

Chapter 8

Chaperonin: Co-chaperonin Interactions



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Abstract Co-chaperonins function together with chaperonins to mediate ATP-dependent protein folding in a variety of cellular compartments. Chaperonins are evolutionarily conserved and form two distinct classes, namely, group I and group II chaperonins. GroEL and its co-chaperonin GroES form part of group I and are the archetypal members of this family of protein folding machines. The unique mechanism used by GroEL and GroES to drive protein folding is embedded in the complex architecture of double-ringed complexes, forming two central chambers that undergo conformational rearrangements that enable protein folding to occur. GroES forms a lid over the chamber and in doing so dislodges bound substrate into the chamber, thereby allowing non-native proteins to fold in isolation. GroES also modulates allosteric transitions of GroEL. Group II chaperonins are functionally similar to group I chaperonins but differ in structure and do not require a co-chaperonin. A significant number of bacteria and eukaryotes house multiple chaperonin and co-chaperonin proteins, many of which have acquired additional intracellular and extracellular biological functions. In some instances, co-chaperonins display contrasting functions to those of chaperonins. Human HSP60 (HSPD) continues to play a key role in the pathogenesis of many human diseases, in particular autoimmune diseases and cancer. A greater understanding of the fascinating roles of both intracellular and extracellular Hsp10 on cellular processes will accelerate the development of techniques to treat diseases associated with the chaperonin family.

Keywords Chaperonins · Co-chaperonins · GroEL · GroES · Hsp60 · Hsp10 · Cpn60 · Cpn10

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Introduction

Chaperonins are ubiquitous ATP-driven protein folding machines characterised by a large multi-subunit ring structure. They prevent aggregation by binding non-native proteins and facilitate folding and unfolding of proteins. They form part of the Hsp60 family of heat shock proteins and are related by homology to the GroEL proteins of *E. coli* (Hartl and Hayer-Hartl 2002; Hemmingsen et al. 1988). The *E. coli* chaperonin GroEL and its co-chaperonin GroES are the quintessential members of this family of protein folding machines (Ellis and Hartl 1996; Hartl and Hayer-Hartl 2002; Horwich et al. 2007). The term ‘chaperonin’ (Cpn) was first coined in 1988 to represent this family of molecular chaperones after finding sequence similarity between Rubisco binding protein (now known as chloroplast Cpn60) and GroEL (Hemmingsen et al. 1988). The Hsp60 family of chaperones is one of the most abundant classes of molecular chaperone present in the plastids, mitochondria, and cytoplasm of all eukaryotes and eubacteria.

The terms GroEL and GroES were initially applied strictly to the two proteins found in *E. coli* and have been extended to include homologues from other bacterial species. The GroEL protein functions as a typical molecular chaperone as it binds and folds proteins, whilst GroES exhibits no autonomous role as a chaperone but modulates the activity of GroEL and is referred to as a co-chaperone. The term chaperonin is applied to bacterial proteins that are homologous to the *E. coli* GroEL and are also referred to as Cpn60, whilst co-chaperonins refer to homologues of *E. coli* GroES, also known as Cpn10. Although the mitochondrial homologues are called Hsp60 and Hsp10, the archaeal chaperonins are referred to as thermosomes (Trent et al. 1991). In the eukaryotic group, chaperonins found in the cytosol were first called TCP-1 and are now also called CCT (chaperonin-containing TCP-1) (Kubota et al. 1994), TRiC (TCP-containing ring complex) (Frydman et al. 1992), and c-cpn (Gao et al. 1992). The human HSP60/HSP10 proteins have been renamed HSPD/E (Kampinga et al. 2009). The chloroplast chaperonin is referred to as Cpn60 protein, and two types of co-chaperonins Cpn10 and Cpn20 are present (Koumoto et al. 2001). Prior to its recognition as chloroplast Cpn60, it was known as Rubisco-binding protein (Barraclough and Ellis 1980).

GroEL and GroES are essential molecular chaperones in *E. coli*, indispensable for viability at all temperatures (Ang and Georgopoulos 1989; Fayet et al. 1989; Tilly et al. 1981). Mitochondrial Hsp60 is similarly essential for the viability of *Saccharomyces cerevisiae* (Cheng et al. 1989; Rospert et al. 1993b), as are the group II CCT subunits (Lin and Sherman 1997; Stoldt et al. 1996). Mitochondrial Hsp60 inactivation results in embryonic lethality in mice (Christensen et al. 2010). Deletion of HSP60 in mouse cardiomyocytes resulted in heart failure due to impaired mitochondrial function (Fan et al. 2020). GroEL is critical for the correct folding of many proteins in the cell, under both normal and stress conditions. The folding of nascent polypeptides often requires the cooperation of both the Hsp70 and Hsp60 families, and these families are also responsible for most of the general folding events in the cell (Fink 1999; Hartl et al. 1992). Whilst CCT is not upregulated during heat shock

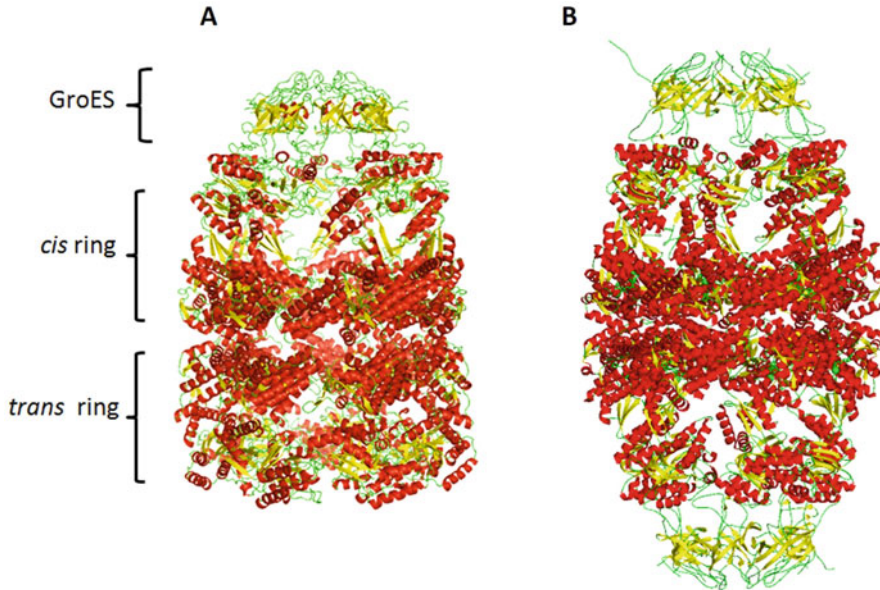


Fig. 8.1 The asymmetric GroEL/GroES complex comprises of two heptameric rings of GroEL stacked back-to-back with the GroES ‘lid’ bound to the *cis* ring to form a bullet-shaped complex, showing the side view (a), and the symmetric GroEL/GroES complex with two heptameric rings of GroEL stacked back-to-back with the GroES bound to both rings to form a football-shaped complex, showing the side view (b). The α -helices are shown in red and β -sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1AON and 4PJ1

(Horwich et al. 2007), GroEL and mitochondrial Hsp60 are heat inducible. In addition to ensuring the correct folding of proteins, chaperonins play a role in the assembly of protein complexes (Seo et al. 2010), trafficking of proteins (Xu et al. 2011) and peptide hormone signalling (Sigal and Williams 1997).

The chaperonins share a common subunit organisation and structure. They are a family of ATPases consisting of twin heptameric rings stacked back-to-back to create a characteristic cylindrical structure and function by assisting in the folding of nascent and misfolded proteins (Hartl and Martin 1995; Houry et al. 1999). Each ring creates a large cavity for unfolded proteins to bind and undergo productive folding to the native state in a highly cooperative and ATP-dependent manner (Bukau and Horwich 1998; Hartl and Hayer-Hartl 2002). Co-chaperonins form a single heptameric ring of 10 kDa subunits and are present in all bacterial and eukaryotic organisms (Hartl 1996). The *E. coli* asymmetric GroEL/GroES complex consists of two stacked heptameric rings of GroEL capped by a single heptameric ring of GroES that forms the lid over the folding cage (Fig. 8.1). The GroEL ring that is bound to GroES and protein substrate is termed the *cis* ring, and opposite ring free of GroES is termed the *trans* ring (Fig. 8.1a) (Xu et al. 1997). Two folding mechanisms have been proposed for GroEL; these are termed the *cis* and *trans* mechanisms (named after the GroEL rings that are bound by GroES), with most

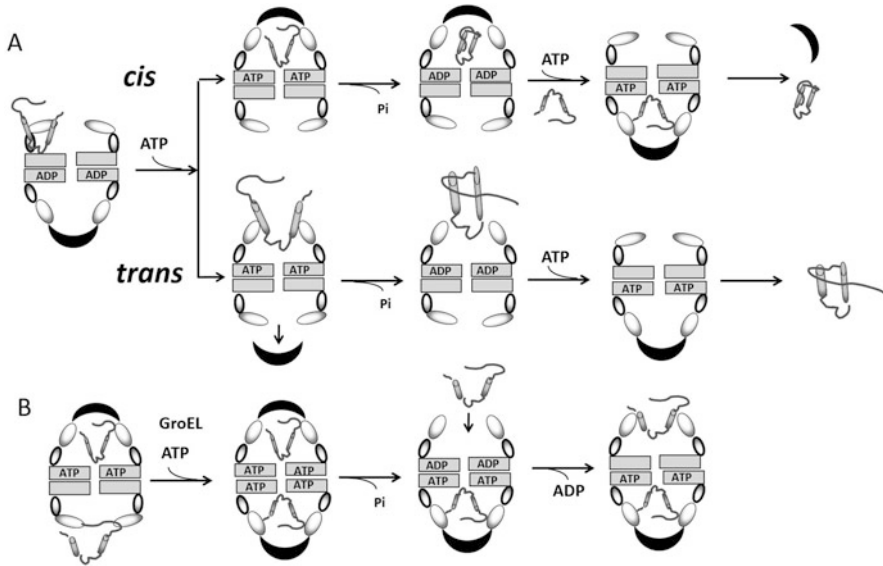


Fig. 8.2 GroEL-/GroES-mediated interaction cycle. The asymmetric (**A**) and symmetric (**B**) GroEL/GroES interaction cycles in the presence of substrate. The binding of ATP to the *cis* cavity causes conformational changes which allows the GroEL-bound substrate to follow either the *cis* or *trans* folding pathway, depending on the properties of the substrate (a). In the *cis* mechanism, the binding of substrate to one GroEL ring is followed by the binding of ATP and GroES to the *cis* ring. The substrate is released into the cavity closed by GroES and allowed to fold. ATP is hydrolysed, and the complex is ready to dissociate. The binding of ATP to the *trans* ring triggers release of substrate and dissociation of GroES from the *cis* ring and allows GroES to bind, releasing substrate into the cavity. Substrates that protrude outside the cavity and require only a portion of the protein to be folded, thus preventing GroES binding, follow the *trans* folding pathway. The subsequent binding of ATP and GroES to the *cis* ring releases the substrate. In the symmetric cycle, the two GroEL rings function simultaneously in substrate folding, with ATP hydrolysis resulting in GroES and substrate release (b). Encapsulation of substrate during the asymmetric folding allows a longer time for folding of the substrate, whilst a short residence time in the symmetric model increases the folding yields (Bigman and Horowitz 2019) [Adapted from Hayer-Hartl et al. (2016) and Kumar et al. (2015)]

GroEL substrates utilising the *cis* mechanism (Fig. 8.2) (Chaudhuri et al. 2001; Farr 2003). The functional cycle requires the binding of chaperonin 10 to one or both chaperonin rings which forms a lid-like structure on top of the cylinder when ATP is bound that causes the chamber to enlarge to allow for protein folding (Chandrasekhar et al. 1986). A vital part of the structure of each subunit is a flexible mobile loop that mediates binding to the chaperonin (Landry et al. 1996). The flexibility and the structure of the complex are conserved amongst co-chaperonins, and sequence variations impose differences in binding affinity (Richardson et al. 2001). Protein substrates first bind to the apical domain and are then dislodged and driven into the cavity by the binding of the co-chaperonin to the same area (Hartl and Hayer-Hartl 2002). The folding process is driven by the binding and hydrolysis of

ATP which triggers a complex set of allosteric signals both within and between the stacked rings (Gray and Fersht 1991; Todd et al. 1993). The *trans* mechanism has been proposed predominantly for large substrates that cannot enter the cavity, and partial substrate folding takes place through binding and release from the *trans* ring (Fig. 8.2) (Chaudhuri et al. 2001; Farr 2003). ATP-independent unfoldase activity has been reported for both GroEL and TRiC that allows the unfolded protein to fold outside the cavity (Priya et al. 2013).

The Gp31 protein from bacteriophage T4, a functional co-chaperonin that promotes the assembly of the T4 major capsid protein, can functionally substitute for GroES resulting in an increase in size and hydrophilicity of the enclosed chamber (Hunt et al. 1997; van der Vies et al. 1994). Another co-chaperonin from bacteriophage RB49, called CocO, is distantly related to GroES (Ang et al. 2001). Both of these bacteriophage co-chaperonins utilise host-encoded GroEL to assemble capsid proteins, and both proteins could functionally replace GroES in *E. coli* (Keppel et al. 2002). Interestingly, the first viral-encoded chaperonin was identified in the genome of *Pseudomonas aeruginosa* bacteriophage EL (Hertveldt et al. 2005) and later demonstrated to have functional properties similar to GroEL except that it does not require a co-chaperonin for activity (Kurochkina et al. 2012). The crystal structure of the GroEL homologue from the bacteriophage EL of *Pseudomonas aeruginosa* revealed that the chaperonin prevented protein aggregation without encapsulation and may represent an earlier version of the protein before the development of encapsulation (Bracher et al. 2020). A wide range of newly identified functions have been attributed to eukaryotic Hsp60, including roles in carcinogenesis, immunity, and cell signalling (Chandra et al. 2007). The roles played by both intracellular and extracellular forms of human HSP10 (HSPE) in pregnancy, cancer, and autoimmune diseases continue to receive attention (Corrao et al. 2010; Jia et al. 2011). The role of Hsp60 in promoting infection by hepatitis B virus (HBV), human immunodeficiency virus (HIV), and influenza A virus was reviewed by Wyzewski et al. (2018).

Whilst the *E. coli* chaperonins are encoded by only two genes, *groEL* and *groES*, both Cpn60 and Cpn10 found in green algae and plants are encoded by numerous genes (Boston et al. 1996; Hill and Hemmingsen 2001; Schroda 2004). Chloroplast chaperonins exhibit greater complexity than those found in bacteria and mitochondria with unique structures and functions (Vitlin Gruber et al. 2013a). It also appears that approximately 30% of bacteria encode more than one *groEL* gene (Hill and Hemmingsen 2001). The biological significance of several chaperonin genes has yet to be revealed (Lund 2009); however, the literature has expanded in recent years in this area of research. Cpn60 subunits with diverse expression profiles have evolved in chloroplasts, with the dominant subunits appearing to play housekeeping roles and minor subunits having more specialised functions, including the folding of specific proteins (Peng et al. 2011).

The chaperonins are subdivided into two distantly related groups. Group I chaperonins are found in eubacteria, mitochondria, and chloroplasts, of which GroEL from *E. coli* is the best studied and understood (Leroux 2001). Group II chaperonins are present in archaeobacteria and in the eukaryotic cytosol (Frydman

2001; Horwich et al. 1993). Although both subgroups form ring-like structures with cavities for sequestered protein folding, group II chaperonins form heterooligomeric complexes (Archibald et al. 1999; Spiess et al. 2004). The group II chaperonins consist of two eight- or nine-membered rings consisting of one to three subunit types in the archaeal thermosome rings (Phipps et al. 1991), whilst TRiC/CCT rings consist of eight subunit types (Frydman et al. 1992; Spiess et al. 2004). An important difference between the two groups is the lack of a GroES homologue in the group II chaperonins (Horwich and Saibil 1998). Group I chaperonins utilise an independently expressed co-chaperonin that functions as a lid to aid the encapsulation of unfolded protein, whilst group II chaperonins have a built-in lid in the form of a particular α -helical protrusion and do not require additional protein subunits to function (Meyer et al. 2003; Vabulas et al. 2010). However, the activity of CCT is regulated by a number of co-chaperones, including prefoldin, phospho- μ -foldin-like proteins, and BAG3 (Fontanella et al. 2010; Martin-Benito et al. 2002; Stirling et al. 2006; Vainberg et al. 1998). Prefoldin produces tentacle-like coils that capture protein substrates and transfers them to group II chaperonins; further studies have highlighted the importance of human prefoldin in proteostasis and the development of various diseases (reviewed by Sahlan et al. 2018). In 2010, a third group was proposed in bacteria and are conserved in the genomes of eleven bacteria (Techtmann and Robb 2010). These novel chaperonins are capable of refolding denatured proteins in a GroES-independent manner. Group III chaperonins are highly divergent and distantly related to group I and group II, and they might represent an ancient horizontal gene transfer event from archaea to bacteria, and this revises the current paradigm for chaperonin classification (Techtmann and Robb 2010). The crystal structure of the thermophilic bacterial group III Cpn from *Carboxydotherrmus hydrogenoformans* revealed that it is mechanistically distinct from group I and II chaperonins, and further evidence suggests that groups I and II may have arisen from a group III Cpn (An et al. 2017).

To date, the structure and mechanism of chaperonin and co-chaperonin functions have centred on the GroEL and GroES system of *E. coli* (Hartl 1996; Hartl and Hayer-Hartl 2002; Horwich et al. 2007). This system has received the most attention and serves as a model for chaperonin and co-chaperonin interactions. The GroEL and GroES folding machine will be discussed in the following section with emphasis on the role of GroES. Group I chaperonins will be the focus of this chapter as the functional activity of group II chaperonins is not assisted by co-chaperonins. The biological impact of chaperonins extends beyond protein folding as they are the dominant immunogens present during human bacterial infections, and there is considerable interest in their role in cancer and autoimmune diseases (Kaufmann 1992; Wiechmann et al. 2017). Data on the extensive roles of both extracellular and intracellular Hsp10 has left no doubt that the functions of this protein extends beyond its role as a co-chaperonin, and these roles have been reviewed by (Corrao et al. 2010). Research on bacterial chaperonins has expanded in recent years as more bacterial genomes have been sequenced. Our understanding of co-chaperonins in other organisms and organelles is gaining momentum, and recent findings on bacterial and eukaryotic co-chaperonins will be addressed.

Activities of the *E. coli* GroEL/GroES Folding Machine

One of the most efficient chaperone systems is the well-characterised *E. coli* chaperonin machine composed of GroEL and its co-chaperonin GroES. Three different functions have been assigned to this folding machine, binding to non-native proteins preventing aggregation (Buchner et al. 1991), facilitating protein folding by encapsulating the protein in a sequestered environment (Weissman et al. 1995), and finally unfolding of kinetically trapped intermediates so that they can refold (Shtilerman et al. 1999; Sparrer et al. 1997; Sparrer and Buchner 1997; Todd et al. 1993). The *groE* genes of *E. coli* were the first chaperonin genes to be discovered. These genes were first identified when temperature-sensitive mutant strains of *E. coli* could not support the growth of bacteriophage λ (Georgopoulos et al. 1972); afterwards, it was determined that the two genes are encoded on the same operon *groE*. The importance of these GroEL and GroES proteins is emphasised by the fact that they are the only chaperones that are essential for the viability of *E. coli* at all temperatures (Fayet et al. 1989). Additionally, host GroEL and GroES play a role in both phage infection and defence strategies of the host (Ang et al. 2000), as well as protecting viral proteins at high temperatures (Chen et al. 2013). *E. coli* GroEL/ES was previously known to play a role in the regulation of sigma-32 by enhancing proteolysis (Guisbert et al. 2004). An additional proteolytic role was demonstrated whereby interaction with the cold shock RNA chaperone (CspC) lead to proteolysis (Lenz and Ron 2014).

It is estimated that under normal cellular growth conditions, 10–15% of all cytoplasmic proteins rely on GroEL in order to fold correctly, and this increases to 30% under conditions of stress (Ewalt et al. 1997). Many of the cytoplasmic proteins that interact with GroEL have been identified (Houry et al. 1999), and GroEL acts downstream of the *E. coli* molecular chaperones, DnaK (prokaryotic Hsp70), and trigger factor, in the folding of 10% of cytosolic proteins (Ewalt et al. 1997; Houry et al. 1999). The mechanism of action is different to that of Hsp70 as the protein is sequestered from its environment. In a proteomic study of *E. coli* proteins, ~250 different proteins interact with GroEL, of these ~85 proteins are dependent on GroEL for folding and 13 of these are essential proteins (Kerner et al. 2005). These 85 proteins were scrutinised further, and ~60% were found to be absolutely dependent on GroEL and GroES for folding, and an additional 8 proteins were classified as obligate substrates (Fujiwara et al. 2010). Most of the substrates are characterised by a size range of 20–50 kDa and complex α/β or $\alpha + \beta$ topologies and tend to populate kinetically trapped folding intermediates (Kerner et al. 2005).

Over the past 30 years, many researchers have demonstrated the abilities of the *E. coli* GroEL and GroES machine to bind and refold a wide range of aggregation-prone proteins both in vivo and in vitro. Early in vitro experiments demonstrating the abilities of *E. coli* GroEL and GroES to refold denatured proteins were carried out using heat-denatured Rubisco enzyme (Goloubinoff et al. 1989), and following this seminal paper, the GroEL-GroES cycle has been scrutinised in vitro. Chaperonins continue to also play an important role in recombinant protein production, and this

has been well documented in the literature. *E. coli* is a frequently used host, and the folding of proteins in the cytoplasm is assisted primarily by Hsp70 and Hsp60 (Vabulas et al. 2010). They aid in functional expression and retain solubility by assisting the refolding of aggregated target proteins. The chaperonin GroEL and its co-chaperonin GroES have been used extensively for this purpose and are often co-expressed with the protein of interest. Some of these proteins include malate dehydrogenase (Hartman et al. 1993; Ranson et al. 1997), citrate synthase (Buchner et al. 1991), rhodanese (Martin et al. 1991), carbamoylase (Sareen et al. 2001), and aconitase (Chaudhuri et al. 2001). The presence of *E. coli* GroEL and GroES significantly improved the yields of soluble protein in most instances; however, large amounts of the chaperonins are often required, exceeding endogenous concentrations. Extensive optimisation of the reaction conditions is also vital, and the requirements of each chaperonin are variable. A greater understanding of the effects of overexpressing chaperonins on cell growth, and conditions for optimum recombinant protein production, needs to be investigated (Gupta et al. 2006). Despite these drawbacks, the *E. coli* chaperonins have been used successfully in biotechnology for the production of a wide range of recombinant proteins. The co-expression of GroEL/ES appreciably enhanced the expression of human tumour necrosis factor, CD 137 ligand (Wang et al. 2012). The solubility of *Plasmodium falciparum* 1-deoxy-D-xylulose-5-phosphate reductoisomerase was significantly increased by the co-production of GroEL/ES (Goble et al. 2013). GroEL immobilised on a sensor has been developed to detect and quantify unfolded therapeutic proteins in solution (O'Neil et al. 2018).

Structure of GroEL and GroES

GroEL and GroES form both GroEL-GroES asymmetric bullet-shaped and GroEL-GroES₂ symmetric football-shaped complexes as one GroEL ring can bind to one GroES heptamer (Fig. 8.1). There has been much debate concerning which of these complexes is essential for protein folding and its mechanism of action (Bigman and Horovitz 2019; Taguchi 2015). The crystal structure of GroEL bound to GroES and ADP was resolved in 1997, which corresponded to the bullet-shaped complex (Xu et al. 1997). Nearly two decades later, the structure of the football-shaped complex was resolved (Fei et al. 2014; Koike-Takeshita et al. 2014). Several crystal structures of GroEL are available (Braig et al. 1994), including GroEL complexed with ATP (Boisvert et al. 1996), and a GroEL-peptide complex (Chen and Sigler 1999), as well as NMR (nuclear magnetic resonance) spectroscopy (Fiaux et al. 2002; Nishida et al. 2006) and cryo-electron microscopy structures (Chen et al. 2006; Ranson et al. 2006). Co-chaperonin structures alone have been reported for GroES (Boudker et al. 1997; Hunt et al. 1996; Seale et al. 1996).

The ability of GroEL and GroES to enhance protein folding is embedded in the unique quaternary structures of these proteins. The arrangement of the GroEL subunits results in an oligomeric structure consisting of 14 subunits arranged in two

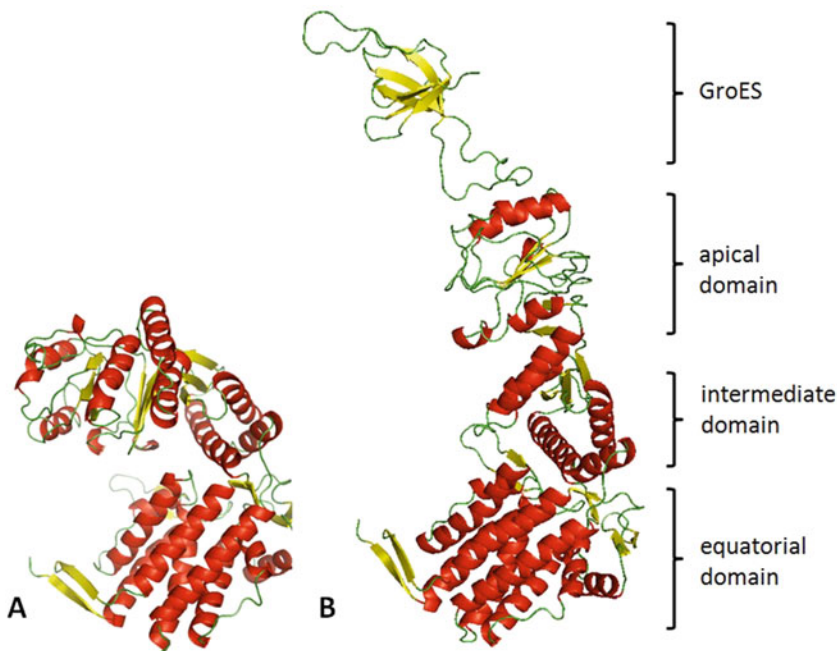


Fig. 8.3 Binding of GroES induces a large conformational change in GroEL. Each subunit of GroEL is divided into three distinct domains: an apical domain, an equatorial domain, and an intermediate domain that connects both domains. Unbound GroEL (**a**) undergoes large rigid body movements of the apical domain upon binding of GroES (**b**). Apical domain is twisted 90° relative to the open ring not bound to GroES. Alpha helices are shown in red and β -sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1AON

inverted rings, whilst the GroES subunits are arranged into a single ring of 7 subunits, and both structures display sevenfold rotationally symmetric ring-shaped oligomers (Fig. 8.1). The GroEL subunits are composed mainly of α -helices, and the arrangement of the subunits into two stacked GroEL rings creates a central channel that is split into two functionally separate cavities at the ring interface (Braig et al. 1993, 1994). Each subunit is divided into three distinct domains: an ATP-binding equatorial domain that mediates interactions between subunits of each ring, a substrate-binding apical domain including co-chaperone binding sites, and an intermediate domain that connects both domains and transmits conformational changes generated by nucleotide binding between the equatorial and apical domains (Fig. 8.3) (Braig et al. 1994; Fenton et al. 1994). The apical domains are positioned on the outside of each ring, the intermediate domains are in the middle, and the equatorial domains are positioned at the interface of both rings. Coalescence of the disordered and flexible C-terminal segments of the subunits in each ring was determined to block the central channel at the equatorial domain causing discontinuity between the cavities turning them into two separate chambers for folding (Chen et al. 1994). An alternative model, based on molecular simulations, suggests that the non-native protein is

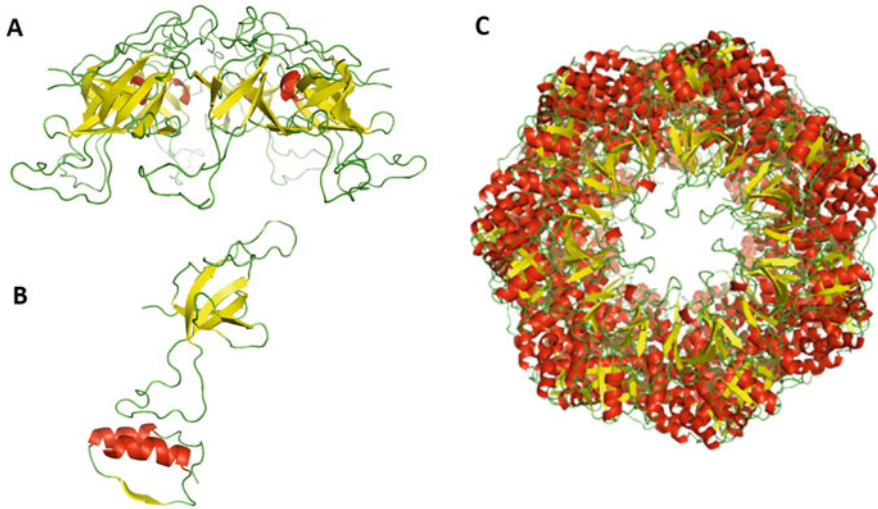


Fig. 8.4 Structure of GroES. Side view of GroES heptameric structure, as it occurs bound to GroEL and ATP, showing the flexible loops that interact with GroEL pointing downwards (a). The backbone structure of the GroES monomer interacting with the top of the apical domain of GroEL (b). A top view of the GroEL/GroES complex (c). Alpha helices are shown in red and β -sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1AON

translocated from one barrel of GroEL to the next until it is fully folded, and this may account for the double-ring structure (Coluzza et al. 2006).

GroES is composed of a seven identical 10 kDa subunits that form a lid-like structure (Hunt et al. 1996; Mande et al. 1996). These subunits form an irregular β -barrel structure formed by five β -strands with anti-parallel pairing of the last β -strand of one subunit with the first β -strand of the following subunit (Landry et al. 1996). Each subunit includes two loop regions, one facing upwards that forms the roof of the lid and one extending downwards from the bottom of the lid that constitutes a highly flexible mobile loop 16 amino acids in length (Fig. 8.4) (Landry et al. 1993). Binding of GroES to GroEL is mediated by the seven flexible loops which are induced to form a β -hairpin structure upon formation of the GroEL/GroES/ATP complex (Fig. 8.4) (Richardson et al. 2001). Mutations in the mobile loop disrupted GroES binding to GroEL (Zeilstra-Ryalls et al. 1994). The contribution of the mobile loop was studied using a synthetic peptide resembling the loop, which lacked structure until induced to form the β -hairpin structure when bound to GroEL (Landry et al. 1996). The functional contribution of the flexibility of the mobile loop to chaperonin function was investigated by restricting the flexibility by the formation of disulphide bonds within the loop, and the results revealed that they play an important role in inducing substrate release into the cavity (Nojima et al. 2012).

The GroEL rings are subject to intra- and inter-ring allostery (Yifrach and Horowitz 1995). The two GroEL rings are staggered such that each subunit contacts two subunits on the other ring that facilitates negative cooperativity between rings (Braig et al. 1994; Roseman et al. 2001). A review of the unfolding and refolding of GroEL in the presence of ligands and different solvents has highlighted differences in behaviour between these two proteins (Ryabova et al. 2013). The crystal structure of the bullet-shaped GroEL-GroES-ADP complex revealed that the apical domains are twisted 90° relative to the open ring not bound to GroES (Fig. 8.3) (Roseman et al. 1996; Xu et al. 1997). The transmission of conformational changes between the apical and equatorial domains of GroEL via the intermediate domain is essential as mutations in this domain compromised the folding capacities of GroEL/GroES (Kawata et al. 1999). Movement of the apical domains upon ATP binding shifts the hydrophobic GroES and substrate binding site from a position facing the cavity to an elevated and rotated position to facilitate the binding of the mobile loop of GroES to cap the folding chamber (Fig. 8.3). Mutational mapping revealed that there is an overlap between substrate and GroES binding to the hydrophobic binding site (Fenton et al. 1994). Another study suggested that rotation of the hydrophobic binding site weakens substrate binding (Ranson et al. 2001). However, mapping the trajectories of domain movements of the GroEL-ATP complex showed that the apical domains are linked by salt bridges that allow the binding sites to separate from each other in an extended conformation, at the same time maintaining the binding surface facing the cavity, providing a potential binding site for GroES which triggers a final rotation that provides the ‘power stroke’ to eject substrate in the chamber (Clare et al. 2012). The effects of interactions between the cavity wall of GroEL and rhodanese were investigated with the result that these interactions slowed down the folding rate of rhodanese (Sirur and Best 2013).

The Role of GroES in the Reaction Cycle

GroES functions as a co-chaperonin of GroEL to mediate the folding of unfolded or partially unfolded proteins. GroEL captures substrates at a site in the apical domain that exposes hydrophobic amino acid residues to facilitate substrate binding towards the ring centre (Fenton et al. 1994). GroES binds at the apical domain of ATP-bound GroEL, at a site that overlaps largely with the substrate binding site, and in doing so displaces the substrate into the binding cavity (Fenton et al. 1994). The distortion of the GroEL ring caused by the binding of GroES causes the hydrophobic residues that bind non-native protein to become inaccessible creating a hydrophilic lined cavity (Xu et al. 1997). The result is the eviction of the protein into the cage for folding, also referred to as the Anfinsen cage (Ellis and Hartl 1996). GroES then forms a lid over the central cavity entrapping the protein. GroES binding is faster than ATP-induced release of the substrate, and this provides a mechanism for the entrapment of proteins in the *cis* cavity (Burston et al. 1995).

Once the substrate is encapsulated in the chamber, the slow rate of ATP hydrolysis dictates the length of time for folding to take place (Fenton and Horwich 2003; Frydman 2001; Hartl and Hayer-Hartl 2002, 2009). GroEL exhibits weak ATPase activity that is lowered in the presence of GroES (Chandrasekhar et al. 1986; Goloubinoff et al. 1989). GroEL assists in the folding of non-native proteins with the consumption of ATP (Xu et al. 1997). Transformational changes in the *trans* ring caused by binding of substrate, ATP, and GroES to the *cis* ring result in the *trans* ring not being able to bind substrate (Tyagi et al. 2009). This phenomenon may be substrate specific, since in some cases GroEL was shown to bind two substrates simultaneously, one in each folding chamber (Lorca et al. 1997; van Duijn et al. 2007). ATP hydrolysis of the GroES-bound ring is required for the binding of ATP to the *trans* ring; negative cooperativity is displayed between the two GroEL rings which favours dissociation of GroES, ADP, and substrate from the *cis* ring (Rye et al. 1997). If the substrate is not folded correctly, it can rebind to another or the same GroEL for successive cycles of folding (Rye et al. 1997). GroES can now bind to the *trans* ring, and this ring then becomes the new *cis* ring in the subsequent round of substrate folding events. Thus both rings alternate to become the *cis* ring during folding cycles, and this has led to the term ‘two-stroke engine’ for the GroEL/GroES folding machine (Lorimer 1996; Xu and Sigler 1998).

The transition between the open conformation, that is receptive to protein binding, and the closed state, in which the protein is isolated, is induced by ATP binding and hydrolysis (Horovitz and Willison 2005). ATP binds with positive cooperativity within rings but with negative cooperativity between rings (Yifrach and Horovitz 1995). Allosteric transitions support the ATP-dependent control of the affinity of GroEL for its substrate and the subsequent folding (Roseman et al. 1996; Saibil et al. 1993; Yifrach and Horovitz 1995). ATP binding initiates bending and twisting of subunit domains that distort the ring structure and exposes the GroES binding sites. ATP binds to a ring with positive cooperativity, and movements of the interlinked subunit domains are concerted. In contrast, there is negative cooperativity between the rings, so that they act in alternation (Horovitz et al. 2001; Rye et al. 1997). GroEL/GroES-assisted protein folding is further complicated by the existence of both bullet-shaped (asymmetric) and football-shaped (symmetric) complexes. Computational analysis of GroEL substrates revealed that both complexes functionally co-exist with a shorter folding rate observed for certain substrates in the football complex, whilst low ATP concentrations favoured the bullet-shaped species (Fig. 8.2) (Bigman and Horovitz 2019). Asymmetric GroEL-GroES complexes appear to persist under physiological conditions as a result of negative cooperativity between GroEL rings, as nucleotide binding in one ring causes suppression of binding in the other ring (Gruber and Horovitz 2016; Inobe et al. 2008). However, the presence of a high concentration of substrate allows the symmetric cycle to dominate, suggesting that GroEL can switch between two types of reaction cycle (asymmetric or symmetric), possibly depending upon the substrate proteins [reviewed by Iizuka and Funatsu (2016)]. Using FRET assays, symmetric GroEL-GroES₂ were determined to be the folding functional form, and these reverted to asymmetric forms as protein substrate levels decreased (Yang et al. 2013). The

advantage of the symmetric complex is that protein folding can occur in both cavities of the football-shaped complex; in fact, the folding of GFP was determined to occur independently in both rings of the symmetric complex (Takei et al. 2012). In an effort to reconcile the two different types of complexes, transient ring separation caused by ATP binding to the *trans* ring allows the GroEL rings to function sequentially (Yan et al. 2018). Understanding the pathways of allosteric communication in GroEL has been the subject of intense research, and this was reviewed by Saibil et al. (2013) and more recently by Lorimer et al. (2018). A comprehensive review of the modes of action of the GroEL/ES folding machine revealed that the environment of the cage can accelerate the folding of some proteins (Hayer-Hartl et al. 2016). The results of numerous studies on the folding kinetics of the encapsulated protein substrate are available, and these vary due to differences in the properties of the protein substrate (Korobko et al. 2020). GroEL encapsulation repaired a folding defect of a maltose-binding protein with a destabilising mutation by restoring or re-establishing fast folding, suggesting an active role of GroEL in a compression effect in the cavity (Ye et al. 2018). Recently, it was determined that the protein stability of thermally unstable dihydrofolate reductase from *Moritella profunda* was greatly reduced after stable encapsulation by GroEL, resulting in unfolded protein that then had the option to fold again (Korobko et al. 2020).

In addition to its role as a lid for the folding chamber in the chaperonin complex, GroES controls the cooperativity by directing conformational changes in GroEL that are orchestrated by the seven mobile loops binding to each of the seven GroEL subunits, followed by release of substrate into the cage (Gray and Fersht 1991; Todd et al. 1994; Yifrach and Horovitz 1995). Interestingly, an alpha haemolysin nanopore was fused with the seven flexible loops of GroES allowing it to function as efficiently as the native GroES (Ho et al. 2015). GroES also plays a key role in controlling the competence and specificity of protein folding by GroEL (Richardson et al. 2001). Based on the GroEL-GroES-ADP complex, the binding of GroES causes large rigid body movements of the apical domains of GroES that result in doubling of the volume of the *cis* ring cavity compared to the *trans* ring (Fig. 8.3) (Xu et al. 1997). This increased volume is capable of binding a native protein of 70 kDa (Houry et al. 1999). Most of the *E. coli* proteins that require GroEL-GroES for folding are ~60 kDa, and larger proteins that cannot be accommodated within the folding cavity can be folded by binding to the uncapped *trans* ring of GroEL (Sigler et al. 1998). Binding of GroES causes a dramatic change in the walls of the cavity as the hydrophobic binding sites are rotated towards the interfaces of adjacent subunits and GroES resulting in a hydrophilic wall, and the intermediate domain twists downwards capping the nucleotide binding site (Xu et al. 1997).

Roles of Bacterial Chaperonins

Due to their importance in protein homeostasis, chaperonins are essential and universally distributed in all bacteria. Bacterial chaperonins are required for the correct assembly of the cell division apparatus (Ogino et al. 2004). In contrast to *E. coli* which possesses a single operon-encoded *groEL* gene with a *groES* gene, nearly 30% of all bacterial genomes contain multiple chaperonin genes (Lund 2009). The mycobacteria were the first bacteria revealed to have multiple chaperonins (Kong et al. 1993; Lund 2001). *M. tuberculosis* encodes two chaperonin genes, *cpn60.1* in an operon with the co-chaperonin gene *cpn10* and *cpn60.2* in a different position on the chromosome (Kong et al. 1993), whilst *M. smegmatis* has three copies of *cpn60* (Fan et al. 2012). In bacteria with multiple *groEL* genes, such as mycobacteria, the essential copy is unexpectedly often not the operon-encoded gene, and this has resulted in much interest and speculation about the functions of these additional chaperonins (Hu et al. 2008; Ojha et al. 2005). It is possible that one copy preserves the essential chaperone function, whilst the others diverge to take on altered roles (Lund 2001). Biophysical studies of the chaperonins from *M. tuberculosis* and *M. smegmatis* provide support of novel functions for Cpn60.1 as Cpn60.2 proteins assemble into oligomers and are able to replace GroEL in *E. coli* when co-expressed with GroES or the cognate Cpn10, whilst neither Cpn60.1 nor Cpn60.3 found in *M. smegmatis* could functionally replace GroEL (Fan et al. 2012). Based on the fact that Cpn60.1 appears to chaperone a discrete set of key enzymes involved in the synthesis of the complex cell wall and differences in protein sequence, this novel mycobacterial chaperonin may provide a unique target for drug development reviewed by (Colaco and MacDougall 2014). As part of the development of GroEL/ES inhibitors as potential antibiotics, clinically significant ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) GroEL/ES formed mixed complexes in the presence of *E. coli* GroEL/ES leading to a loss of viability in some cases, using chaperonin-deficient *E. coli* (Sivinski et al. 2021).

One of the five GroEL paralogs in *Sinorhizobium meliloti* is required for NodD protein folding (Ogawa and Long 1995), whilst *Bradyrhizobium japonicum* possess at least five *groESL* operons that can partially compensate for the lack of one or other genes (Fischer et al. 1993). These duplicated proteins have evolved specific roles in different bacteria, but the mechanism involved in functional divergence has not been elucidated (Wang et al. 2013). *Mycococcus xanthus* DK1622 displayed functional divergence with respect to substrate specificity, and this was as a result of differences in the apical and C-terminal regions of the two GroEL proteins (Wang et al. 2013). Interestingly, monomeric Cpn60 from *Thermus thermophilus* was able to support protein folding independently of both ATP and a co-chaperonin (Taguchi et al. 1994). The crystal structures of the *T. thermophilus* Cpn60/Cpn10 complex alone (Shimamura et al. 2003) and with bound proteins have been reported (Shimamura et al. 2004). Despite a destabilised structure, Cpn60 proteins from *M. tuberculosis*

also displayed activity in the absence of ATP or co-chaperonin (Qamra and Mande 2004). *Chlamydia* harbours three putative chaperonins, and ChGroEL has been associated with increased pathology and is the primary chaperonin, whereas the other two paralogues perform novel *Chlamydia*-specific functions during infection (Illingworth et al. 2017). Further functions of the multiple chaperonins in bacteria were reviewed by (Kumar et al. 2015).

Cpn60s are dominant immunogens present during bacterial infections. Moreover, Cpn60s of *M. tuberculosis* are potent inducers of host inflammatory responses and behave as antigens and cytokines (Qamra et al. 2005). The host immune response to exogenous chaperonins may be both protective and damaging (Ranford and Henderson 2002). It has been hypothesised that due to sequence conservation, the host immune response mounted against bacterial co-chaperonins may result in cross-reactivity to human Cpn60 causing an autoimmune reaction (van Eden et al. 1998). There is convincing evidence for the case in the development of atherosclerosis (Wick 2006). The roles of chaperonins in disease, including models and potential treatments, are addressed in a review by (Ranford and Henderson 2002).

Immunisation of mice with GroEL conferred full protection against *Bacillus anthracis* infection, whilst DnaK was ineffective (Sinha and Bhatnagar 2010). More recently GroEL was evaluated as an ideal vaccine candidate against *Streptococcus agalactiae*, responsible for significant economic losses in the fishing industry (Li et al. 2019). Extracellular leptospiral GroEL may play a role in the adhesion of leptospires to host tissues and induce cytokine secretion during infection (Ho et al. 2021).

Specific Functions of Bacterial Co-chaperonins

In addition to co-chaperonin activity, a number of diverse roles played by bacterial co-chaperonins are emerging, in particular during host-pathogen interactions. The possible reasons for numerous chaperonins in bacteria were reviewed by Lund in 2009, and the evolution of so many different functions is highlighted by Henderson and Martin (2011) (Henderson and Martin 2011; Lund 2009). Despite the conservation of the GroEL-ES system in prokaryotes, it is absent in several members of the class of mollicutes, which are bacteria lacking a cell wall (Schwarz et al. 2018). Most bacterial Cpn10 proteins are stimulators of the immune system, and the response varies between different species, with human and *E. coli* Cpn10 proteins being poor immunogens and *M. tuberculosis* and *M. leprae* Cpn10 proteins being strong immunogens (Cavanagh and Morton 1994). These proteins also play a role in apoptosis, cytokine secretion, and cellular growth and development (Cavanagh 1996). Cpn10 of *M. tuberculosis*, a secreted protein with cell signalling functions, is an important virulence factor during infection, and it plays a key role in the pathology of spinal tuberculosis by inhibiting the growth of osteoblasts (Meghji et al. 1997; Roberts et al. 2003). Structures have been reported for *M. leprae*, and *M. tuberculosis* Cpn10 proteins and immunodominant epitopes have been mapped

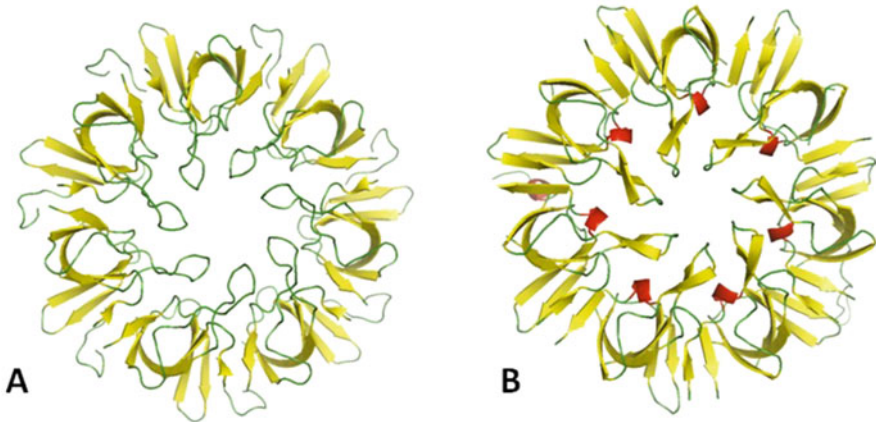


Fig. 8.5 The overall structures of *M. tuberculosis* Cpn10 (a) and *T. thermophilus* Cpn10 (b) conform to the GroES-fold. Differences are evident in the mobile loops, and a partially helical structure is present in the *T. thermophilus* Cpn10 monomer. Breaks are evident in the structures due to a lack of electron density in the highly flexible mobile loops. Alpha helices are shown in red and β -sheets in yellow. The images were generated using PyMol (DeLano Scientific) from coordinates in PDB: 1HX5 and WNR

to the mobile loop (Mande et al. 1996; Roberts et al. 2003). Further structural analysis of *M. tuberculosis* Cpn10, in the presence of divalent cations, showed the existence of a heptamer (Taneja and Mande 2001, 2002). The crystal structure of *T. thermophilus* HB8 Cpn10 showed disordered loops in five subunits (Numoto et al. 2005). Comparison of *M. tuberculosis* Cpn10 to that of *T. thermophilus* HB8 Cpn10 revealed a similar overall structure; however, the dome loops and mobile loops are different (Fig. 8.5). The Cpn10 from *Aquifex aeolicus* has a 25-residue C-terminal extension present in each monomer, that is absent from any other known Cpn10 protein, that is not essential for function but plays a role in preventing aggregation at high temperatures (Chen et al. 2008; Luke et al. 2005).

Roles of Eukaryotic Group I Chaperonins

In eukaryotes, group I Hsp60 is found in the mitochondria and also in chloroplasts of plants. It interacts with its co-chaperonin Hsp10 or Cpn10 to promote protein folding in the cell. Most mitochondria and chloroplasts in higher plants appear to possess multiple chaperonin subunit types (Hill and Hemmingsen 2001). A number of novel functions and interacting molecules have been assigned to Hsp60 (Czarnecka et al. 2006). Some of these are associated with carcinogenesis as its role in the survival and proliferation of tumour cells has increased (Cappello et al. 2008; Czarnecka et al. 2006). Human HSPD has received considerable interest as an anticancer drug target. It is highly expressed in ovarian tumours and knockdown of HSPD disrupted

mitochondrial functioning resulting in impeded cell proliferation (Guo et al. 2019). A review of small molecule modulators of Hsp60 function was conducted to identify potential anticancer drugs (Meng et al. 2018). The mitochondrial Hsp60 protein is essential for the folding of proteins imported into the mitochondria and prevention of denaturation during stress (Cheng et al. 1989; Levy-Rimler et al. 2001). They are also characterised by a host of additional functions, including extracellular functions. Hsp60 found in the cytosol and the extracellular space possesses various moonlighting functions (Henderson et al. 2013). Mutations of HSPD are linked to severe genetic diseases (Bross et al. 2007; Hansen et al. 2007; Magen et al. 2008). It also plays a role in the production of pro-inflammatory cytokines (Chun et al. 2010). In addition, it plays both pro-apoptotic and anti-apoptotic roles, depending on localisation (Knowlton and Gupta 2003; Xanthoudakis et al. 1999). The first assessment of proteins that interact with the human HSPD/E complex was conducted using HEK293 cells and revealed that half of the mitochondrial proteins associate with this complex (Bie et al. 2020). Not surprisingly, a number of these proteins are associated with human disease, whilst 19 highly abundant proteins occupied approximately 60% of the chaperonin capacity (Bie et al. 2020). A number of reviews have been written on Hsp60 chaperonopathies, diseases that arise from abnormal chaperonins (Cappello et al. 2008, 2011, 2013, 2014; Macario and Conway de Macario 2005, 2007). A review of Hsp60 in the pathogenesis of diabetes mellitus suggests that the chaperonin may provide the connection between mitochondrial stress and inflammation (Juwono and Martinus 2016). Future research is needed to understand the role of post-translational modifications of Hsp60 in chaperonopathies, and this is highlighted in a recent review by Caruso Bavisotto et al. (2020).

A structural study of HSPD/E revealed that both full football-shaped (double ring) and half-football-shaped complexes (single ring) are functional and co-exist, potentially forming two separate folding cycles that may be optimised for different substrate sets (Gomez-Llorente et al. 2020). The recent cryo-EM structure of apo HSPD1 reveals predominantly single ring assemblies and increased flexibility of the apical domain in comparison to GroEL (Klebl et al. 2021). The mitochondrial Hsp60 in mammalian cells is transformed to a double-ring structure in the presence of ATP and/or HSP10 (Levy-Rimler et al. 2001). X-ray crystallography confirmed that it is capable of forming a football-shaped complex in the presence of both HSP10 and ATP (Nisemblat et al. 2015). The mitochondrial chaperonin complex that is composed of a single ring of seven subunits and a ring of Hsp10 subunits cannot exploit binding of ATP to the *trans* ring as a mechanism for releasing *cis* GroES (Nielsen and Cowan 1998). This complex may have evolved an intrinsically lower affinity for the co-chaperonin, but the presence of a higher affinity mobile loop on Hsp10 may offset the low affinity (Nielsen and Cowan 1998). Despite the fact that mitochondrial Hsp60 can functionally replace GroEL, it is incapable of interacting with GroES (Nielsen et al. 1999). The elements that dictate the specificity of mitochondrial Hsp60 for Hsp10 appear to lie in the apical domain (Parnas et al. 2012). Analysis of *in vivo* substrates of yeast mitochondrial chaperonins revealed divergent chaperonin requirements, indicating that Hsp60 and Hsp10 do not always operate as a functional unit (Dubaque et al. 1998). Yeast mitochondrial Hsp60 can bind to

single-stranded DNA *in vitro* and play a role in the structure and transmission of nucleoids (Kaufman et al. 2003). A number of parasites affecting human health have demonstrated an upregulation of Hsp60, which is possibly linked to diverse environmental conditions encountered during its transition from a mammalian to an insect vector (Maresca and Carratu 1992). Induction of Hsp60 was found to occur during the entire course of infection of *Trypanosoma brucei*, a protozoan parasite responsible for causing sleeping sickness in humans (Radwanska et al. 2000). The crystal structure of *P. falciparum* mitochondrial Cpn60 bound to ATP revealed that large conformational changes can occur in the apical domain regulating substrate binding, whilst a unique insertion in the equatorial domain increased interactions between the rings (Nguyen et al. 2021).

The chloroplast type I chaperonin complex (Cpn60) is structurally similar to GroEL and also forms two stacked heptameric rings (Tsuprun et al. 1991); however, these are composed of two different subunit types, Cpn60 α and Cpn60 β (Martel et al. 1990) which are ~50% identical to each other (Hill and Hemmingsen 2001). *Arabidopsis thaliana* encodes several Cpn60 α and Cpn60 β families and both are required for plastid division (Suzuki et al. 2009). A unique chaperonin subunit in *A. thaliana* confers substrate specificity, whilst the dominant subunits retain house-keeping functions (Peng et al. 2011). The unicellular green algae *Chlamydomonas reinhardtii* encodes three CPN60 subunits, CPN60 α 1, CPN60 β 2, and CPN60 β 2 (Schroda 2004). Hetero-oligomeric chloroplast chaperonins are unstable in the presence of ATP, and *C. reinhardtii* CPN60 subunits revealed ATP-induced disassembly (Bai et al. 2015). The crystal structure of apo *C. reinhardtii* CPN60 β 1 appears similar to GroEL but with a larger binding cavity and a wider ATP binding pocket, which may justify the structural instability upon ATP hydrolysis (Zhang et al. 2016; Zhao and Liu 2018).

Specific Functions of Eukaryotic Group I Co-chaperonins

A single copy of the Cpn10 co-chaperonin is present in the mitochondria of yeast and mammals (Hansen et al. 2003; Rospert et al. 1993a). The chloroplast co-chaperonins are varied with *cpn10* encoding the conventional 10 kDa protein that is similar in structure and function to GroES, as well as *cpn20* encoding tandem fusions of Cpn10 domains that form tetrameric ring structures that function with GroEL and Cpn60 (Bertsch et al. 1992; Koumoto et al. 2001; Sharkia et al. 2003). Of the five co-chaperonin homologues present in *A. thaliana*, three reside in the chloroplast; Cpn10-2 and Cpn20 form functional homo-oligomers, whilst Cpn10-1 requires the integration of Cpn20 to form a functional hetero-oligomeric complex (Vitlin Gruber et al. 2014). Interestingly, *C. reinhardtii* has three co-chaperonins, Cpn10, Cpn20, and Cpn23, that are individually non-functional (Tsai et al. 2012). They are also structurally different, and the sequence encoding the roof-like β -hairpin in the co-chaperonin complex is absent, though Cpn10 and Cpn23 maintain this sequence (Zhao et al. 2019). In studies using recombinant co-chaperonins of

A. thaliana and *C. reinhardtii*, hetero-oligomeric ring complexes formed by combinations of Cpn10, Cpn20, and Cpn23 were able to serve as co-chaperonins, in order to perhaps modify the chaperonin folding cage for specific client proteins (Tsai et al. 2012). The symmetrical match of Cpn60, with sevenfold symmetry, to the chloroplast co-chaperonin, may be solved by forming hetero-oligomers of Cpn20 and Cpn10 or by splitting the Cpn20 (Tsai et al. 2012). In fact, a symmetrical match is not an absolute requirement for chaperonin function, and the flexibility and plasticity of this interaction were demonstrated by forming concatamers of six to eight covalently linked 10 kDa domains and three to four covalently linked Cpn20 subunits, which could help the chaperonin to refold a denatured protein in vitro (Guo et al. 2015). A previous study determined that a minimum of four active GroES subunits are necessary to contact GroEL for the formation of a stable GroEL/ES complex, whilst five subunits allow for an active complex that can fold proteins (Nojima et al. 2008). Despite the fact that human mitochondrial Cpn60 can bind *A. thaliana* Cpn20, it does not lead to productive protein folding, demonstrating different modes of binding of co-chaperonins to chaperonins, some of which are not functional (Bonshtien et al. 2009). The high-resolution structure of *C. reinhardtii* hetero-oligomeric Cpn60, in complex with hetero-oligomeric chloroplast Cpn10, revealed that the overall structure was similar to that of GroEL/ES but with an uneven spread of roof-forming domains in the co-chaperonin and possible varied surface properties of the chaperonin that may enable the system to fold specific substrates (Zhao et al. 2019).

Just as Hsp10 may have many other roles in mammalian cells, it seems that Cpn20 may have many additional roles in plants. Analysis of the stromal proteome of *A. thaliana* chloroplasts indicates that the steady-state levels of Cpn20 exceed those required to function with Cpn60, implying that there may be further roles for Cpn20 (Peltier et al. 2006). Additional roles have been revealed in *A. thaliana*, including the identity of Cpn20 as a negative regulator of abscisic signalling (Zhang et al. 2013) and as a mediator of iron superoxide dismutase activity in chloroplasts (Kuo et al. 2013).

Whilst our understanding of the roles of HSPE in disease continues to receive research attention, little is known about the roles of its homologues in virulence and pathogenicity of protozoan parasites affecting human health, and they may interact with the human chaperone system. The first protozoan CPN10 protein characterised was from *Leishmania donovani* and was shown to interact with CPN60.2 with increased concentrations detected during the amastigote stage of the life cycle (Zamora-Veyl et al. 2005). Cpn20 proteins were known to exist only in chloroplasts; however, sequencing of the malarial genome revealed a single Cpn20 protein which correlates with the algal origin of the apicoplast (Janouskovec et al. 2010; Sato and Wilson 2005). Since the *P. falciparum* genome encodes only one *cpn20* gene, it functions as a homo-oligomeric co-chaperonin that can functionally replace GroES (Vitlin Gruber et al. 2013b). Characterisation of HSP10 from *Strongyloides ratti*, an intestinal nematode infecting humans, revealed a strong immunogenic response, and the inability to bind to *S. ratti* HSP60 provided evidence of a role in host-parasite interactions (Tazir et al. 2009).

The structure of HSPE has been solved, and mutations in the first and last β -strands altered both the oligomeric and folded states (Guidry et al. 2003). In contrast to HSPD, HSPE stimulates the production of anti-inflammatory cytokines and exerts immunosuppressive activity (Johnson et al. 2005). One of the first extracellular heat shock proteins to be isolated was a circulating immunosuppressive protein, termed early pregnancy factor (EPF), which was later identified as HSPE after the isolation and demonstration of its role as a co-chaperonin for Hsp60 (Cavanagh and Morton 1994; Morton et al. 1977). The isolation of EPF was also the first evidence that heat shock proteins could function as cell signalling agonists (Morton et al. 1977). EPF appeared in the maternal serum within 24 hours after fertilisation in some mammals and has been found to exhibit growth factor qualities and anti-inflammatory properties essential for protecting the embryo from the mother's own immune system (Athanasas-Platsis et al. 2004; Morton et al. 1977; Quinn et al. 1990). The relationship between HSPE and EPF is discussed in a review by (Corrao et al. 2010). Recombinant HSPE has been used for the treatment of rheumatoid arthritis (Vanags et al. 2006) and multiple sclerosis (Broadley et al. 2009). HSP10 is essential for the regulation of histone transcription and cell proliferation (Ling Zheng et al. 2015). Selective overexpression of HSP10 in metastatic lymph nodes suggest that it acts autonomously from HSP60 (Cappello et al. 2005). Elevated levels of Hsp10 correlate with poor prognosis in oral squamous cell carcinoma (Feng et al. 2017). Extracellular Hsp10 influences endothelial cell differentiation (Dobocan et al. 2009). There is growing evidence to suggest that extracellular Hsp10 plays an active role in cell signalling (David et al. 2013).

Conclusion

Despite the fact that HSPD/E can replace GroEL/ES, continued research has shown them to be mechanistically different. Divergence from the *E. coli* archetype is also apparent in chloroplasts and other bacteria. Defective chaperonins cause chaperonopathies. However, both wild-type and mutant Hsp60 are associated with a number of disease affecting human health, and the search will continue for small molecules that can modulate the activity of Hsp60 as therapeutic strategies. Research on the influence of the cellular environment on the GroEL/ES folding machine and factors affecting the rate of protein folding will continue to enhance our understanding of this system. The moonlighting functions of bacterial chaperonins and co-chaperonins will continue to evolve. The structural states of Hsp10, including mixed oligomeric or fragmented, appear to influence the function as well as location. Hsp10 often functions as an antagonist to Hsp60 and possibly other molecular chaperones. Further knowledge of the extracellular functions of Hsp10, including secretion pathways and cell signalling, will definitely be of benefit in the development of treatments for cancer and autoimmune diseases related to this protein.

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