSSDLD⁵⁴⁹ might also contribute to protein stability. Accordingly, we mutated these charged residues to Gly as shown in Fig 5A. Glycine, which is uncharged, converted the 16Mer complexes to single ring, octomeric forms. We discovered that charged residues in these positions are responsible for maintaining thermal stability at high temperature. The destabilized mutants were also "detuned" in their optimal temperature for activity, and a wide range of optimal temperatures was covered (Figure 5B).

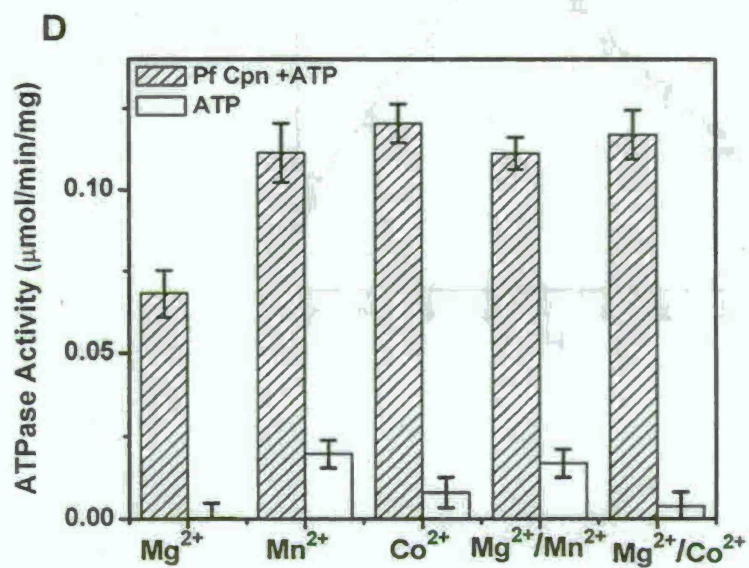
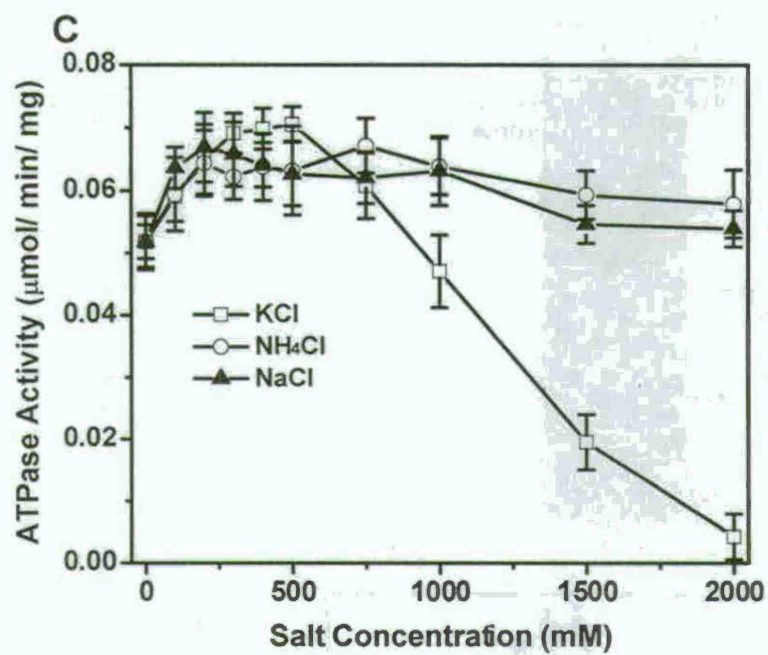
Accomplishments

- Establishment of interaction chain for protein folding by Surface Plasmon Resonance of small Heat Shock Proteins and chaperonins.
- Identification of key residues in thermal stability interaction at C-terminal of the chaperonin.
- Construction of a variant of *P. furiosus* chaperonin that is active at 40⁰C by adding a synthetic C-terminal subdomain

Key experiments establishing the thermal stability of HSP 60 wild type and mutants and cooperative protein folding of HSP60 and sHSP from *P. furiosus* are shown in Figures 1-5 below:

Figure 1. Biochemical studies of the recombinant Pf Cpn. A. SDS-PAGE (5 μ g) and Native gel (20 μ g) analysis of Pf Cpn. The native gel markers from top to bottom are IgM Hexmer, IgM Pantamer, Apoferritin band 1, Aproferritin band 2 and B-phycoerythrin. B. ATPase activities of Pf Cpn at various pH levels. C. ATPase activities in different salts. D. ATPase activities of Pf Cpn at various divalent cations. All cations were in a concentration of 10 mM. E. Thermal stability of Pf Cpn (0.02 mg/ml at 25 mM Hepes pH7.2, 300 mM KCl, 10 mM $MgCl_2$) at high temperatures. The samples were heated for specified times and assayed for their ATPase activities at 90°C. All data were the average values from three experiments.





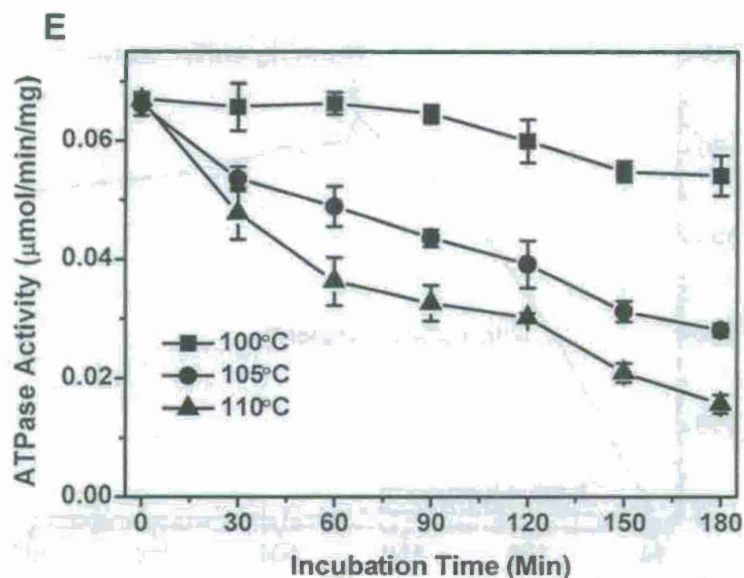
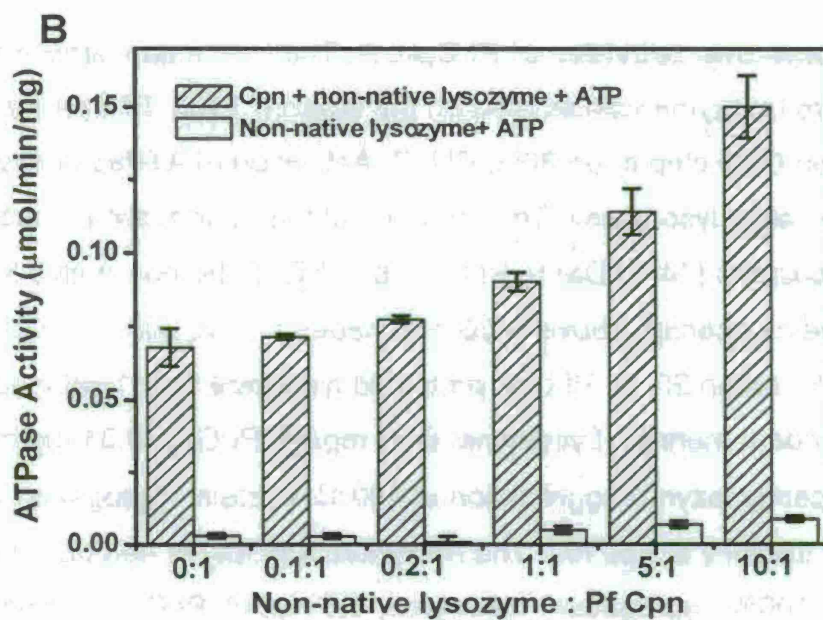
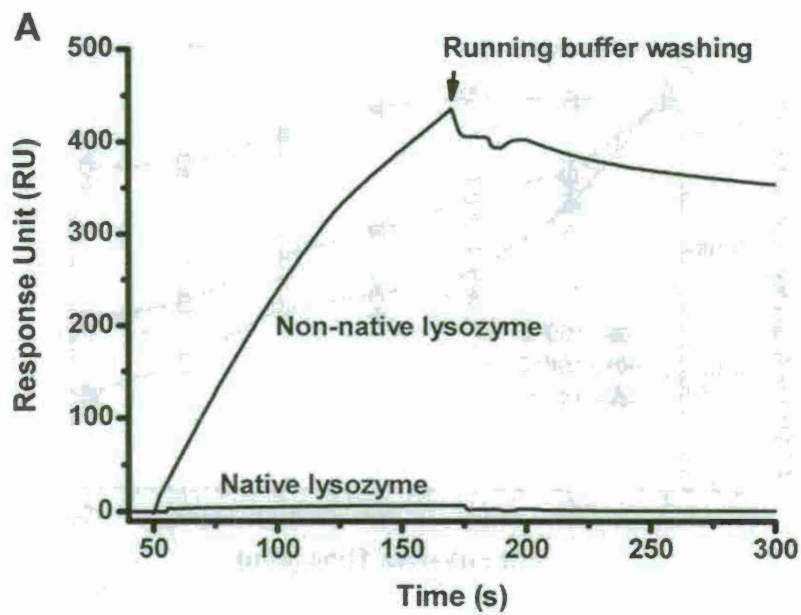


Figure 2. Chaperone activities of Pf Cpn. A. The interaction between Pf Cpn and non-native lysozyme was studied on the Biacore T100. Pf Cpn was immobilized on CM5 chip to be 3000 RU. B. Activation of ATPase activity by a series of non-native lysozymes. The numbers at the bottom are the molar ratio of non-native lysozyme (14.4 kDa) to Pf Cpn (960 KDa). The non-native lysozyme were prepared as described above in 25 mM Hepes pH7.2, 300 mM KCl, 10 mM $MgCl_2$, 0.001% tween 20. C. Pf Cpn protected lysozyme from heat inactivation in an ATP dependent manner. Lysozyme, 0.01 mg/ml; Pf Cpn, 0.34 mg/ml. D. Pf Cpn suppressed lysozyme aggregation at 100°C. Protein aggregation was monitored as turbidity at 450 nm. The maximum turbidity of denatured lysozyme was taken as 100% aggregation. Lysozyme, 0.5 mg/ml; Pf Cpn, 1 mg/ml.



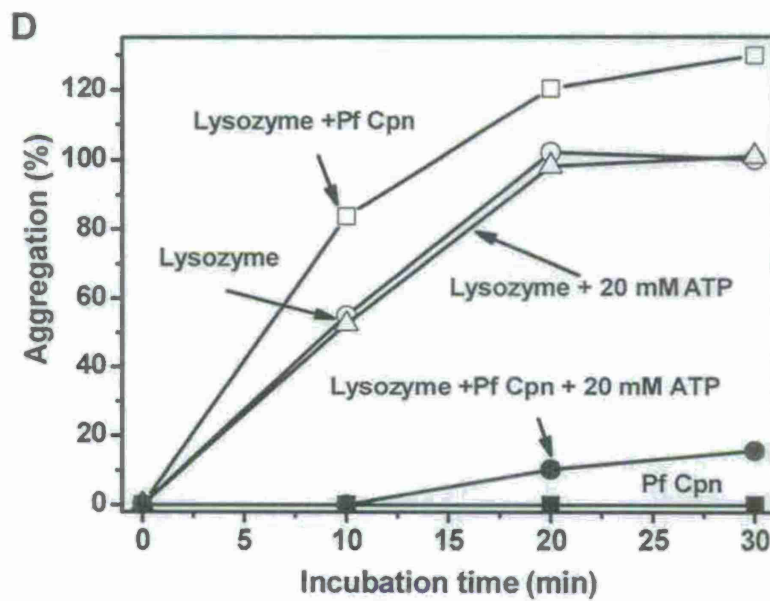
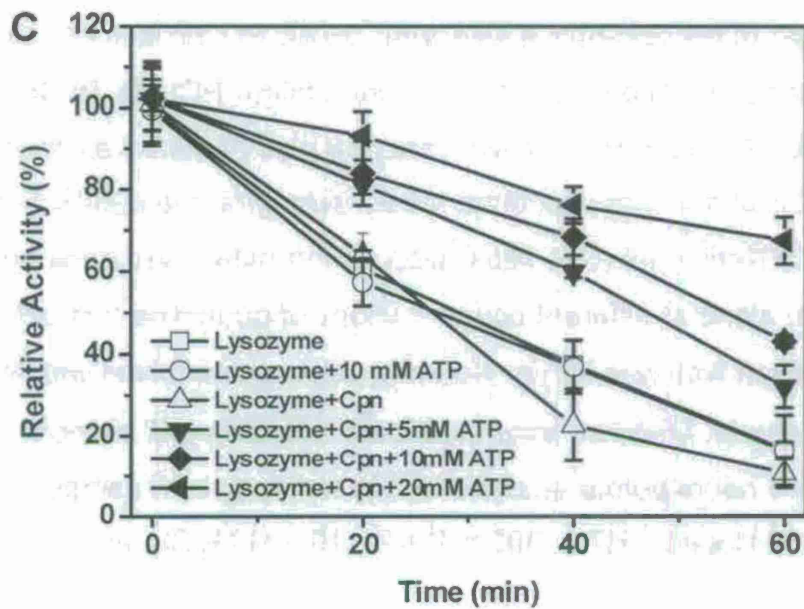


Figure 3. Binding of non-native lysozyme enhances the stability of Pf Cpn.

ATPase activities of the non-native lysozyme bound Pf Cpn and Pf Cpn alone at different temperatures (A) and with the addition of Gdn-HCl (B). Pf Cpn in these assays was at 0.02 mg/ml. Non-native lysozyme was prepared as described above at 0.01 mg/ml. All of the data was obtained by averaging the results from three experiments. (C) Native gel (6%) separation of non-native lysozyme bound Pf Cpn and Pf Cpn alone at different concentrations of guanidine hydrochloride. Pf Cpn loaded to each well was 30 μ g. Non-native lysozyme mixed with Pf Cpn was 8 μ g for each sample. Mixtures were incubated with Gdn-HCl at room temperature for 3 hours before analysis by 6% native gel. All samples solution contained 25 mM Hepes, pH7.2, 300 mM KCl, 10 mM MgCl₂, and 0.001% tween 20.

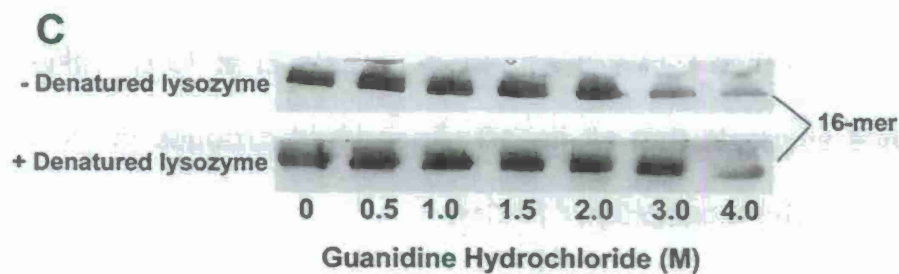
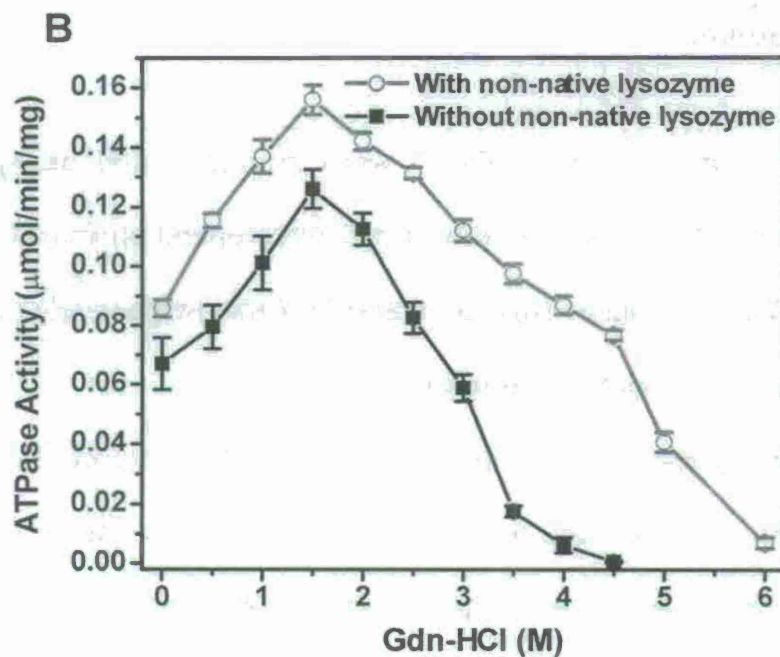
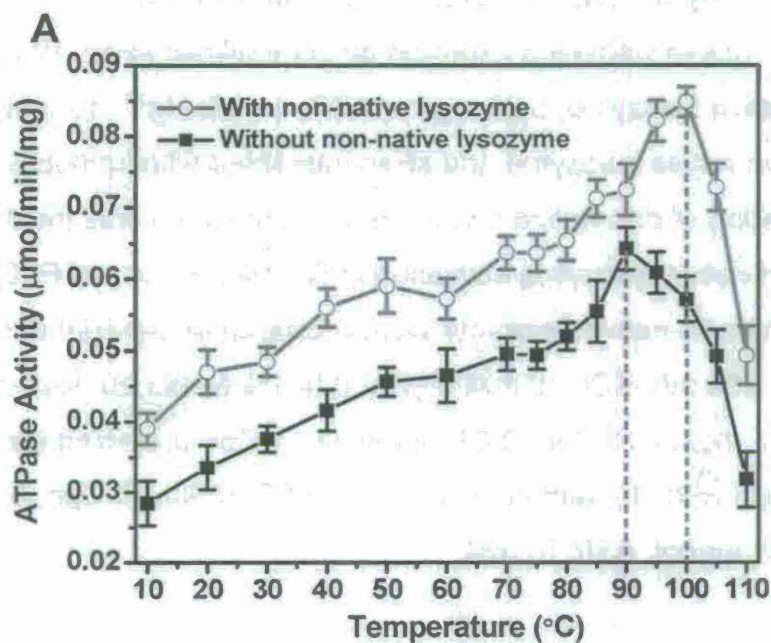
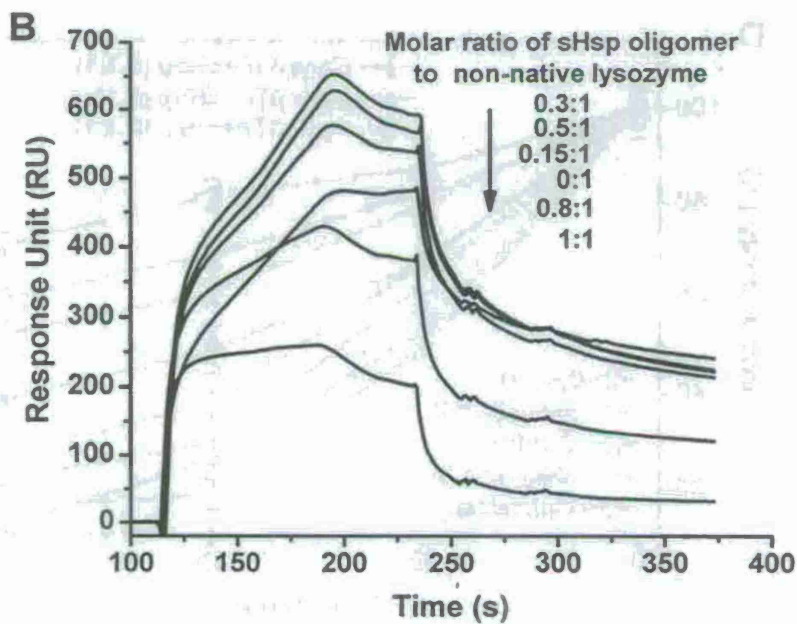
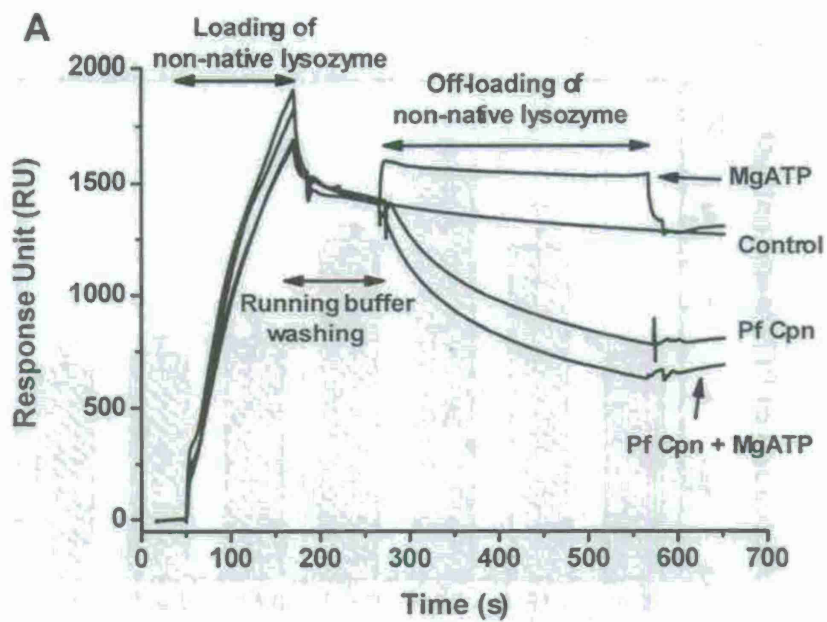


Figure 4. The cooperation between Pf Cpn and Pf sHsp. A. Pf Cpn facilitated release of non-native lysozyme captured by immobilized sHsp. Pf Cpn, 0.02 mg/ml; non-native lysozyme, 0.05 mg/ml; ATP, 1 mM; Mg^{2+} , 10 mM. B. The mixtures of non-native lysozyme and sHsp interacted with immobilized Pf Cpn. The concentration of non-native lysozyme in the mixtures was fixed at 0.025 mg/ml. C. Non-native lysozyme activation of ATPase activity of Pf Cpn at a series of Pf sHsp. The non-native lysozyme were prepared as described above in 25 mM Hepes pH7.2, 300 mM KCl, 10 mM $MgCl_2$, 0.001% tween 20. Non-native lysozyme, 0.01 mg/ml; Pf Cpn, 0.02 mg/ml. D. Pf Cpn protected lysozyme from heat inactivation with different concentrations of Pf sHsp. Pf Cpn, 0.06 mg/ml; lysozyme, 0.01 mg/ml; ATP 10 mM.

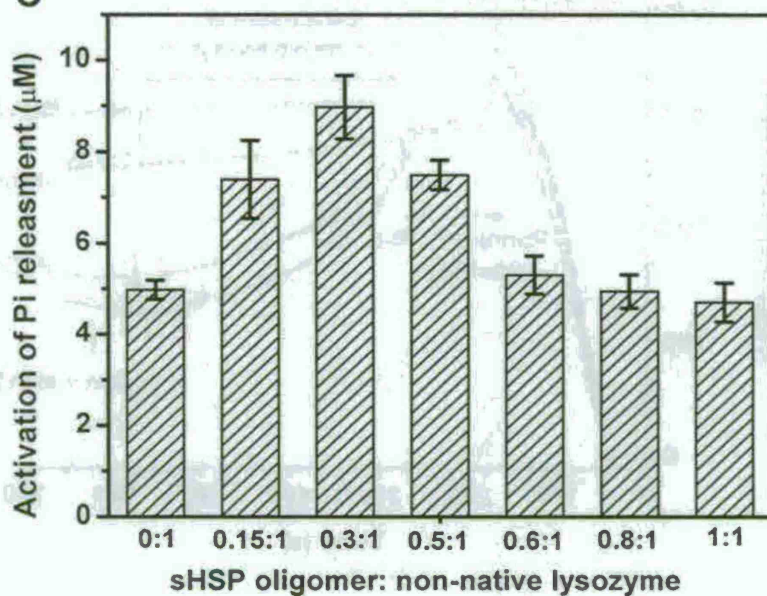
Figure 5. Mutant constructs of PfCpn and their effect quaternary structure of the chaperonin.

Mutants Pf Cpn CD3 and ED2G were produced and assayed (Fig 3A). Compared to WT, these two mutants showed decreased optimal temperatures of 73.3°C and 72.3 and shorter $t_{1/2}$ of 57.5 and 55.7 min respectively suggesting that these 4 residues contribute to thermostability.

Finally, all negatively charged residues in the C-terminal were mutated to Gly to produce the MA ("mutate all") variant (Fig. 3A). As shown in Fig. 3B, MA assembled to form only 8-mers with no detectable 16mers or filaments. MA had an optimal ATPase temperature of 40.1°C and denatured within 1 min at 100°C. It was less stable than E2A with shorter $t_{1/2}$ (Table 2) at 70°C. Taken together; this evidence suggests that all negatively charged residues in the C-terminus contribute to the thermostability of Pf Cpn.



C



D

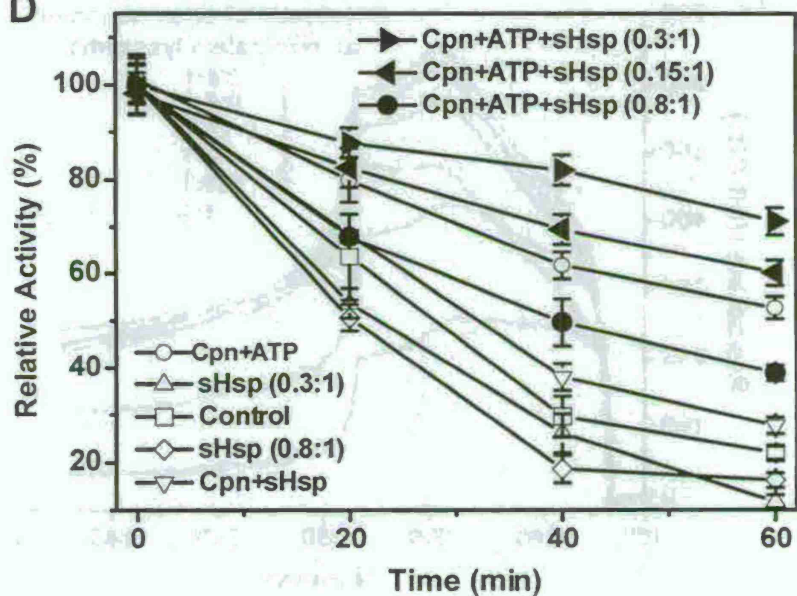
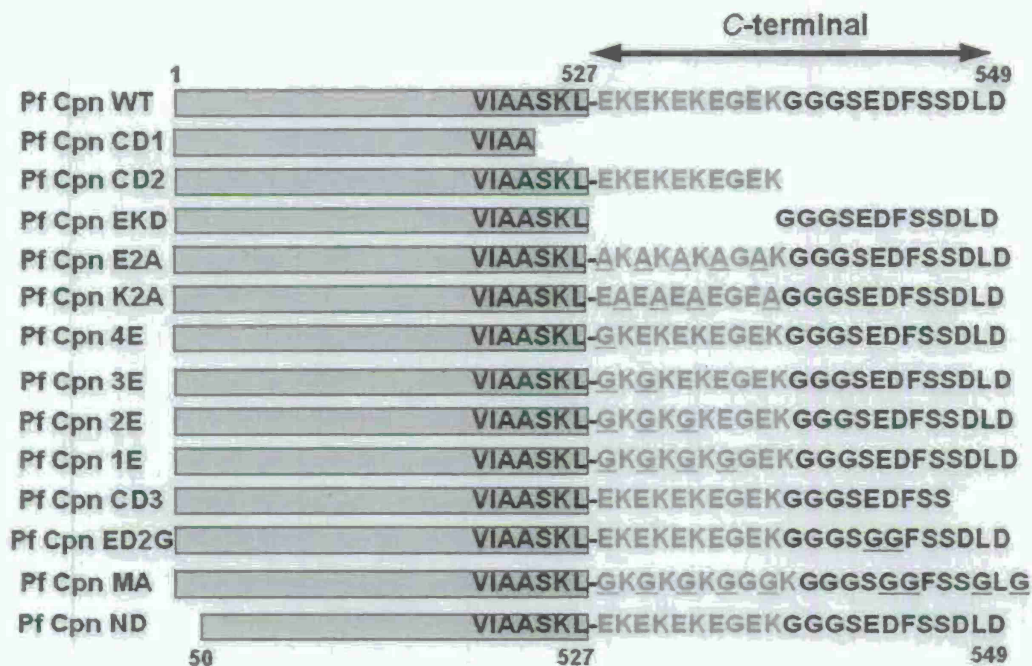


Figure 5 Schematic of C-terminal mutants (A) and sizing analysis by Native-PAGE (B). Pf Cpn WT and mutants were prepared in buffer A (25 mM HEPES-KOH, pH 8.0, 300 mM KCl, 1 mM MgCl₂). Samples were analyzed by 3-6% native PAGE and 10% SDS PAGE. Each lane contains 20 µg of protein.

A



B



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14. ABSTRACT Recombinant hyperthermophile HSP60s were fully characterized in vitro. The recombinant Pfu HSP60 has optimal ATPase activity at 90C and pH of pH of 7.0 and from 300 to 500 mM KCl. Biacore (SPR) binding assays showed that the denatured target proteins could be loaded on to immobilized chaperones, and suggesting possible applications of affinity refolding of non-native proteins following recombinant expression. HSP60 is an effective ATP-dependent protein foldase even in 1-4 M guanidine chloride at 90C. Binding of denatured lysozyme conferred higher thermo-stability on the chaperonin, and increased the optimal ATPase temperature from 90C to 100C. The HSP60 and sHSP were found to be extremely effective at disrupting stable cross-beta structures in prion amyloid proteins. Transient treatment with 2M-guanidine resulted in a modified HSP60 with 30% lower basal ATP hydrolysis but unaltered folding capacity. This suggests that recombinant HSP60, resembling the native HSP60, can be engineered by reassembly following exposure to hot guanidine.						
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