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Mycobacterium tuberculosis GroEL Homologues Unusually Exist as Lower Oligomers and Retain the Ability to Suppress Aggregation of Substrate Proteins

Rohini Qamra[1], Volety Srinivas[2] and Shekhar C. Mande[1]*

[1]*Laboratory of Structural Biology, Centre for DNA Fingerprinting and Diagnostics ECIL Road, Nacharam Hyderabad 500 076, India*

[2]*Centre for Cellular and Molecular Biology, Uppal Road Hyderabad 500 007, India*

**Corresponding author*

Chaperonin-60s are large double ring oligomeric proteins with a central cavity where unfolded polypeptides undergo productive folding. In conjunction with their co-chaperonin, Chaperonin-60s bind non-native polypeptides and facilitate their refolding in an ATP-dependent manner. The ATPase activity of Chaperonin-60 is tightly regulated by the 10 kDa co-chaperonin. In contrast to most other bacterial species, *Mycobacterium tuberculosis* genome carries a duplicate set of *cpn60* genes, one of which occurs on the *groESL* operon (*cpn60.1*), while the other is separately arranged on the chromosome (*cpn60.2*). Biophysical characterization of the mycobacterial proteins showed that these proteins exist as lower oligomers and not tetradecamers, an unexpected property much different from the other known Chaperonin-60s. Failure of the *M. tuberculosis* chaperonins to oligomerize can be attributed to amino acid mutations at the oligomeric interface. Rates of ATP hydrolysis of the *M. tuberculosis* chaperonins showed that these proteins possess a very weak ATPase activity. Both the *M. tuberculosis* chaperonins were partially active in refolding substrate proteins. Interestingly, their refolding activity was seen to be independent of the co-chaperonin and ATP. We hypothesize that the ATP independent chaperones might offer benefit to the pathogen by promoting its existence in the latent phase of its life cycle.

Keywords: chaperonin; protein folding; oligomer; aggregation; *M. tuberculosis*

Introduction

Molecular chaperones are a diverse set of proteins that mediate the correct folding, assembly, transport and degradation of other proteins *in vivo*.[1] Chaperonins form a sub-group of molecular chaperones that are found in all domains of life. Group I chaperonins comprise a family of highly conserved proteins, approximately 60 kDa in molecular mass that form a cylindrical assembly of two heptameric rings. Unfolded polypeptides bind and undergo productive folding within the large central cavity of this assembly.[2] The *Escherichia coli* chaperonin, GroEL, has provided a paradigm in understanding protein folding mechanisms mediated by the chaperonins.[3] GroEL promotes *de novo* folding of ~10–15% of all proteins in bacterial cytosol in co-ordination with the heptameric co-chaperonin, GroES.[4] ATP-dependent conformational changes in GroEL have been shown to be necessary for proper chaperonin function *in vivo*.[1,5]

Chaperonins in all bacteria are encoded by essential *groEL* and *groES* genes (also called *cpn60* and *cpn10*), arranged on the bicistronic *groESL* operon.[6] The operon arrangement of the two genes is highly conserved among all known bacterial species. Interestingly, *Mycobacterium tuberculosis* contains two copies of the *cpn60* genes.[7] One of these genes, *cpn60.1*, is organized on the operon with *cpn10*, while the second copy, *cpn60.2*, is arranged separately on the genome.[7] A similar arrangement of *cpn10* and *cpn60* genes has been described in *Streptomyces spp*.[8] and *Mycobacterium leprae*.[9]

The *E. coli* chaperonin, GroEL, is known to be essential at all temperatures of growth.[10] Ubiquitous presence of chaperonins, and their high

Abbreviations used: Cpn60s, chaperonin-60s; bis-ANS, 1,1-bis (4-anilino) naphthalene-5,5′-disulfonic acid; CD, circular dichroism.

E-mail address of the corresponding author: shekhar@cdfd.org.in

conservation suggests a similar indispensable role that these proteins play in all life forms. A high sequence similarity that *M. tuberculosis* Chaperonin-60s (Cpn60s) exhibit with *E. coli* GroEL, suggests that these might also perform a similar role in this organism. The existence of a duplicate set of *cpn60* genes in *M. tuberculosis*, however, has been perplexing. Plant chloroplasts also possess two copies of Cpn60s that are known to form a hetero-oligomeric assembly.[11] The two *M. tuberculosis* GroEL homologues may similarly be interdependent for the assembly of the protein into a functional hetero-tetradecamer. Duplication therefore might suggest either an interdependence of the gene products or alternatively, the necessity for a redundant function.

While reasons for gene duplication remain unclear, both the Cpn60s of *M. tuberculosis* have been shown to be highly antigenic in nature, eliciting strong B-cell and T-cell immune responses. Cpn60s have also been proposed to play roles as virulence determinants and as inducers of host inflammatory responses.[12] Moreover, both Cpn60.1 and Cpn60.2 of *M. tuberculosis* have been shown to be potent cytokine inducers.[13] The Cpn60s might therefore represent important components of *M. tuberculosis*, playing roles as immunomodulators, and perhaps also required for proper protein folding and transport.

Biochemical properties of *M. tuberculosis* Cpn60s, especially their role as molecular chaperones, have not been characterized yet. Moreover, the presence of two copies of these genes in the *M. tuberculosis* genome is intriguing. To address these questions, we have expressed the *M. tuberculosis cpn60* genes in *E. coli* in an attempt to elucidate their role in protein folding.

Results

Cloning, expression and purification of *M. tuberculosis* Cpn60.1 and Cpn60.2

The full length chaperonin *cpn60.1* and *cpn60.2* clones were obtained and overexpressed in *E. coli* as described in Materials and Methods. The two chaperonins were expressed in large quantities and exhibited a protomer molecular mass of ~60 kDa. As judged by SDS-PAGE, both the proteins were more than 98% pure after the metal affinity purification.

Quaternary structure determination

A tetradecameric structure for wild-type Cpn60 has been established for a number of chaperonins from different species.[14,15] In a few cases, however, Cpn60 homologues have also been reported to exist as heptamers.[16] The native molecular mass of the purified *M. tuberculosis* proteins, Cpn60.1 and Cpn60.2, was established by size exclusion chromatography. Intriguingly, the purified Cpn60.1 and Cpn60.2 eluted as sharp homogenous peaks at volumes corresponding to molecular mass of approximately 110 kDa when compared to standard molecular weight markers (Figure 1). Neither Cpn60.1 nor Cpn60.2 eluted at the position where *E. coli* GroEL and canonical Cpn60 14-mers would appear. The sharp peaks were suggestive of the globular state of the purified proteins. Elution profile on the size exclusion chromatography of the two Cpn60s lacking the $(His)_6$-tag was consistent with that of the $(His)_6$-tagged proteins, suggesting that the presence of $(His)_6$-tag did not interfere in oligomerization (data not shown).

Osmolyte stabilizers, such as glycerol, are known to promote stability of the oligomeric state of proteins. Similarly, the presence of ATP and the co-chaperonin, Cpn10 may also be crucial to the oligomeric assembly of the mycobacterial Cpn60s. *M. tuberculosis* Cpn10 has already been demonstrated to exist as a heptamer in the presence of divalent cations.[17] Purification of Cpn60.1 and Cpn60.2 was performed in the presence of glycerol, or in the presence of ATP-γ-S and *M. tuberculosis* Cpn10. Their oligomeric state was then tested on Native-PAGE. Proteins purified under these conditions, however, did not exhibit oligomerization (Figure 2). Thus, purification of *M. tuberculosis* Cpn60s, either in the presence of co-chaperonin or other osmolyte stabilizers did not seem to promote the oligomeric assembly of these proteins. The size exclusion chromatography and Native-PAGE data hence suggest that the *M. tuberculosis* GroEL homologues might intrinsically exist as lower oligomers.

Reconstitution of *M. tuberculosis* Cpn60s

In order to functionally reconstitute the chaperonins in their canonical oligomeric forms, we subjected the purified Cpn60.1 and Cpn60.2 to *in vitro* reconstitution experiments under a variety of conditions. It has previously been reported that urea-dissociated *E. coli* GroEL 14-mers are capable of reassembling into the tetradecameric state upon removal of the chaotrope.[18] Reconstitution of the *E. coli* GroEL tetradecamers from the disassembled monomers requires the presence of Mg^{2+} and adenine nucleotides. Presence of ammonium sulphate has also been reported to be essential for the oligomer reassembly.[19] When subjected to similar refolding conditions, neither of the two mycobacterial GroEL homologues spontaneously assembled into tetradecamers (Figure 3(a)). Reconstitution was also attempted in the presence of glycerol. However, none of the various combinations of nucleotides, GroES, ammonium sulphate, glycerol, K^+ or Mg^{2+}, promoted oligomeric assembly of the mycobacterial chaperonins (Figure 3(a)).

GroES has previously been shown to facilitate the oligomeric assembly of GroEL.[20] Reconstitution of Cpn60s was thus performed in the presence of the cognate Cpn10. Presence of the *M. tuberculosis* co-chaperonin, Cpn10 in the reconstitution mix however, had no effect on the oligomeric assembly of the purified proteins (Figure 3(b)). Failure to

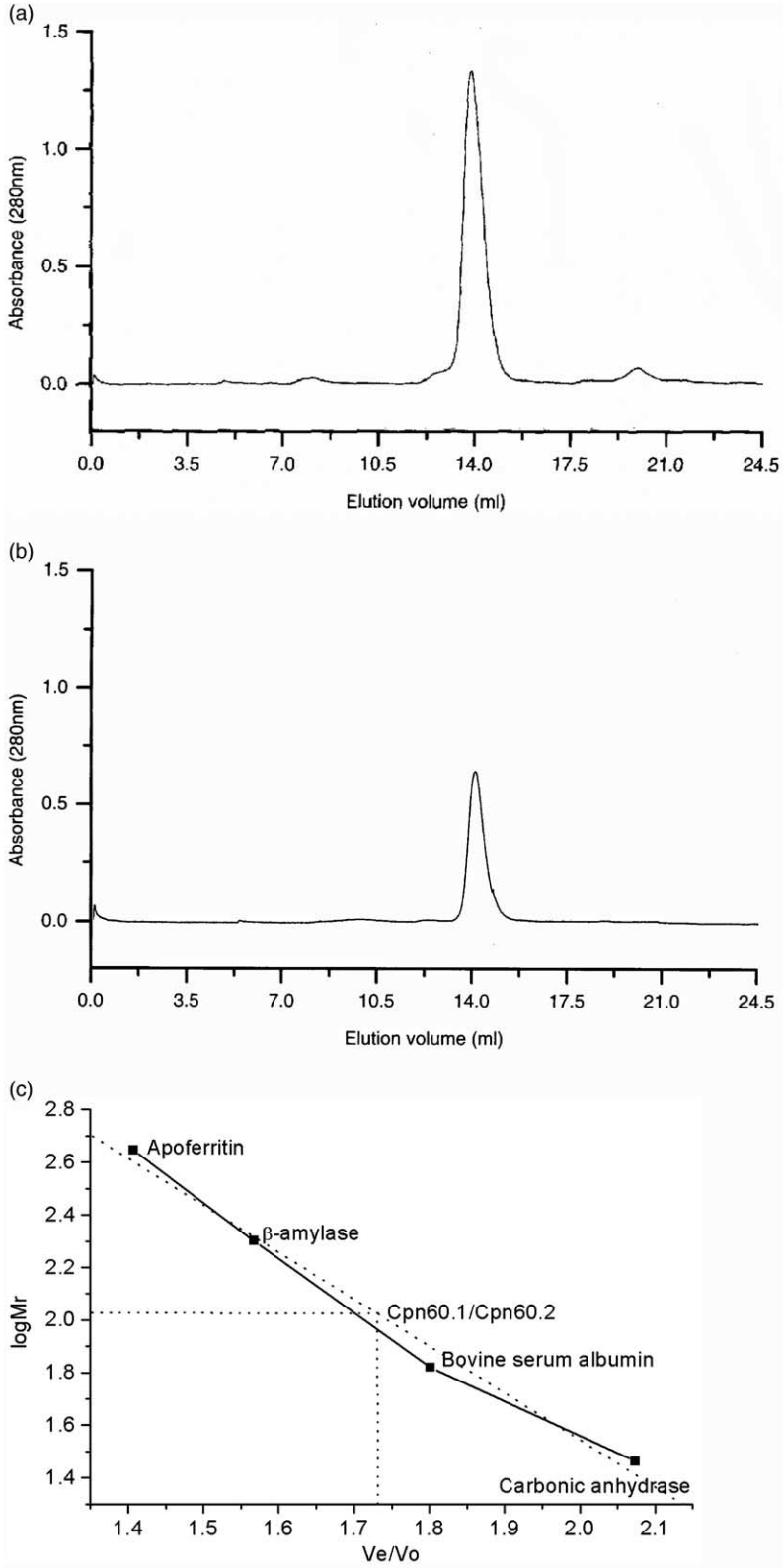
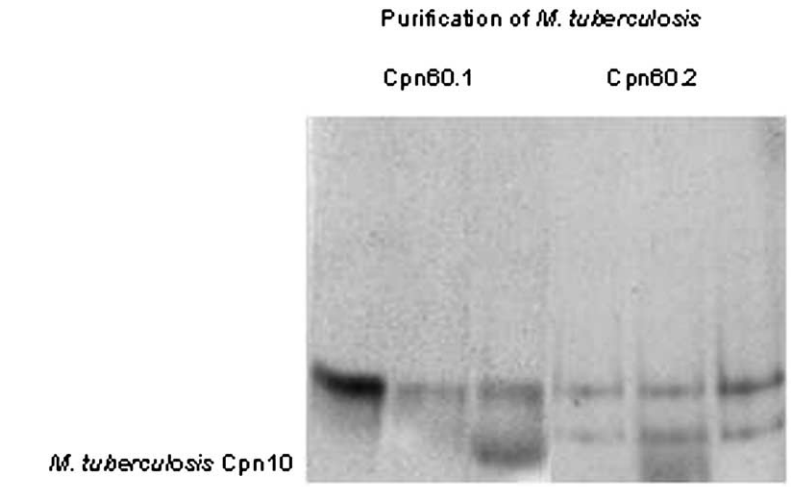
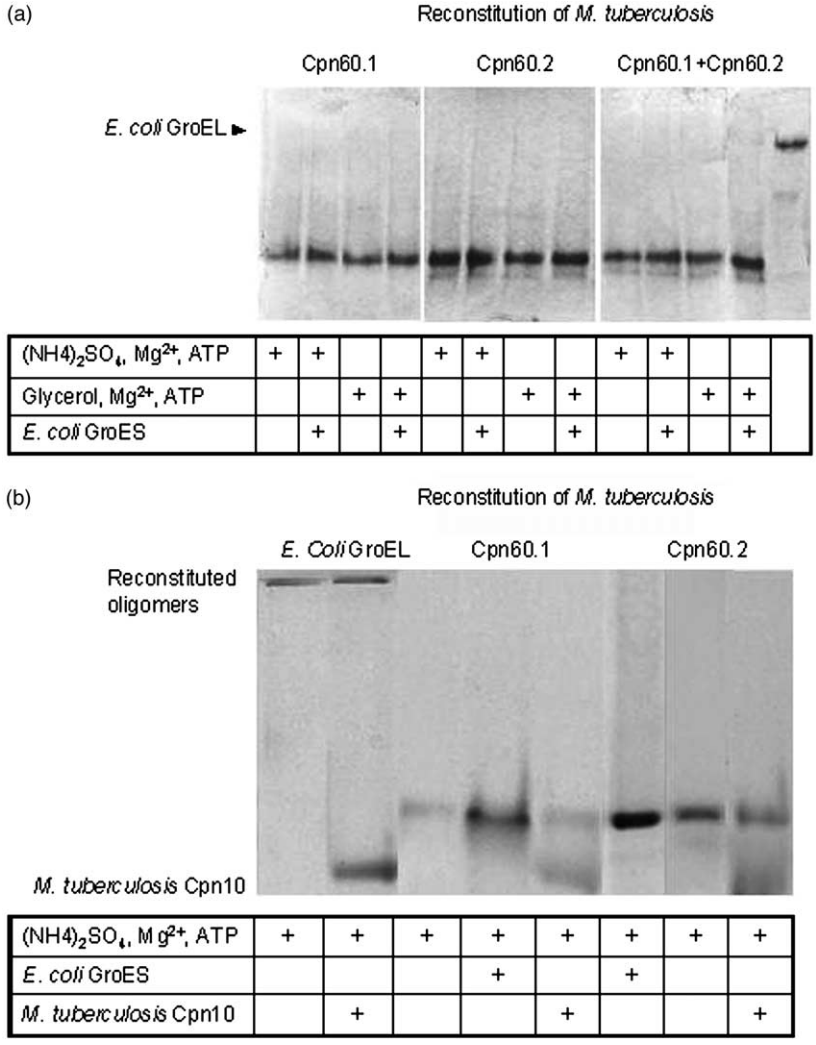


Figure 1. Gel filtration chromatograms of *M. tuberculosis* Cpn60s. Elution profile of the purified (a) Cpn60.1 and (b) Cpn60.2 on a Superdex-200 HR 10/30 column. Both Cpn60.1 and Cpn60.2 elute as 110 kDa species. (c) Elution of standard molecular mass marker proteins shown as a function of $\log(M_r)$. V_e and V_o correspond to elution and void volumes, respectively.



M. tuberculosis Cpn10			+		+	
Glycerol	+			+		
ATP-γS		+				+

Figure 2. Native gel electrophoresis of purified *M. tuberculosis* Cpn60s demonstrating their lower oligomeric state. Cpn60.1 and Cpn60.2 were purified in the presence of glycerol, ATP-γ-S and *M. tuberculosis* Cpn10. The purified Cpn60s migrate as lower oligomers, showing the proteins' inability to assemble into oligomers in the presence of these supplements.



$(NH4)_2SO_4$, Mg^{2+}, ATP	+	+			+	+			+	+			
Glycerol, Mg^{2+}, ATP			+	+			+	+			+	+	
E. coli GroES		+		+		+		+		+		+	

$(NH4)_2SO_4$, Mg^{2+}, ATP	+	+	+	+	+	+	+	+
E. coli GroES				+		+		
M. tuberculosis Cpn10		+			+			+

Figure 3. Reconstitution of *M. tuberculosis* Cpn60s demonstrating their lower oligomeric state. (a) *In vitro* reconstitution of Cpn60 oligomer was attempted in the presence of ammonium sulphate and glycerol as described in Materials and Methods. Presence of either ammonium sulphate or glycerol in the reconstitution mix does not alter the oligomeric state of Cpn60.1 (lanes 1–4) or Cpn60.2 (lanes 5–8) independent of the presence of *E. coli* GroES. Co-existence of the two Cpn60s (lanes 9–12) also does not promote the oligomeric assembly of the mycobacterial Cpn60s. Lane 13 indicates the oligomeric state of the wild-type *E. coli* GroEL. (b) *In vitro* reconstitution of Cpn60 oligomers was attempted in the presence of the cognate co-chaperonin, *M. tuberculosis* Cpn10. *E. coli* GroEL monomers subjected to reconstitution in the presence of ammonium sulphate (lane 1) and *M. tuberculosis* Cpn10 (lane 2) readily reassemble as tetradecamers. On the other hand, *M. tuberculosis* Cpn60.1 and Cpn60.2, subjected to reconstitution in the presence of ammonium sulphate (lanes 3 and 7), *E. coli* GroES (lanes 4 and 6) and *M. tuberculosis* Cpn10 (lanes 5 and 8), clearly show that the two proteins are unable to reconstitute as tetradecamers.

reconstitute the *M. tuberculosis* chaperonins in canonical oligomeric states thus suggested that their existence as lower oligomers might be an intrinsic property of the two chaperonins.

Plant chloroplast chaperonins are known to oligomerize as heterotetradecamers of two different polypeptide chains.[11] In order to test the possibility whether the two *M. tuberculosis* chaperonins were similarly interdependent on each other for reassembly into tetradecameric state, reconstitution of the two proteins was attempted in the presence of each other. Incorporation of both Cpn60.1 and Cpn60.2 in the reaction mix, however, did not alter the oligomeric state of the reconstituted proteins (Figure 3(a)). Moreover, the oligomeric state remained unaltered irrespective of the presence or absence of the nucleotides or GroES. These results thus further corroborate that the *M. tuberculosis* GroEL homologues are lower oligomeric proteins, unlike their *E. coli* counterpart.

Circular dichroism measurements of *M. tuberculosis* Cpn60s

The above data indicate that the two *M. tuberculosis* GroEL homologues possess an unusual quaternary structure. The possibility that their lower oligomeric state might have arisen due to the loss of secondary and tertiary structure was tested by measuring the CD spectrum of the proteins. The far UV-CD spectrum of *M. tuberculosis* Cpn60.1 and Cpn60.2 (Figure 4) is characteristic of highly helical proteins with signature bands for helical structure at 208 nm and 222 nm.[21] The data suggest that the *M. tuberculosis* chaperonins are in a significantly folded conformation. The unusual quaternary structure of the *M. tuberculosis* chaperonins thus does not appear to be due to the absence of secondary structure of the proteins.

Oligomeric state of *M. tuberculosis* Cpn60s *in vivo*

As mentioned above, Cpn60s from other prokaryotes exist as tetradecamers in their cellular environment and the same oligomeric state is also observed *in vitro*. Our *in vitro* purification and reconstitution results show that the *M. tuberculosis* Cpn60s exist as lower oligomers, a state surprisingly different from that observed for other Cpn60s. To discount the possibility that the unusual behaviour of chaperonins as lower oligomers was a result of their heterologous expression, we assessed the oligomeric state of these proteins in their homologous cellular environment. This was tested by resolving the cellular fraction of *M. tuberculosis* proteins on Native-PAGE followed by Western blot analysis (Figure 5). Upon immunoblotting, both Cpn60.1 and Cpn60.2 appeared as lower oligomers, and not tetradecamers, reconfirming our *in vitro* data (Figure 5). In conclusion therefore, the absence of the oligomeric state of the *M. tuberculosis* Cpn60s, *in vivo* and *in vitro*, indicates the natural tendency of these proteins to exist as lower oligomers.

Importance of Ala2 and Glu76 in oligomerization

Multiple sequence analysis of 43 Cpn60 protein sequences suggested that residues at positions 2 and 76, which occur at the inter-monomer interface

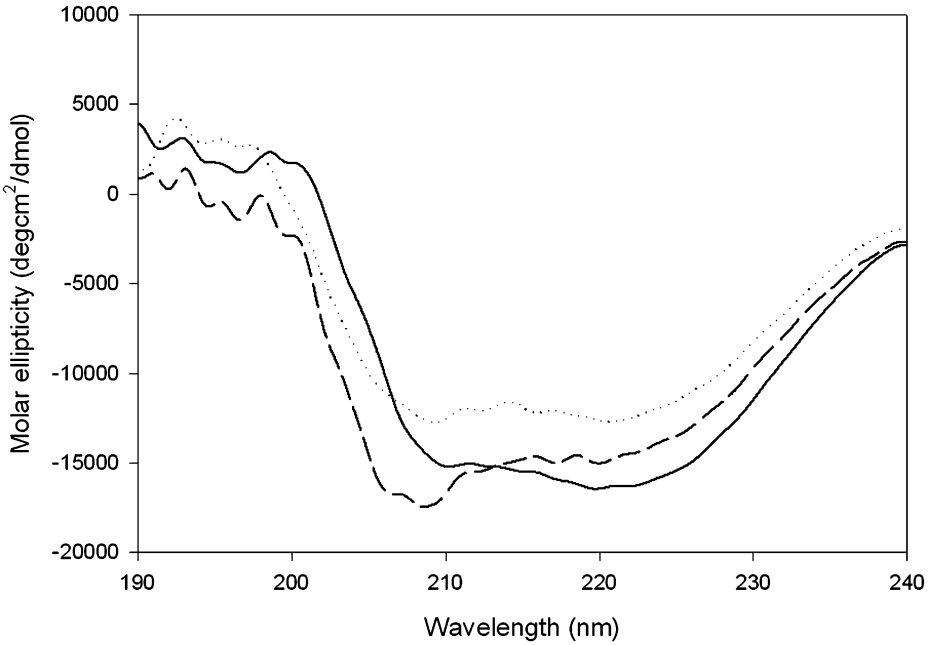


Figure 4. Far UV-CD spectrum of *E. coli* GroEL, *M. tuberculosis* Cpn60.1 and Cpn60.2. Mean residue ellipticity *versus* wavelength was measured at 25 °C in 10 mM Tris–Cl (pH 8.0). Signature bands for helical structure are seen at 208 nm and 222 nm, suggesting a well-formed secondary structure for the Cpn60s. The continuous line represents the CD spectrum of *E. coli* GroEL, the dotted line represents that of Cpn60.1 and the broken line that of Cpn60.2.

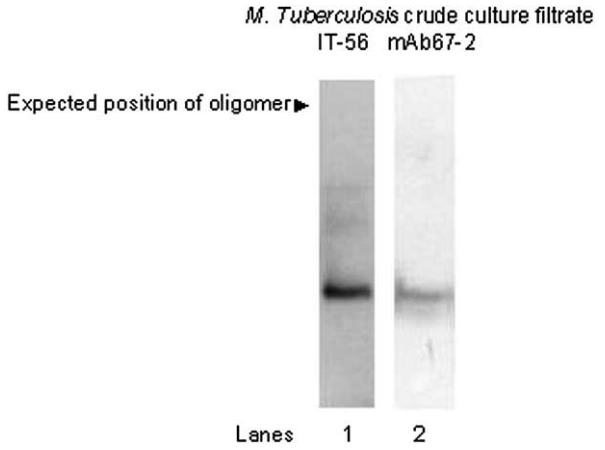


Figure 5. Western blot analysis of *M. tuberculosis* culture proteins demonstrating lower oligomeric state of Cpn60s. Culture extracts of *M. tuberculosis* were subjected to 6% Native-PAGE and probed with monoclonal antibodies, IT-56 and mAb67-2, against Cpn60.1 (lane 1) and Cpn60.2 (lane 2), respectively. Western blot analysis indicates that Cpn60s of *M. tuberculosis* exist as lower oligomers *in vivo*. Expected position of the oligomeric species is indicated.

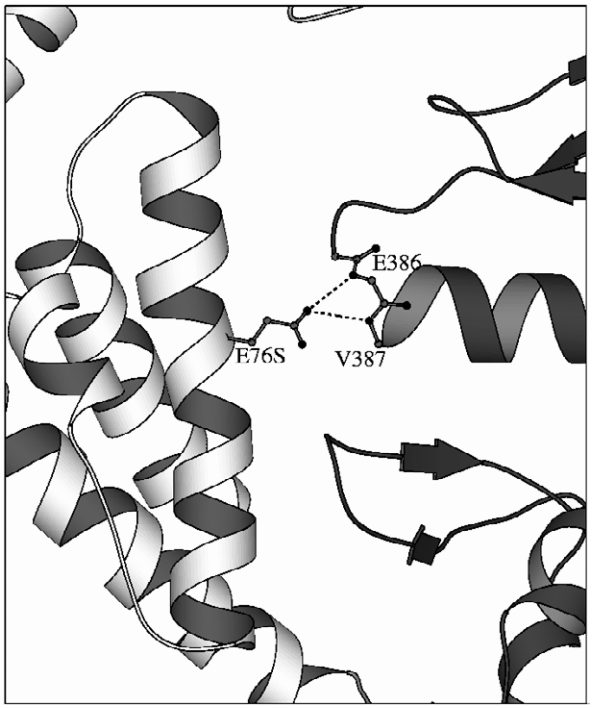


Figure 6. Crucial side-chain–main-chain interaction at the *E. coli* GroEL interface involving Glu76. Interaction between the side-chain carboxyl of E76 (chain A) and main-chain amide of E386 (chain B) in *E. coli* GroEL has been suggested to be important for the oligomerization of the protein. A natural occurrence of serine at position 76 in *M. tuberculosis* Cpn60.1 may disrupt this interaction and is hypothesized to contribute to the loss of its oligomeric structure. The two polypeptide chains have been coloured in different shades of grey. Other crucial interface residues important for maintenance of the oligomeric state are indicated in the Supplementary data. This Figure was generated using MOLSCRIPT.[47]

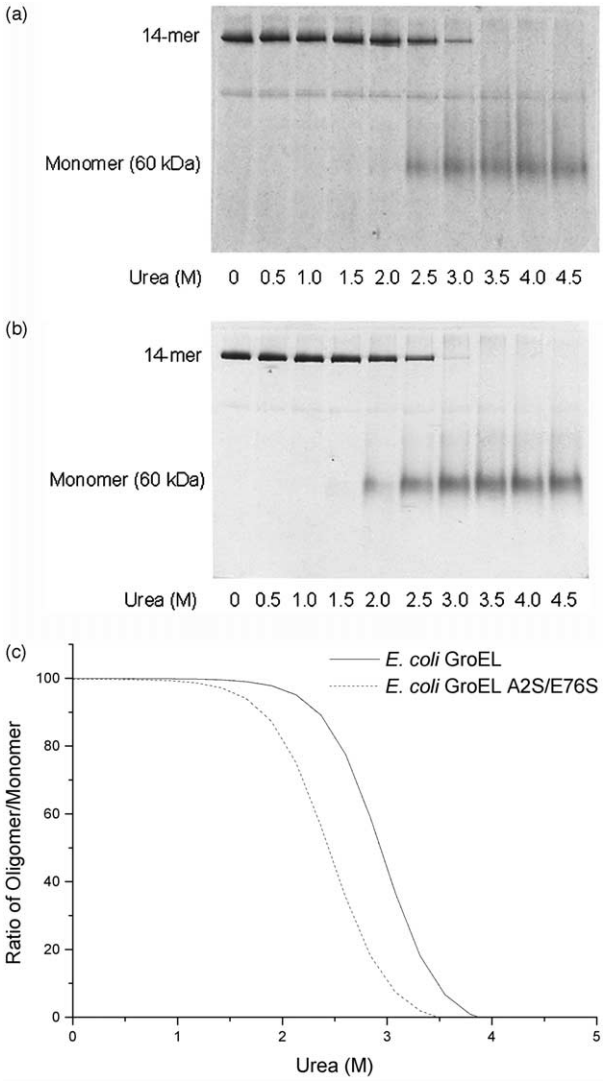


Figure 7. Effect of urea concentration on the dissociation of *E. coli* GroEL mutant. Urea-induced dissociation of the (a) wild-type and (b) A2S/E76S GroEL mutant was analysed on a 6% Native-PAGE. (c) Ratio of the oligomeric to monomeric forms of GroEL plotted against urea concentration indicates that the mutant dissociates into monomers at 2.5 M urea concentration, in comparison to 3 M for the wild-type.

in the tetradecameric GroEL structure, are strictly conserved as alanine and glutamate (Figure 6 and Supplementary data). These positions are, however, occupied by serine in *M. tuberculosis* Cpn60.1. To assess the importance of these residues in maintenance of the oligomeric structure, we generated an A2S/E76S mutant of *E. coli* GroEL.

The monomer–tetradecamer equilibrium of the wild-type as well as that of the A2S/E76S mutant was analysed as a function of urea concentration on Native-PAGE. In accordance with an earlier report, the wild-type protein dissociates into monomers at a urea concentration greater than 3 M (Figure 7).[22] The mutant, however, disassociated into monomers at a much lower concentration of urea than the

wild-type GroEL (Figure 7). The results thus indicate that the thermodynamic stability of the GroEL oligomeric assembly is significantly reduced as a consequence of the mutations A2S and E76S. A natural occurrence of serine at residue positions 2 and 76 in Cpn60.1 might thus be responsible for the lower oligomeric state of this protein.

ATPase activity of the mycobacterial Cpn60s

E. coli GroEL possesses a weak K^+ stimulated ATPase activity, an essential component of the chaperonin-mediated protein folding reaction.[23] Due to their unusual quaternary structure, measuring the ATPase activity of the recombinant *M. tuberculosis* Cpn60 proteins was an important test for their functional characterization. Figure 8 compares the ATPase activity of *M. tuberculosis* Cpn60.1 and Cpn60.2 with that of *E. coli* GroEL. *E. coli* GroEL catalysed the hydrolysis of ATP with a k_{cat} of 2.6 min^{-1} at 37 °C. Cpn60.1 and Cpn60.2 exhibited a k_{cat} of 0.16 min^{-1} and 0.28 min^{-1} respectively. The data thus suggest a very weak ATPase activity in the GroEL homologues of *M. tuberculosis*.

Bis-ANS fluorescence assay for Cpn60s

Loss of oligomerization would have led to the exposure of large hydrophobic regions on the surface of *M. tuberculosis* Cpn60s that were otherwise buried. Also the apical domain in a GroEL oligomer is known to possess an exposed hydrophobic area where substrate polypeptides are known to bind in the central cavity.[3] Presence of such hydrophobic patches on the surface of *M. tuberculosis* Cpn60s was probed by binding to 1,1-bis (4-anilino) naphthalene-5,5′-disulfonic acid (bis-ANS).

As shown in Figure 9, fluorescence intensity of bis-ANS was significantly enhanced in the presence of Cpn60s, suggesting binding of bis-ANS to the hydrophobic surfaces of these proteins. The fluorescence enhancement in Cpn60.1 was comparable to that observed for *E. coli* GroEL. In comparison, the increase observed for Cpn60.2 was much lower, suggesting smaller percentage of hydrophobic surface exposed in Cpn60.2. The results clearly indicate the presence of hydrophobic surfaces on the Cpn60s, a larger surface in Cpn60.1 than in Cpn60.2.

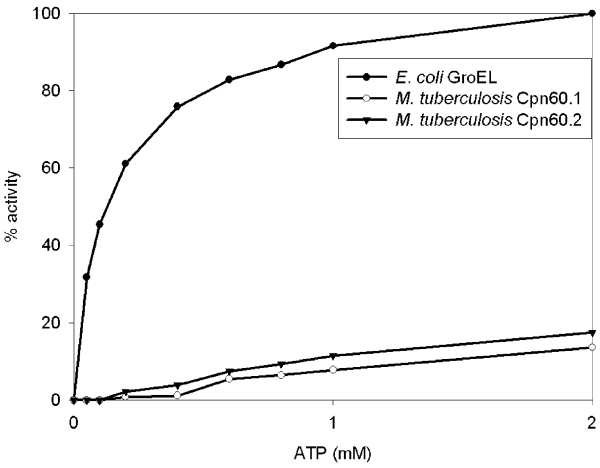


Figure 8. ATPase activity of *M. tuberculosis* Cpn60s and *E. coli* GroEL. ATP hydrolysis by *E. coli* GroEL, *M. tuberculosis* Cpn60.1 and Cpn60.2 was measured as a function of ATP concentration at 37 °C in the presence of 10 mM $MgCl_2$ and 100 mM KCl for 15 minutes. Cpn60.1 and Cpn60.2 clearly show very little ATPase activity compared to GroEL.

Effect of *M. tuberculosis* Cpn60s on citrate synthase aggregation

E. coli GroEL is known to facilitate refolding of denatured citrate synthase by suppressing its aggregation.[24] Exposed hydrophobic patches on *M. tuberculosis* Cpn60s suggested that these might exert similar effect on substrate proteins. Figure 10(a) compares the ability of Cpn60.1 and Cpn60.2 to prevent the aggregation of citrate synthase at 43 °C measured by light scattering. Cpn60.1 was more potent in suppressing aggregation when used in equimolar ratio (Figure 10(a)). In molar excess, however, both the chaperonins were observed to completely prevent aggregation of citrate synthase.

Effect of *M. tuberculosis* Cpn60s on refolding of rhodanese

In order to assess the ability of the *M. tuberculosis* Cpn60s to facilitate refolding of substrate proteins we tested *in vitro* refolding of rhodanese. Cpn60.2 was seen to be able to promote the refolding of guanidine-HCl-denatured rhodanese (Figure 10(b)). The percentage recovery was however less than that obtained by spontaneously refolded rhodanese. Intriguingly, this ability of Cpn60.2 was independent of the presence or absence of the co-chaperonin or ATP. Cpn60.1 did not seem to promote the refolding of rhodanese. This might have been due to the tight binding of Cpn60.1 to the substrate preventing its release. It is known that a GroEL bound polypeptide is initiated to refold by hydrolysis of ATP. In the absence of ATP, however, the polypeptide remains tightly bound to GroEL. Cpn60.1 might similarly bind denatured rhodanese, thus preventing its successful refolding.

Effect of *M. tuberculosis* Cpn60s on citrate synthase refolding

The ability of *M. tuberculosis* chaperonins to refold a wider range of substrate proteins was tested by *in vitro* refolding of citrate synthase. Cpn60.1 was capable of promoting refolding of citrate synthase, to an extent comparable to that obtained by Cpn60.2 (Figure 10(c)). Moreover, the presence or absence of co-chaperonin or ATP did not affect the extent of recovery of citrate synthase activity. Our results therefore suggest that the *M. tuberculosis* Cpn60s are efficient in preventing

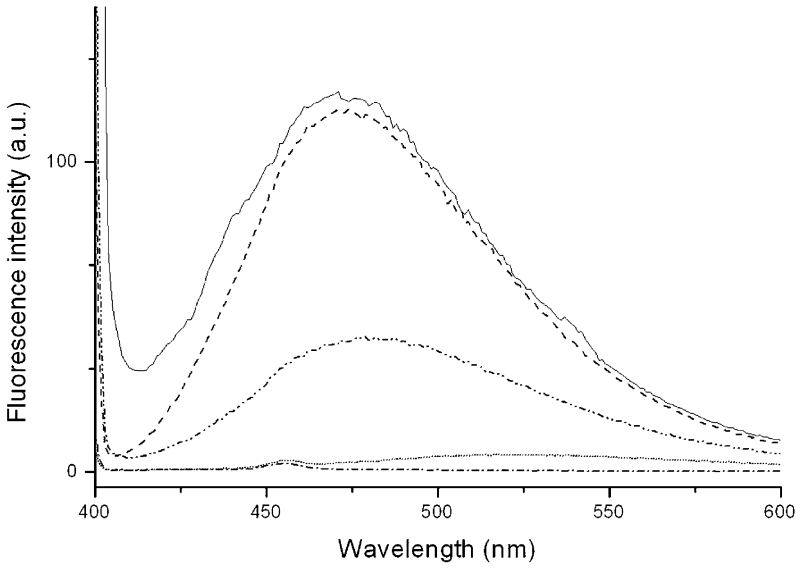


Figure 9. Fluorescence intensity enhancement of bis-ANS upon binding to *M. tuberculosis* Cpn60s The fluorescence intensity of bis-ANS is observed to increase significantly in the presence of both Cpn60.1 (broken line) and Cpn60.2 (dashed-double-dotted line), suggesting the presence of hydrophobic surfaces on the proteins. The continuous line represents the enhancement seen for *E. coli* GroEL in presence of bis-ANS. Fluorescence intensity for the buffer (dotted line) and bis-ANS (dashed-dotted line) were measured as controls.

aggregation of denatured proteins, but do not possess ATP-dependent chaperoning activity.

Discussion

GroEL is the major heat shock protein present in all forms of life guiding several essential steps during synthesis, folding, transport and degradation of proteins. In *E. coli*, *groEL* is known to be an essential gene for growth at all temperatures.[10] Upon thermal stress, nearly 15% of the normal protein mass of the cell consists of the GroEL and GroES proteins.[25] Over 150 homologues of Cpn60 sequences are currently available, with a pair-wise similarity extending from 40% to 100% at the amino acid level.[26] High conservation of Cpn60s across species suggests that they play an important role in the physiology of all species.

Heat shock proteins, including Cpn60s, are not only induced under thermal stress, but also show elevated expression levels under a variety of other unnatural conditions. Not surprisingly therefore, heat shock proteins are induced within pathogenic organisms upon invasion of host cells, presumably contributing to their survival within the hosts.[27] Study of regulation of heat-shock proteins in *M. tuberculosis* has shown enhanced expression of both the Cpn60s upon thermal shock to the bacteria,[28] as well as upon phagocytosis by macrophages.[27] It is therefore reasonable to believe that Cpn60s of *M. tuberculosis* contribute to its defensive response against external stress conditions.

Here we present interesting biochemical characteristics of the two Cpn60s of *M. tuberculosis*. A major surprising outcome of the present study has been that the two Cpn60s are distinct from the canonical GroEL homologues. The most distinctive feature of *M. tuberculosis* Cpn60s is their oligomeric nature, where unlike the known Cpn60s, they do not form a 14-meric assembly. The proteins rather exist as lower oligomers irrespective of the presence or absence of nucleotides. Several attempts to reconstitute the *M. tuberculosis* Cpn60.1 or Cpn60.2 under a variety of conditions did not yield higher oligomers. The lower oligomeric state of *M. tuberculosis* Cpn60s also appears to be their natural state of existence as shown by our Western blot analysis on Native-PAGE. Current understanding of the chaperonin function assumes a strict oligomeric assembly of GroEL, a tetradecameric state, which is the functional unit for protein refolding.[29,30] Thus, the functional relevance of the lower oligomeric state of mycobacterial chaperonins is still not clear.

Interestingly, many of the highly conserved residues, listed with "conservation index" better than 0.4 of Cpn60s are observed to be different in *M. tuberculosis* chaperonins.[26] For example, a crucial glutamate at position 76, involved in an important side-chain–main-chain interaction across two monomers, is conserved in all Cpn60 sequences (Figure 6, see Supplementary data for the multiple sequence alignment). The conserved glutamate is, however, replaced by a serine in *M. tuberculosis* Cpn60.1. Several other alterations have been observed to occur at the interface of Cpn60.1 (Supplementary data). Similarly, the presence of an alanine at position 2 has earlier been reported to be crucial for maintenance of the oligomeric state of GroEL. Mutation of alanine to serine at this position was shown to weaken the intersubunit interactions in GroEL, destabilizing its oligomeric structure.[31] Interestingly, this position is occupied by a serine in *M. tuberculosis* Cpn60.1. Thus, we hypothesize that crucial changes in the interface residues might have resulted in the loss of oligomerization of the *M. tuberculosis* Cpn60.1. In an attempt to verify our hypothesis, site-directed mutagenesis of these residues was performed in *E. coli* GroEL. Urea-induced unfolding transition show that the mutation A2S/E76S indeed destabilizes the

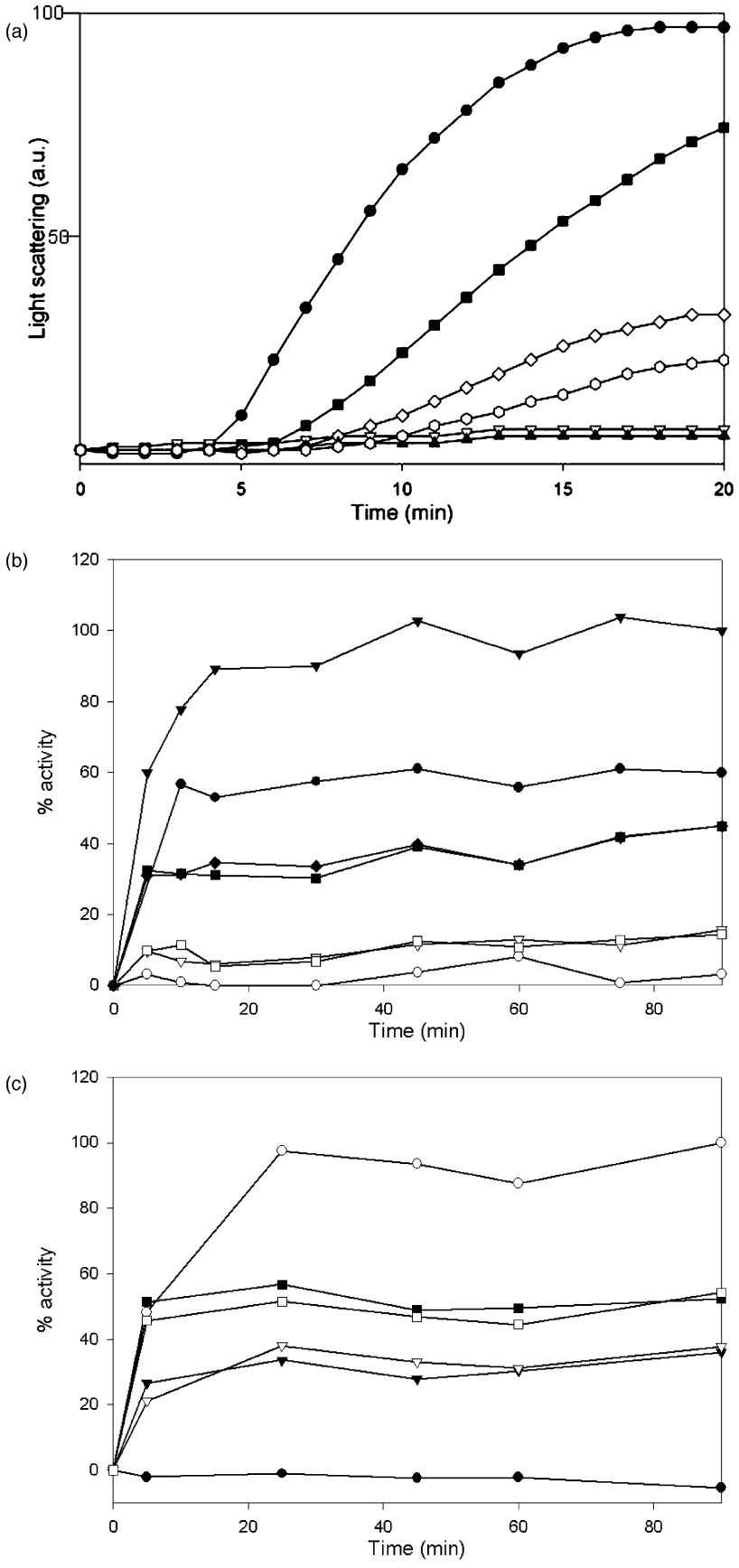


Figure 10. Assessment of chaperoning ability of Cpn60.1 and Cpn60.2. (a) Aggregation of citrate synthase as a function of time: aggregation of citrate synthase at 43 °C in the absence (●) and the presence of equimolar ratios of *M. tuberculosis* Cpn60.1 (▽); and Cpn60.2 (◇), measured as a function of light scattered at 465 nm. Increase in molar excess ratio of Cpn60.2 to twofold (○) or 14-fold (▲) resulted in suppression of aggregation of citrate synthase to a better extent. Both the Cpn60s are clearly able to prevent light scattering by citrate synthase when present in molar excess. Citrate synthase in the presence of BSA (■) was taken as a control. (b) Effect of *M. tuberculosis* Cpn60s on refolding of chemically denatured rhodanese. Refolding of denatured rhodanese was monitored spontaneously (●) or in the presence of *E. coli* GroEL, GroES and ATP (▼); GroEL, in the absence of GroES or ATP (○); *M. tuberculosis* Cpn60.1 in the presence (▽) or absence (□) of Cpn10 and ATP and *M. tuberculosis* Cpn60.2 in the presence (■) or absence (♦) of Cpn10 and ATP. Activity obtained when rhodanese was refolded in the presence of *E. coli* GroEL for 90 minutes was considered to be 100%. (c) Effect of *M. tuberculosis* Cpn60s on refolding of chemically denatured citrate synthase. Refolding of denatured citrate synthase was monitored in the presence of *E. coli* GroEL, GroES and ATP (○); GroEL, in the absence of GroES or ATP (●); *M. tuberculosis* Cpn60.1 in the presence (▽) or absence (▼) of Cpn10 and ATP and *M. tuberculosis* Cpn60.2 in the presence (□) or absence (■) of Cpn10 and ATP. Activity obtained when denatured citrate synthase was refolded in the presence of *E. coli* GroEL for 90 minutes was considered as 100%.

oligomeric structure of GroEL. Similarly, other identified positions in Cpn60.1, which largely occur at the oligomeric interface in GroEL, might further contribute to the loss of 14-meric state of Cpn60.1.

Another interesting observation in the present study is the loss of ATPase activity of *M. tuberculosis* Cpn60s. The protein folding cycle of GroEL has been reported to be largely dependent on the ATPase activity of the protein. We present evidence that the *M. tuberculosis* chaperonins have lost their ATPase activity. Interestingly however, despite the loss of canonical oligomeric state and ATPase activity, the *M. tuberculosis* Cpn60s retain their ability to suppress aggregation of substrate proteins. However, the extent to which the two Cpn60s suppress aggregation is different, possibly due to variation in the exposed hydrophobic surfaces of the two proteins. Our results indicate that Cpn60.1 is more potent in preventing aggregation than Cpn60.2. For substrate proteins such as rhodanese, Cpn60.2 is able to bind these proteins reversibly, giving them a chance to fold to their native conformation. Cpn60.1, on the other hand, remains tightly bound to the substrate protein. The substrate protein is thus prevented from aggregating but does not get refolded. Substrates such as citrate synthase on the other hand, by reversible binding to Cpn60s, are refolded more efficiently.

The ability of *M. tuberculosis* Cpn60s to promote refolding in the absence of ATP is similar to that observed for other known chaperones. For example, functionally active monomeric minichaperones containing part of the polypeptide binding domain of GroEL are known to be effective *in vitro* and *in vivo*.[32,33] Also monomeric Cpn60 from *Thermus thermophilus* has been shown to possess the ability to suppress aggregation and promote protein folding with no requirement for ATP or GroES.[34] Thus, *M. tuberculosis* Cpn60s in a similar fashion might have evolved to function in lower oligomeric state with no ATP requirement.

A recent study surprisingly shows the presence of the mycobacterial Cpn60s in the plasma membrane fraction although the major functional role of these proteins lies in the cytosol.[35] Moreover, various other reports suggest alternative roles for the GroEL homologues. For example, a GroEL homologue in Buchnera, an intracellular symbiotic bacterium of aphids, is not only a molecular chaperone but also a phosphocarrier protein, suggesting that the protein plays a role in a signal transducing system.[36] The *M. leprae* Hsp65 (Cpn60.2) has been shown to display proteolytic activity that is catalytically related to the HslVU protease.[37] By similarity, the *M. tuberculosis* Cpn60.2 too may have gained such a role.

Judicial utilization of energy sources in a cell is important to the organism for its survival. The protein folding cycle by the Cpn60s in all life forms is an ATP-dependent process utilizing seven ATP molecules in each reaction cycle.[38] Ability of an organism to promote protein folding without energy utilization would prove to be economical. We hypothesize that high demands of energy resulting from its extremely slow rate of metabolism might have led *M. tuberculosis* to devise an altered route to protein folding, avoiding the usual energy-dependent pathway. Cpn60s through such a route thus continue to fold substrate proteins within the cytosol, sparing ATP for other crucial processes of the cell. We thus suggest that loss of an oligomeric state of Cpn60s, yet retaining their chaperoning role, might have been designed in evolution to save the energy sources of the bacterium. It is pertinent to note in this context that other ATP-dependent enzymes, such as RecA, also have reduced ATPase activity in *M. tuberculosis*.[39] Further analysis of the role of GroEL homologues would lead us to a better understanding of the importance of the presence of two Cpn60s in *M. tuberculosis*.

Materials and Methods

Cloning, expression and purification of *M. tuberculosis* Cpn60s

The genes coding for the *M. tuberculosis* GroEL homologues, *cpn60.1* (Rv3417c) and *cpn60.2* (Rv0440) were PCR amplified from the *M. tuberculosis* H37Rv cosmid library kindly provided by Stewart Cole.[40] The two *cpn60* fragments were cloned into *E. coli* expression vectors, pET3a and pET28a (Novagen), respectively, through an intermediate sub-cloning step in pBluescript (SK+) (Stratagene). Primers used for amplification carried $(His)_6$-tag, which was thus incorporated in the proteins at the N-terminal and the C-terminal of the *cpn60.1* and *cpn60.2* gene products, respectively. *E. coli* BL21 (DE3) over-expressing the two genes were lysed by sonication. The resuspension buffer was supplemented with 10 mM sarkosyl during lysis of the Cpn60.1 expressing culture. The proteins were purified by affinity chromatography over Ni-NTA agarose column. Purification of Cpn60s was also carried out in the presence of 10% (v/v) glycerol, 1 mM ATP-γ-S or 10 μM *M. tuberculosis* Cpn10. These supplements were present in the buffers throughout the purification procedure. The *M. tuberculosis* proteins were also cloned in the expression vector without the $(His)_6$-tag. The *E. coli* GroEL and GroES were overexpressed in *E. coli* harbouring the plasmid pKY206 (kindly provided by Dr K. Ito, Kyoto University) and purified using minor modifications of the published procedure.[41] Purification of *M. tuberculosis* Cpn10 was performed as described.[17]

Size exclusion chromatography

Size exclusion chromatography was performed at room temperature using the FPLC system (Pharmacia Amersham) equipped with Superdex-200 HR 10/30. The column was equilibrated with at least three bed volumes of 50 mM Tris–Cl (pH 8.0), 150 mM NaCl prior to each run. A typical flow rate of 0.35 ml/minute was maintained. Absorbance at 280 nm was measured to monitor elution of proteins from the column.

Reconstitution of Cpn60 oligomers

Reconstitution was performed essentially as described earlier with slight modifications.[19] Briefly, 10 μM Cpn60 (protomers) was incubated with 4 M urea on ice for 90 minutes. Reconstitution was initiated by a rapid tenfold dilution of the monomeric Cpn60 in the buffer containing 50 mM Tris–Cl (pH 8.0), 10 mM $MgCl_2$, 5 mM ATP, 1 M ammonium sulphate or 10% glycerol and *E. coli* or *M. tuberculosis* Cpn10 in a 1 : 1 molar ratio. The reconstitution mix was incubated at 25 °C for two hours and then analysed on a 6% (w/v) Native-PAGE.

Circular dichroism measurement

Circular dichroism (CD) spectra of *E. coli* GroEL and *M. tuberculosis* Cpn60s were recorded using a Jasco J-715 spectropolarimeter at room temperature. The proteins in 10 mM Tris–Cl buffer (pH 8.0) were used at a concentration of 1 mg/ml. Far UV-CD spectrum was recorded using a 0.01 cm path length cuvette.

Protein analysis by Native-PAGE and immunoblotting

Native proteins of *M. tuberculosis* were resolved on a 6% Native-PAGE. The proteins were then transferred onto HybondC (Amersham) nitrocellulose sheet by the method described by Towbin *et al*.[42] Monoclonal antibodies, IT-56 (anti-Cpn60.1) and mAb67-2 (anti-Cpn60.2), kindly provided by John Belisle (Colorado State University) and A. H. Kolk, respectively, were used for detection of proteins. HRP-labelled goat anti-rabbit IgG (anti-anti-Cpn60.1) and goat anti-mouse IgG (anti-anti-Cpn60.2) were used at dilutions of 1 : 2000 and 1 : 10,000, respectively. The proteins were visualized using standard protocols.

Site-directed mutagenesis of *E. coli groEL*

Point mutants of the *E. coli groEL* were generated using the Quik-Change site-directed mutagenesis kit (Stratagene). The point mutant A2S was generated and used as a template for generation of the double mutant A2S/E76S. The forward and reverse primers for mutagenesis of A2S were 5′-TAAAGATAATGGCATCTAAAGACG TAAAATTC-3′ and 5′-GAATTTTACGTCTTTAGATGC CATTATCTTTA-3′. For the E76S mutation, the forward and reverse primers were 5′-GCAGATGGTGAAA TCAGTTGCCTCTAAA-3′ and 5′-TTTAGAGGCAAC TGATTTCACCATCTGC-3′, respectively. The underlined nucleotides indicate codons for the mutated residues. The mutant was purified using similar protocol as that for the wild-type GroEL.

Analysis of urea-promoted dissociation of the *E. coli* GroEL mutant

Urea-induced dissociation of *E. coli* GroEL and its mutant was performed as reported.[31] Briefly, 10 μl samples containing 10.6 μg of the wild-type or mutant GroEL in 100 mM Tris–Cl (pH 8.0) supplemented with 10 mM $MgCl_2$, 100 mM KCl and 4.5 mM dithiothreitol were pre-incubated for five minutes at 25 °C. This protein was then mixed with an equal volume of 0 to 9 M urea. The samples were incubated for 40 minutes at room temperature and then analysed on a 6% Native-PAGE.

ATP hydrolysis by Cpn60

The Cpn60 ATPase activity was quantified by a colorimetric assay performed in microtitre plates as described.[43] Briefly, the reaction buffer containing 100 mM Tris–Cl (pH 8.0), 10 mM KCl, 10 mM $MgCl_2$ and 2.5 μM GroEL was incubated with varying concentrations of ATP at 37 °C for 15 minutes. The enzymatic reaction was terminated by addition of 200 μl of the acidic solution of malachite green, ammonium molybdate and polyvinylalcohol. The activity was measured as the amount of inorganic phosphate (Pi) liberated at 655 nm in an ELISA plate reader. The values obtained were corrected by subtracting the blank readings. A standard curve with monobasic potassium phosphate was run concurrently with each experiment and thus nanomoles of Pi released were calculated.

Bis-ANS fluorescence assay

Binding of bis-ANS to *M. tuberculosis* Cpn60s was monitored by exciting the probe at 395 nm and recording the emission spectra in the range of 400–600 nm. The protein and bis-ANS were used at a concentration of 20 μM. Fluorescence intensity measurements were carried out at room temperature on a Varian Eclipse spectrofluorimeter. The fluorescence of buffer (100 mM Tris–Cl, pH 8.0) and bis-ANS alone were measured as controls.

Aggregation of citrate synthase

Citrate synthase (0.015 mg/ml) was incubated at 43 °C in 50 mM Hepes–KOH buffer (pH 7.5), in the presence or absence of 14-molar excess ratios of *M. tuberculosis* Cpn60.1 or Cpn60.2. Aggregation was monitored for 20 minutes on a Hitachi F-4000 spectrofluorimeter with emission and excitation wavelengths set at 465 nm and corresponding band passes set at 3.0 nm. Temperature of the sample was maintained using a Julabo circulating water-bath. Internal temperature of the cuvette was monitored using a Physitemp type T microcouple.

Chemical denaturation and refolding of citrate synthase

Denaturation and refolding of citrate synthase was carried out as described.[44] Briefly, 15 μM citrate synthase from pig heart was denatured by 6 M guanidine-HCl in 100 mM Tris–Cl buffer (pH 8.0) containing 20 mM DTT. The enzyme was incubated in the denaturant for two hours at room temperature. Renaturation was carried by a 100-fold dilution into 100 mM Tris–Cl buffer (pH 8.0), 10 mM KCl, 10 mM $MgCl_2$ containing the Cpn60, Cpn10 and ATP to final concentrations of 1 μM, 2 μM and 2 mM, respectively. Aliquots were withdrawn at different time points and tested for the recovery of activity at room temperature.

Citrate synthase activity was measured as described by Srere.[45] The reaction was monitored as a decrease in absorbance at 233 nm due to cleavage of Acetyl-CoA and utilization of oxaloacetate. The reaction mix contained 100 mM Tris–Cl (pH 8.0), 0.15 mM Acetyl CoA and 0.1 mM oxaloacetate. The reaction was initiated by addition of citrate synthase to a final concentration of 3 nM. The activity assay was performed at 25 °C and the absorbance recorded using a Unicam-UV spectrophotometer.

Chemical denaturation and refolding of rhodanese

Denaturation of rhodanese to a final concentration of 9 μM was carried out in 100 mM Tris–Cl (pH 8.0) containing 6 M guanidine-HCl and 1 mM dithiothreitol (DTT) for two hours at room temperature. Refolding was initiated at 37 °C by a rapid dilution in 50 mM Tris–Cl (pH 8.0) supplemented with 20 mM $MgCl_2$, 10 mM KCl, 50 mM $Na_2S_2O_3$ and 5 mM DTT in the presence or absence of the different Cpn60s. The final concentration of rhodanese in the refolding mix was 108 nM. The protomer concentrations of Cpn60s and Cpn10 were maintained at 2.5 μM and 2.4 μM, respectively. 2 mM ATP was added to the refolding solution, containing the chaperonins, just before initiation of protein refolding. An aliquot from the refolding mix was withdrawn at different time points and assayed for the recovery of activity as described.[46] The activity was spectrophotometrically measured as the formation of ferrithiocyanate complex at 460 nm.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2004.07.066

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