Tuberculosis (2005) **85**, 385–394

ELSEVIER

Tuberculosis

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The unusual chaperonins of *Mycobacterium tuberculosis*

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KEYWORDS
M. tuberculosis;
Chaperonins;
Oligomer;
Protein folding

Summary Heat shock proteins (Hsps), also known as molecular chaperones, are a diverse set of proteins that mediate the correct folding, assembly, transport and degradation of other proteins. In addition, Hsps have been shown to play a variety of important roles in immunity, thereby representing prominent antigens in the humoral and cellular immune response. Chaperonins form a sub-group of molecular chaperones that are found in all domains of life. Chaperonins in all bacteria are encoded by the essential *groEL* and *groES* genes, also called *cpn60* and *cpn10* arranged on the bicistronic *groESL* operon. Interestingly, *Mycobacterium tuberculosis* contains two copies of the *cpn60* genes. The existence of a duplicate set of *cpn60* genes in *M. tuberculosis*, however, has been perplexing. Cpn10 and Cpn60s of *M. tuberculosis* have been shown to be highly antigenic in nature, eliciting strong B- and T-cell immune responses. Recent work has shown intriguing structural, biochemical and signaling properties of the *M. tuberculosis* chaperonins. This review details the recent developments in the study of the *M. tuberculosis* chaperonins.

Introduction

Heat shock proteins (Hsps) are among the most highly conserved protein families in nature. Although originally identified by their enhanced expression under thermal stress and consequently referred to as Hsps, increased synthesis of Hsps occurs under a variety of conditions of stress such as hypoxia, nutrient deprivation, oxygen radicals,

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doi:10.1016/j.tube.2005.08.014

metabolic disruption, viral infection, phagocytosis and transformation.[1–4] Most of these proteins are expressed at significant levels in all eukaryotic and prokaryotic cells under normal conditions and are often essential for cellular growth at physiologically relevant temperatures.

Up to 5% of the total intracellular protein mass is normally constituted by Hsps. However, under conditions of stress, Hsps can constitute as much as 15% of the prokaryotic cellular protein mass. Increase in the cellular content of these proteins enables cells to protect themselves from the various lethal assaults. The protection that Hsps offer to cells is primarily through their properties as molecular chaperones. Hsps perform important functions in the folding of cellular proteins, their translocation across different compartments within a cell and also in the assembly of protein complexes. Under conditions of stress, Hsps intercept the uncontrolled protein unfolding in cells thereby playing an important role in cell protection following various stress stimuli.

One particular form of stress occurs upon invasion of a host by a pathogen. The alterations in the cellular environment of the pathogen result in an increased production of Hsps. Pathogen-derived Hsps form a major group of immunodominant antigens inducing strong humoral and cellular immune responses in the infected host.[5]

Hsps thus play a dual role in cells, primarily as molecular chaperones and also as immunodominant antigens upon infection in the host.

Significance of heat shock proteins in immunity

Various families of Hsps have been shown to elicit strong immune responses in the host. Among these the Hsp90, Hsp70, Hsp60 and Hsp10 classes have been shown to play key roles in eliciting immune responses. The chaperonins, Hsp60 and Hsp10, are among the most potent stimulators of the immune system. This review stresses upon the importance of the chaperonins, Hsp60 and Hsp10, their role as molecular chaperones and as host immune stimulators, with recent developments in our understanding of the structural aspects and immunopathology of these proteins from *Mycobacterium tuberculosis*.

Role as antigens

Encounter with the host upon infection presents a form of stress to the pathogen resulting in an enhanced expression of Hsps. The host, on the other hand, reacts to the invasion by activating its own protein degrading machinery. The pathogen-derived proteins are degraded into peptides within the host antigen-presenting cells (APCs) and are presented to the immune system. Efficient presentation of the pathogen-derived determinants by host APCs therefore promotes recognition of infected cells by the immune system.[5,6] Since Hsps constitute the bulk of the proteins present in the pathogen upon infection, the Hsp-derived peptides over-represent the repertoire of peptides presented to the host immune system. The foreign Hsps thus serve as important antigens of the pathogen eliciting strong immune responses from the host. The cell–cell signaling activity of Hsps, which has recently been recognized,[7] is also likely to contribute to this immunogenicity and will be discussed later in this article.

In addition to their abundance in the infected host, high sequence homology that the Hsps share across different species also contributes to their strong cross-reactivity. High conservation results in the presence of cross-reactive epitopes on different Hsps. The immune system once primed for Hsps of a particular pathogen, due to generation of immunological memory, therefore also cross-reacts with Hsps of other organisms, including those of the host.[8] The immune system of an infected individual is therefore geared to react quickly to any subsequent infections. A recent study also suggests that an immune network among Hsp60, Hsp70 and Hsp90, is responsible for the T-cell immunity in the arthritogenic response in adjuvant induced arthritis.[9] Thus, due to their wide distribution in nature, and a high homology among different species, Hsps represent important immunogenic components of different pathogens.

Role as antigen presenters

In addition to their role as antigens, Hsps mediate other important roles that engage the immune system. Many Hsps have been shown to be associated in vivo with a large repertoire of cellular peptides generated by degradation of proteins within a cell. The Hsp–peptide complexes are taken up by the APCs through the cell surface receptors. The peptides thus get presented by the Major-Histocompatibility Complex (MHC) class I and class II molecules, which in turn stimulate the $CD8^+$ and $CD4^+$ T cells.[10] Moreover, recent studies in murine tumor models showed that transfection with mycobacterial 60kDa heat shock protein

(Hsp60) reduces the tumorigenicity of a murine macrophage tumor cell line. Hsp60 has thus been suggested to play a role in delivery of immunodominant tumor antigens to the cell surface.[11] These observations together have led to the postulation of an interesting role of Hsps as antigen presenters and carriers.

Mycobacterial chaperonins as antigens

Reactivity to Hsps has been shown to predominate in mycobacterial infections. The *Mycobacterium leprae* and *M. tuberculosis* Hsp10s have previously been shown to be important T-cell antigens.[12,13] Approximately one-third of the *M. leprae* reactive T cells have been shown to cross-react with Hsp10. Specific immune responses to Hsp10 include production of antibodies, T-cell proliferation and delayed-type hypersensitivity.[14] Moreover, immunodominant T-cell epitopes have recently been mapped to regions of the *M. leprae and M. tuberculosis* Hsp10.[15–17] Interestingly, although the mycobacterial Hsp10s have been found to be strongly immunogenic, their homologues from *Escherichia coli* and humans do not exhibit strong immunogenicity.[18] The conformational differences between mycobacterial and human Hsp10 might be responsible for differences in immunoreactivity of these proteins.[19–21]

Hsp60 has been shown to be an immunodominant target of the humoral and T-cell response in mice and humans.[3] Hsp60-specific antibodies have been detected in patients with tuberculosis and leprosy, and also in mice after infection with *M. tuberculosis*.[22,23] Moreover, $CD4^+$ $\alpha\beta$-T cells specific for the mycobacterial Hsp60 have been found in patients with leprosy or those vaccinated with *M. bovis* BCG.[24] Surprisingly, about 20% of all mycobacterium-reactive $CD4^+$ $\alpha\beta$-T cells in mice immunized with killed *M. tuberculosis* are specific for Hsp60.[25] These studies suggest a protective role for Hsp60-specific T cells in mycobacterial infection.

Extensive sequence homology between Hsps from *M. tuberculosis* and humans has led to the postulation of the involvement of Hsps in autoimmune disorders. Increased levels of antibodies to Hsp60 of *M. tuberculosis* have been demonstrated in various autoimmune disorders such as systemic sclerosis, rheumatoid arthritis, psoriasis and ankylosing spondylitis.[26–28] Similarly, Hsp10 has also been implicated as an immunomodulator in experimental arthritis. Mycobacterial Hsp10 has been shown to delay the onset and severity of adjuvant-induced arthritis in rodents when administered after disease induction.[29] Thus, the chaperonins of *M. tuberculosis* also appear to suppress the immune system probably by modulating T-cell function.

From the preceding discussion it is clear that Hsps play an important role in host immunity as well as pathogen virulence. While Hsps induce expression of pro-inflammatory cytokines through intercellular cell adhesion molecules on host endothelial cells, these proteins promote antigen presentation to the immune system by chaperoning peptides to the APCs. Their role as antigen presenters is a result of a very different role that the Hsps play in the intracellular milieu, that of molecular chaperones. As chaperones, the Hsps promote the correct folding and assembly of other cellular proteins under normal and stress conditions. This they perform by interacting with the unfolded or misfolded polypeptides and preventing their aggregation, thus providing the nascent polypeptides a chance to fold into the correct conformation.

Chaperonins as cytokines

A paradigm revolution has occurred in the field of molecular chaperone biology with the realization that in addition to their protein folding actions many molecular chaperones also have the capacity to act as intercellular signals for a range of cell populations including myeloid cells.[7,30,31] The cell–cell signaling role of the mycobacterial chaperonins will be discussed later. As a flavor of the literature it has been shown that Gram-negative Cpn60 proteins can stimulate the formation of osteoclasts and the destruction of bone.[32] *Chlamydia pneumoniae* induces the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in human endothelial cells as well as the production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) upon infection of human monocyte-derived macrophages.[33,34] Similarly, human Hsp60 has been shown to elicit a potent proinflammatory response resulting in the induction of TNF-α and nitric oxide (NO) formation in cells of the innate immune system. This response has been shown to be Toll-like receptor 4 (TLR4)-dependent thereby suggesting that Hsp60 also serves as a cell signaling molecule.[35] It is this novel property of the molecular chaperones that presumably accounts, at least in part, for the unexpected immunogenicity of these cell stress proteins.

Heat shock proteins as molecular chaperones

Protein folding in vivo

Anfinsen and co-workers established that the primary structure of a protein contains all the information necessary to direct the native secondary and tertiary fold.[36] Many proteins can fold spontaneously in vitro as long as they do not undergo aggregation or get involved in inappropriate interaction with other proteins. Under physiological conditions, however, the protein folding process is prone to the production of a variety of misfolded species. It is thus necessary to prevent aggregation of newly synthesized proteins and circumvent any off-pathway folding intermediates. An important step towards the molecular understanding of how proteins fold inside cells came with the discovery of specialized protein components, molecular chaperones, that play essential roles in enabling polypeptides to reach biologically active forms in a variety of cellular compartments.[37–39]

Molecular chaperones

Although a protein's tertiary structure is determined by the primary sequence of the polypeptide, the tertiary structure is achieved, in most cases, with the aid of helper proteins. Molecular chaperones recognize non-native states of other proteins and, by controlled binding and release, assist these substrate proteins to fold properly.[38] While molecular chaperones assist the non-covalent assembly of proteins in vivo, they themselves are not permanent components of these proteins.[40] The functions of these proteins as molecular chaperones depend upon their ability to recognize and bind to hydrophobic regions of proteins that might become exposed during the synthesis of proteins under normal growth conditions.[41]

Heat shock proteins as molecular chaperones

Many molecular chaperones are members of the heat shock family of proteins. Under normal conditions these proteins play an important role in co-translational folding, assembly and transport of proteins. Under conditions of stress, the Hsps bind and stabilize cellular proteins at intermediate stages of folding and assembly, thereby preventing their misfolding. The overexpression of these under conditions of stress thus reflects their importance is preventing protein aggregation in vivo.

The chaperonins

The Hsp60 chaperones, also known as the chaperonins, are a subgroup of molecular chaperones found in all domains of life. These proteins have been shown to mediate the ATP-dependent folding of many proteins in vivo and in vitro.[42] Chaperonins consist of ~60 kDa subunits arranged in two stacked rings forming a large central cavity where unfolded polypeptides bind and undergo productive folding[43] (Fig. 1). Each subunit of the chaperonin is divided into three domains designated apical, intermediate and equatorial. The equatorial domain forms the interface between individual rings and is the site of ATP binding and hydrolysis. Substrate protein binds at the central face of the apical domain while a short intermediate domain connects it to the equatorial domain.[44,45] Thus the functional properties of chaperonins appear to arise from a common domain architecture and oligomeric assembly.

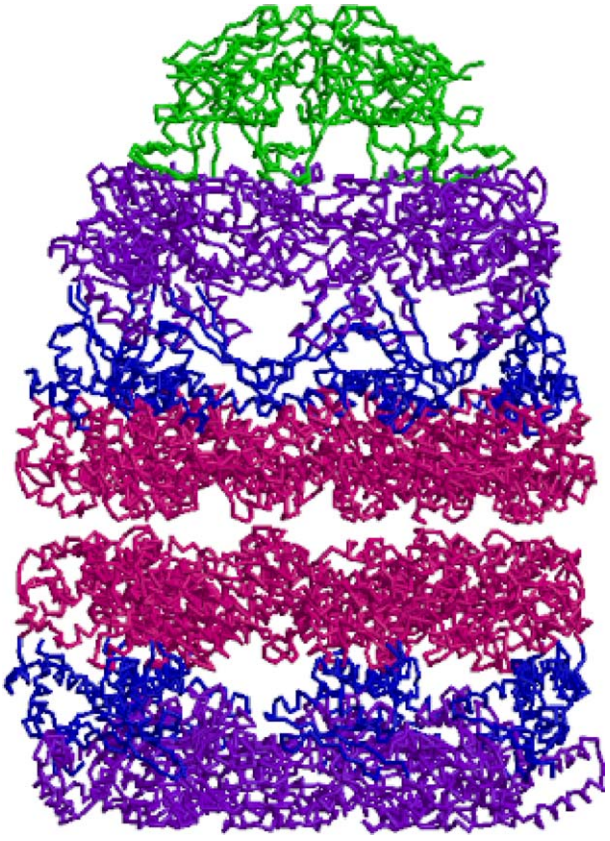

Figure 1 Structure of the $GroEL_{14}$–$GroES_7$–ADP_7 complex. The apical, intermediate and equatorial domains are colored violet, blue and purple, respectively. Non-native polypeptide binds to the apical domain of GroEL. GroES is colored green.[44] The GroES-bound ring of the GroEL tetradecamer is referred to as the *cis*-ring, while the opposite ring is referred to as the *trans*-ring.

The bacterial chaperonin, GroEL, is one of the best characterized molecular chaperones. About 10–15% of all the newly synthesized polypeptide chains interact with GroEL under normal conditions. This number, however, rises to more than 30% upon exposure to stress.[46] GroEL binds to collapsed and partially structured folding intermediates in the central channel, which is enclosed by the co-chaperonin GroES thereby preventing the aggregation of polypeptides in an ATP-dependent manner.[47]

GroEL

Crystal structures of the unliganded GroEL and the GroEL–GroES complex show a cylindrical arrangement with subunits of GroEL assembled into two heptameric rings stacked back-to-back to form the native 14-mer (Fig. 1). Each subunit of GroEL is folded into three distinct domains.[44,45,48,49]

The equatorial domain is the largest and the most well ordered of the three domains and provides most of the intra-ring and all of the inter-ring contacts across the equatorial plane. This domain also provides the ATP binding site as established by mutational analysis and the crystal structure. The apical domain forms the opening of the central channel and is involved in interactions with GroES and substrate proteins. The binding of GroEL to non-native proteins is largely non-specific and hydrophobic in nature.[50,51] Link between the apical and equatorial domains is mediated by the intermediate domain.

GroES

GroEL's co-chaperonin partner, GroES, is a dome-shaped structure existing as a single seven-membered ring of 10 kDa subunits.[19,52–54] Each subunit has a core β-barrel structure with two large protrusions extending from it. These loops are highly disordered and flexible in the unbound GroES.[55] One of the loops, the dome loop, contributes to the top of the dome. The second, known as the mobile loop, interacts in a 1:1 stoichiometry with residues in the apical domain of a GroEL subunit. The mobile loop becomes highly structured upon interaction with GroEL and forms the GroEL–GroES interface contributed mainly by aliphatic side chains from GroEL and GroES.

The GroESL complex and the protein folding cycle

Interaction between GroEL and GroES is necessary for the folding of a variety of polypeptides in an ATP-dependent manner. Interaction of GroES with one of the GroEL rings leads to the formation of the asymmetric complex, with the GroES bound ring referred to as the *cis* ring and the unbound ring known as the *trans* ring (Fig. 1).

The GroES ring caps the apical surface of the GroEL *cis* ring closing off the end of the central channel.[56] Upon this interaction the interior cavity of GroES becomes continuous with that of GroEL. The cavity, now double the original size, is capable of accommodating a globular protein or a molten globule intermediate of >70 kDa. Major conformational changes occur in the apical domains of the GroEL *cis* ring upon binding of the substrate. The domain movements in the GroEL *cis* ring are driven by the binding and hydrolysis of ATP and are essential for the completion of the chaperonin reaction cycle.

Binding and hydrolysis of ATP by one of the chaperonin rings controls the release of the co-chaperonin GroES from the opposite ring. ATP binds cooperatively to the subunits of one ring in GroEL triggering a conformational change that reduces substrate affinity in the ATP-bound ring.[57,58] While inside the cavity the protein attempts to refold in a time period of 10–20 s following which the seven ATP molecules in the *cis* ring are hydrolyzed.[59,60]

ATP hydrolysis in the *cis* ring primes the ring to release the bound substrate. The energy of γ-phosphate is used in a forceful release of the bound substrate from the central cavity of GroEL.[61] The actual release is triggered as a result of negative cooperativity between the two GroEL rings. Binding of ATP to the *trans* ring results in a reduced GroES affinity in the *cis* ring thereby allowing the substrate protein to be released from the *cis* ring.[62,63] Communication between the two rings is responsible for the release of GroES and the bound substrates.

Chaperonins of *Mycobacterium tuberculosis*

The GroES and GroEL proteins are encoded by genes arranged on the bicistronic *groESL* operon. This chromosomal arrangement allows for co-ordinated expression of the *groES* and *groEL* genes, which is in accordance with their biological function as molecular chaperones. Most eubacteria contain a single copy of the *groEL*-like gene. Two *groEL*-like genes were, however, found to exist in the genome of *M. leprae*[64] as also in *M. tuberculosis*.[65] Existence of a duplicate set of *groEL*-like genes on the genome has been found to be common to certain

of the high G+C bacteria, which include the mycobacteria.

Genes encoding the two chaperonins in *M. tuberculosis* exist on separate parts of the genome. The genes have been referred to as *cpn60.1* and *cpn60.2*.[65] While *cpn60.1* is arranged on a putative operon with the *groES* (*cpn10*), the second gene, *cpn60.2*, exists in a different part of the chromosome. The two *cpn60* gene products display 61% sequence identity. Upon comparison with other members of the Cpn60 family, *M. tuberculosis* Cpn60.2 consistently shows 5–10% greater sequence identity and similarity than Cpn60.1. Cpn60.1 and Cpn60.2 display 53% and 59% sequence identity with respect to *E. coli* GroEL, respectively.[65]

Co-chaperonin, Cpn10

The *cpn10* gene, present upstream of *cpn60.1*, codes for the 10 kDa co-chaperonin partner protein, Cpn10. The *M. tuberculosis* Cpn10 has been shown to be one of the most abundant proteins found in the bacterium culture filtrates.[66] Recently Fossati et al.[67] showed that the secretion of the *M. tuberculosis* Cpn10 in the macrophage phagosome results from the dissociation of the Cpn10 heptamers to monomers. The association between subunits of Cpn10 heptamers has previously been shown to be very labile. Earlier studies have shown that the *M. tuberculosis* Cpn10 heptamer dissociates into monomers in dilute solutions. The dissociated monomer unusually adopts a partially helical structure promoting its secretion outside the bacillus into the macrophage phagosome.[67]

Secretion of the *M. tuberculosis* Cpn10 outside the pathogen has previously been shown to elicit immune stimulating activity in the host. Cpn10 has also been shown to be strongly recognized by T cells from patients suffering from tuberculosis[68] and has been successfully used as an experimental vaccine against auto-immune diseases such as adjuvant arthritis.[29]

Cpn10 has been postulated to be a major factor responsible for bone resorption in Pott's disease.[69] Pott's disease, also known as tuberculosis of the spine, is characterized by massive resorption of the spinal vertebrae and is one of the most striking pathologies resulting from local infection with *M. tuberculosis*. Regions of Cpn10 responsible for its osteolytic and osteoblast antiproliferative activities have been identified to exist in the loop spanning residues 65–70 as well as the mobile loop of this protein.[69]

The crystal structure of Cpn10 showed unusual quaternary association of its subunits. Unlike the canonical heptameric association of the 10 kDa monomers, the *M. tuberculosis* Cpn10 was found to exist as a tetradecamer with the two dome-shaped heptamers complexed through the loops at their bases.[54] Occurrence of electron density in the interior of the enclosed cavity of the Cpn10 tetradecamer was suggestive of the encapsulation of partially disordered protein substrates within the cavity. This binding of substrates has been hypothesized to promote the transportation of folded proteins out of the Cpn60 cavity in a protected environment formed by the Cpn10 tetradecamer.

The crystal structure of Cpn10, determined by Taneja and Mande,[20,53] reiterated the heptameric state of the protein as has previously been demonstrated for the *E. coli* and *M. leprae* Cpn10s.[19,52] The crystal structure of the heptameric Cpn10 revealed accumulation of intense negative charge at the tip of the dome loop, thereby suggesting possible metal binding sites, and hence a decreased flexibility of the dome loop (Fig. 2). It has earlier been demonstrated that the divalent cations lead to substantial conformational changes in the *M. tuberculosis* Cpn10, particularly in the dome loop, reducing its plasticity and thereby conferring stability on the heptameric assembly of the molecule.[20] The mobile loop in GroES undergoes a tremendous loss of flexibility upon interaction with GroEL during the GroEL–GroES complex formation. The Cpn10 structure indicates that while the flexible dome loop of *M. tuberculosis* Cpn10 is partially stabilized by the binding of metal ions, the highly flexible mobile loop also attains a partially stable conformation.[21]

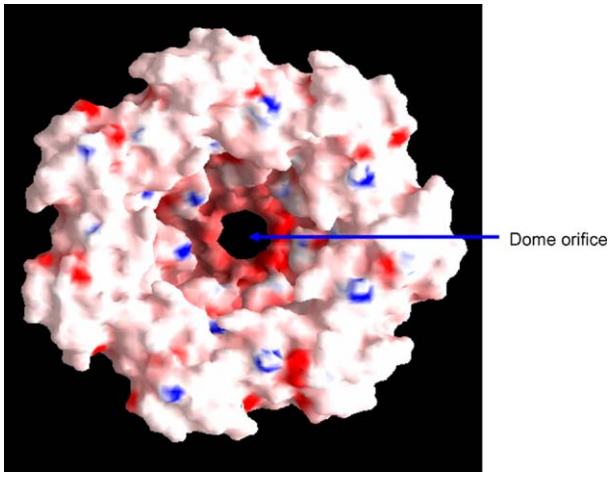

Figure 2 Molecular surface of GroES showing accumulation of negative charges at the dome orifice. Red surface indicates negatively charged region, while the blue regions indicate positively charged regions. Divalent cations have been shown to bind to the dome orifice. (Figure prepared using GRASP.)[70]

The osteolytic activity of Cpn10 has been shown to be present in the mobile loop and the region spanning residues 65–70. This region has been suggested to be comprised of a single conformational unit[69] that is in agreement with the *M. tuberculosis* Cpn10 crystal structure determined by Taneja and Mande.[53] Unlike the currently available crystal structures of Cpn10 from *E. coli*, the mobile loop in *M. tuberculosis* chaperonin is present as a single conformational unit with residues 65–70. It is therefore possible that these two regions of the secreted antigen possess osteolytic activity, as proposed previously by Meghji et al.[69]

Molecular dynamics studies on *M. tuberculosis* Cpn10 show that the presence of calcium ions enhances the accessibility of the 65–70 loop and furthermore increases the flexibility of the mobile loop. The simulations are suggestive of metal ions as key players in controlling the flexibility of the loop.[71] The structural disposition of these regions in the crystal structure were suggestive of an alternate oligomeric state of the protein as being responsible for the osteolytic activity of Cpn10. Monomerization of the protein and its partial helix formation have recently been identified as the major factors responsible for the secretion of the protein into the extracellular medium.[54]

We have hypothesized that upon secretion into the extracellular medium the Cpn10 of *M. tuberculosis* becomes available for chelation of calcium at bone joints. The overall depletion of calcium ions from the bone matrix might therefore lead to severe deformities in the bone. Interference with Ca^{2+}-dependent signaling pathways might also be responsible for the biological activity of Cpn10.[20]

Chaperonin, Cpn60

The content of the Cpn60 proteins of *M. tuberculosis* increases to 1–10% under conditions of stress such as are likely to occur during infection.[14] Both Cpn60s of *M. tuberculosis* have a role to play as important virulence factors in tuberculosis though the two proteins differ in their cytokine inducing potency and efficacy. The advantage that the duplication of the chaperonin genes offers to the pathogen, however, remains unknown.

Cpn60.1 and Cpn60.2 were demonstrated to activate secretion of pro-inflammatory cytokines, IL-β and TNF-α from human monocytes.[72–74] The role of Cpn60.1 as an important immunogen and a potent cytokine inducer promoting the synthesis and secretion of a range of pro-inflammatory cytokines and the anti-inflammatory cytokine IL-10 was recently re-established.[74] Moreover, Cpn60.2 has been shown to play a major role in autoimmunity and arthritis and also as an antigen of $\gamma\delta$-T cells.[75] Cpn60s of *M. tuberculosis* therefore serve an important role as potent cytokine stimulators.

Our recent study shows interesting biochemical characteristics of the two Cpn60s of *M. tuberculosis*. We have demonstrated that the two *M. tuberculosis* Cpn60s behave as dimers in vivo and in vitro.[76] Neither Cpn60.1 nor Cpn60.2, could associate into higher homo- or hetero-oligomers suggesting their stability as lower oligomeric species.[76] This unusual existence as dimers is intriguing as most of the other known Cpn60s have been shown to exist as tetradecamers, the heptameric ring being the functional unit of the chaperonin. The oligomeric ring in the canonical Cpn60s provides a central cavity within the chaperonin important for occlusion of the substrate protein from the cellular environment, thus promoting its refolding in an ATP dependent manner. Sequence and structural analysis revealed the presence of natural differences at the interface residues in Cpn60.1 of *M. tuberculosis* possibly leading to the loss of tetradecameric state of this protein. X-ray studies on one of the Cpn60s of *M. tuberculosis*, Cpn60.2, revealed the dimeric state of this protein (Fig. 3). The crystal structure showed the exposure of large hydrophobic surfaces on the protein.[77] The hydrophobic patches on the protein surface are hypothesized to serve a role in binding unfolded polypeptides thereby preventing their misfolding and aggregation.

As mentioned earlier, Hsps have also been shown to be associated in vivo with a large repertoire of cellular peptides. The Hsp-bound peptides are presented to the immune system in complex with the MHC Class I molecules. This interesting role of Hsps as antigen presenters and carriers has been demonstrated for the eukaryotic Hsps. Exposure of hydrophobic surfaces on Cpn60.2, as revealed by its

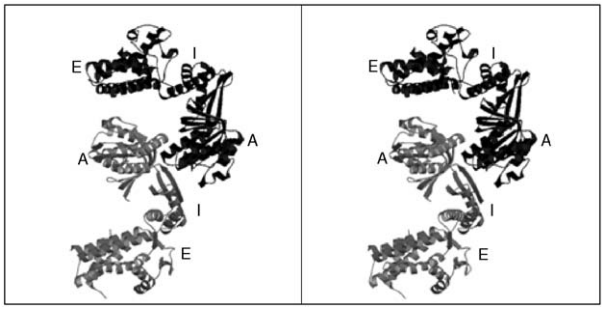

Figure 3 Crystal structure of the *M. tuberculosis* Cpn60.2. Stereo-view of the dimeric Cpn60.2 shows the three domains as observed in GroEL. The apical, intermediate and equatorial domains are indicated.

crystal structure, leads us to hypothesize that *M. tuberculosis* Cpn60.2 might serve such a role. This might be achieved by binding endogenous peptides by non-specific interactions with the exposed hydrophobic regions. We have shown that Cpn60.1 is more potent in preventing aggregation of substrate proteins than Cpn60.2. As Cpn60.1 also exists as a dimer, the mechanism of binding of the substrate polypeptides might be similar to that observed for Cpn60.2. This observation leads us to believe that Cpn60.1, like Cpn60.2, might bind to substrate proteins through exposed hydrophobic regions and might also contribute to the presentation of immunogenic peptides. Cpn60s of *M. tuberculosis* might serve dual roles, as potent immunogenic molecules and as a carrier of immunogenic peptides.

Cpn60s have been shown to predominantly activate a T-cell response. In fact regions of Cpn60 responsible for eliciting T-cell response have been identified. The crystal structure of Cpn60.2 has provided us with an opportunity to map the immunodominant epitopes on the structure of Cpn60.2. While most of the immunodominant peptides were mapped in the exposed equatorial domain, only one immunodominant epitope could be mapped onto the apical domain.[77] Since recognition of T-cell epitopes involves processing of polypeptides prior to their loading onto the MHC molecules, these epitopes are not required to be surface exposed. Occurrence of the immunodominant epitopes on the exposed equatorial domain is thus intriguing.

Another intriguing feature exhibited by the *M. tuberculosis* Cpn60s was the lack of ATPase activity of these proteins. The protein folding cycle of GroEL has been reported to be largely dependent on its ATPase activity.[78] Loss of ATPase activity by the *M. tuberculosis* Cpn60s, and the absence of the canonical oligomeric state, suggests that the *M. tuberculosis* chaperonins have a completely different folding mechanism than that of the prototypic GroEL. The *M. tuberculosis* Cpn60s were shown to promote refolding of substrate proteins independent of ATP. We believe that the ability of *M. tuberculosis* Cpn60s to promote protein folding without utilization of energy would prove to be economical to the bacterium. We hypothesize that *M. tuberculosis*, due to high demands of energy resulting from its extremely slow rate of metabolism, has devised an altered route to protein folding avoiding the usual energy dependent pathway. Loss of an oligomeric state of Cpn60s of *M. tuberculosis*, yet retaining their chaperoning role is suggestive of a design in evolution to save the energy sources of this bacterium.

Thus there appears to be the beginning of a paradigm revolution in chaperonin biology. The initial paradigm of Cpn60 (GroEL) was of a double ring structure requiring ATP hydrolysis and a co-chaperonin to fold proteins, with all the functions of this protein occurring intracellularly. It has emerged over the last decade that Cpn60 and Cpn10 can also act as intercellular signaling proteins.[31] The mycobacterial chaperonins are not fitting into the accepted paradigm on the basis, both of their structure, which is very unlike GroEL, and their cell–cell signaling activity. In spite of 61% sequence identity, the two *M. tuberculosis* Cpn60 proteins have very different cell signaling properties in terms of potency and cellular receptors.[74] Perhaps the most striking intimation that we know almost nothing concerning the biology of the Cpn60 proteins is the finding that the *M. tuberculosis* Cpn60.1 protein can inhibit experimental asthma in the mouse in a therapeutic manner (as opposed to blockade by immune vaccination).[79] In contrast, the Cpn60.2 molecule is completely inactive in this model. Other studies which examined a variety of Cpn60 proteins (including *M. tuberculosis* Cpn60.2) in the same model of asthma found that the only active protein was the Cpn60.2 protein of *M. leprae*.[80] These two mycobacterial Cpn60.2 proteins share $>95\%$ sequence identity revealing that tiny changes in the sequence of these mycobacterial chaperonins can exert profound effects on their biological activity. We desperately need to discover the Rosetta stone of the chaperonins to decipher how their sequences, and their tertiary and quaternary structures confer folding activity and cell–cell signaling activity on them and to determine how folding and cell signaling activity relate to each other. It is hoped that the study of the mycobacterial chaperonins will decipher this molecular language.

Acknowledgements

Financial support from the CSIR, India, the Department of Biotechnology, India, and the Wellcome Trust, UK, is greatly acknowledged.

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