ANNUAL REVIEWS

Annual Review of Biophysics

Chaperonin Mechanisms: Multiple and (Mis)Understood?

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Annu. Rev. Biophys. 2022. 51:115–33

First published as a Review in Advance on January 4, 2022

The *Annual Review of Biophysics* is online at biophys.annualreviews.org

https://doi.org/10.1146/annurev-biophys-082521-113418

Keywords

chaperonins, molecular chaperones, protein folding, allostery, molecular recognition

Abstract

The chaperonins are ubiquitous and essential nanomachines that assist in protein folding in an ATP-driven manner. They consist of two back-to-back stacked oligomeric rings with cavities in which protein (un)folding can take place in a shielding environment. This review focuses on GroEL from *Escherichia coli* and the eukaryotic chaperonin-containing t-complex polypeptide 1, which differ considerably in their reaction mechanisms despite sharing a similar overall architecture. Although chaperonins feature in many current biochemistry textbooks after being studied intensively for more than three decades, key aspects of their reaction mechanisms remain under debate and are discussed in this review. In particular, it is unclear whether a universal reaction mechanism operates for all substrates and whether it is passive, i.e., aggregation is prevented but the folding pathway is unaltered, or active. It is also unclear how chaperonin clients are distinguished from nonclients and what are the precise roles of the cofactors with which chaperonins interact.

Contents

1. INTRODUCTION

Chaperonins are large oligomeric assemblies that assist in protein folding in vitro and in vivo in an ATP-dependent manner (40, 46, 101, 116, 130). They have been a source of fascination for over three decades owing to their machine-like properties and as systems whose understanding has required a convergence of studies of protein folding, allostery, and molecular recognition, together with cellular mechanisms of proteostasis and evolution. The chaperonins can be divided into two groups: group I, which is found in prokaryotes, mitochondria, and chloroplasts, and group II, which is found in the eukaryotic cytosol and archaea. This review focuses on open questions regarding the mechanisms of GroEL and the chaperonin-containing t-complex polypeptide 1 (CCT; also known as TRiC or, less frequently, TCP-1), which are the best-studied members of groups I and II, respectively, and have become paradigms for molecular machines.

Group I chaperonins consist of two back-to-back stacked identical (as in GroEL from *Escherichia coli*) or nonidentical (as in chloroplast chaperonins) homo-heptameric rings (**Figure 1*a–c***). The two rings can dissociate into single rings during their reaction cycle, as was reported for the human mitochondrial Hsp60 (34, 83) and, more recently, for GroEL from *E. coli* (135). Group I chaperonins function together with a homo-heptameric ring-shaped cochaperonin, which is GroES in *E. coli* (47). GroES binds to GroEL in an ATP-dependent manner to form either 1:1 GroEL–GroES bullet-shaped or 1:2 GroEL–$GroES_2$ football-shaped complexes (for reviews, see 87, 112) (**Figure 1*c***). The binding of GroES to GroEL generates a compartment, sometimes termed an Anfinsen cage (25), in which proteins can fold (or unfold) in isolation from bulk solution.

Group II chaperonins consist of two identical eight- or nine-membered rings. The octameric versions can be made up of one, two, or eight types of different but homologous subunits, as in the cases of the archaeal group II chaperonins from *Methanococcus maripaludis* (91) and *Thermoplasma acidophilum* [i.e., the thermosome (23)] and CCT, respectively (**Figure 1*d–f***). The chaperonin CCT, the most complex of all, is found in the eukaryotic cytosol (53) and contains eight different subunits that are arranged in a fixed order around the ring (55, 64). Group II chaperonins contain a built-in lid formed by a helical protrusion (59) (**Figure 1*d***) found in the apical domains of their subunits instead of the detachable lid provided by the GroES-like cochaperonin, which they lack.

The subunit architectures of group I and II members are similar. Each subunit contains apical, intermediate, and equatorial domains (**Figure 1*a,d***). The apical domains are involved in binding of polypeptide substrates and the cochaperonin (in group I) and contain the helical extension (in

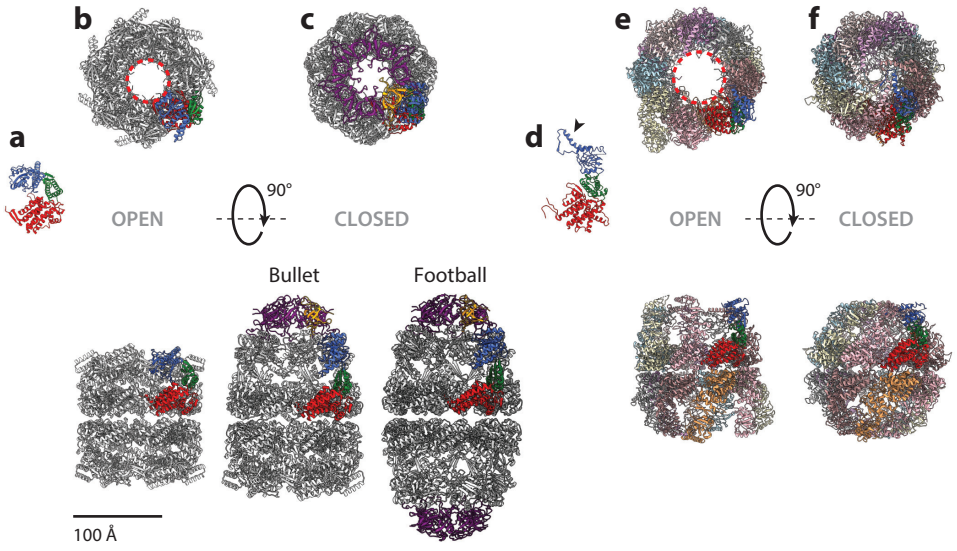

Figure 1

Chaperonin structures. (*a*) The atomic structure of a GroEL monomer showing the equatorial (*red*), apical (*blue*), and intermediate (*green*) domains. (*b*,*c*) Two orthogonal views of the atomic structure of GroEL in (*b*) its apo form and (*c*) its bullet- or football-shaped states, the latter formed by binding of GroES (*purple* except for one of its subunits in *orange*) to one (asymmetric form; bullet) or both (symmetric form; football) rings. The atomic structures correspond to GroEL [Protein Data Bank (PDB) ID 1XCK], the GroEL–GroES complex (PDB ID 1AON), and the GroEL–$GroES_2$ complex (PDB ID 4PKO). The red dotted circle in panel *b* marks the surface formed by the apical domains that is responsible for interactions with protein substrates. (*d*) The atomic structure of one of the chaperonin-containing t-complex polypeptide 1 (CCT) subunits (CCT6; PDB ID 6QB8). The three domains are colored as in panel *a*. The black arrowhead indicates the helical extension responsible for closure of the cavity (see panel *f*). (*e*,*f*) Two orthogonal views of the atomic structure of the CCT oligomer (*e*) in its open conformation (PDB ID 6QB8) and (*f*) in its closed conformation (PDB ID 4V8R). The eight different subunits are in different colors. The red dotted circle in panel *e* marks the surface formed by the apical domains that is responsible for part of the interactions with protein substrates. Scale bar = 100 Å for panels *b*, *c*, *e*, and *f*.

group II). The intermediate domains connect the apical and equatorial domains and are involved in intraring communication and control of the ATPase activity. The equatorial domains contain the ATP binding site and are involved in the ring–ring interactions, which differ in the two groups of chaperonins. In members of group II, the back-to-back stacking of the two rings is in register such that each subunit in one ring is in contact with only one subunit in the opposite ring. In group I, however, one ring is rotated relative to the other so that the stacking is out of register, and each subunit in one ring is in contact with two subunits in the other ring.

Most members of groups I and II display nested allostery, which involves positive intraring and negative inter-ring cooperativity in ATP binding with respect to ATP (37, 103). In the case of GroEL, it was proposed (141) that the intraring positive cooperativity in ATP binding is due to a concerted Monod-Wyman-Changeux (MWC)-type (79) switch, whereas the inter-ring negative cooperativity follows the sequential Koshland-Némethy-Filmer (KNF) model (63). The concerted nature of GroEL's intraring transition, which was confirmed in later work (24), has been attributed to steric hindrance (71) and key salt bridges (48), in particular, those between Arg197 and Glu386 in adjacent subunits (140) and between Asp83 and Lys327 within each subunit.

Indeed, removal of both of these salt bridges by mutation was shown to reduce substantially the intraring cooperativity in GroEL (26). These mutations increase the flexibility of the apical domains of GroEL in a manner reminiscent of CCT, which, unlike GroEL, displays conformational heterogeneity in its apo state and undergoes sequential intraring allostery in ATP binding and hydrolysis (98).

Given that protein substrates bind to specific subunits of CCT, as has been shown, for example, for actin (5, 68), via some yet-to-be-determined combinatorial code, it follows that sequential allostery can lead to an ordered domain-by-domain release of substrates and, thus, to more efficient folding. Such a release mechanism has been demonstrated in the case of the Asp155→Ala GroEL mutant with sequential intraring allostery (58, 88) but has not yet been tested for CCT. One focus of this review is to consider our current understanding of substrate recognition and selection mechanisms of GroEL and CCT. We also discuss the controversies regarding the mechanisms by which GroEL assists in folding and the role of the football-shaped species in this process (**Figure 2**). Finally, the functional roles of the cofactors and dynamics of CCT are discussed in more detail.

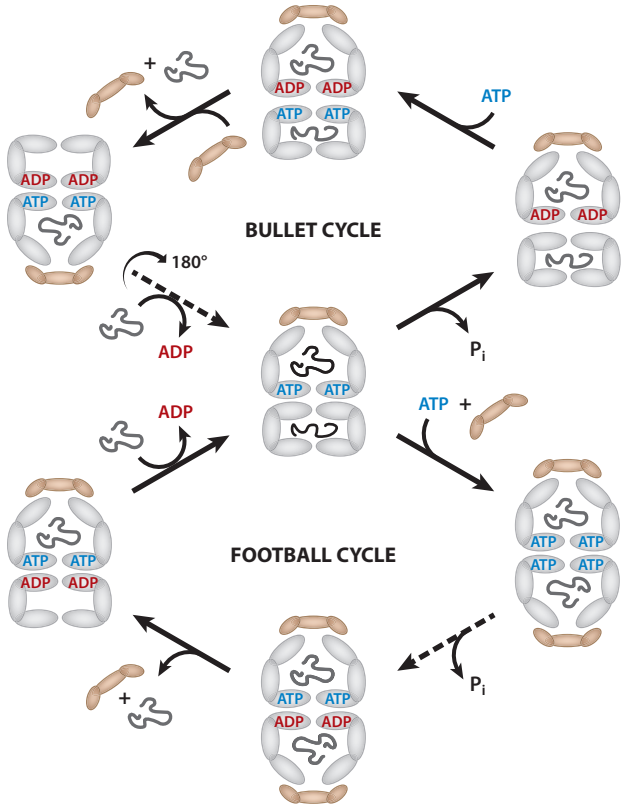

Figure 2

The reaction cycle of GroEL. Both the bullet-shaped GroEL–GroES and the football-shaped GroEL–$GroES_2$ complexes can assist in protein folding via different reaction cycles. In the reaction cycle of the bullet form, the rate-determining step (*dashed arrow*) is ADP departure from the *trans* ring opposite GroES. In the reaction cycle of the football form, the rate-determining step is ATP hydrolysis in one of the GroES-bound rings. The change in the rate-determining step results in different encapsulation times in the bullet (approximately 10 s) and football (approximately 1 s) forms.

2. THE INTERACTOMES OF GroEL AND CCT

The interactome of GroEL has been established with some confidence (31, 57), although the factors that distinguish GroEL substrates from all other *E. coli* proteins are still not fully clear. By contrast, there is still little agreement regarding the identity of CCT's substrates in vivo (22, 36, 134), and it remains controversial whether its substrate specificity is narrow or broad. Additional complexities in the case of CCT are its interactions with multiple cofactors and a potential combinatorial code for substrate binding according to which different substrates bind to different combinations of subunits (36). Advances and open questions regarding the interaction networks of GroEL and CCT are discussed, in turn, below.

2.1. GroEL Substrates

An early study showed that approximately 50% of the soluble proteins of *E. coli* in their unfolded state can bind to GroEL (123). Back-of-the-envelope calculations indicated, however, that the amount of GroEL and GroES in an *E. coli* cell under normal conditions is sufficient to assist in the folding of only approximately 5% of its proteins (69). Consequently, it was clear already early on that structural or sequence motifs that facilitate substrate binding to GroEL do not determine its selectivity. Such binding motifs include hydrophobic and positively charged residues (50); GroES mobile loop–like sequences (given that the mobile loops of GroES compete with polypeptide substrates for binding to the cleft between helices H and I in the apical domains of GroEL) (107); and size, which cannot exceed approximately 60 kDa (132) if encapsulation is required. The identity of the proteins that actually interact with GroEL in vivo was determined by isolating GroEL–GroES complexes from *E. coli* cells that were lysed in the presence of glucose and hexokinase. This enzyme rapidly converts ATP to ADP, thereby preventing GroES cycling and ensuring that substrate encapsulation occurred in vivo and not during cell lysis. Mass spectrometry analysis of the isolated complexes led to identification of approximately 250 proteins that interact with GroEL in vivo under normal growth conditions (57). Subsequent work based on the findings of Kerner et al. (57) led to the identification of a set of 57 proteins that are obligate substrates (31), to which another 20 proteins were later added (84). The set of GroEL interactors has a small but significant overlap (4) with the set of proteins found to aggregate in the presence of a temperature-sensitive variant of GroEL under nonpermissive conditions (15).

Attempts to understand what distinguishes the set of obligate substrates from other *E. coli* proteins led to the observation that this set is enriched in proteins with the $(\beta\alpha)_8$ TIM barrel fold (57). Approximately 44% of GroEL's obligate substrates share the TIM barrel fold, compared to only approximately 7% of all *E. coli* lysate proteins with an identifiable structure. Nevertheless, many *E. coli* proteins with a TIM barrel fold are not substrates. DapA and YagE, for example, are both TIM barrels with sequences that are 27% identical and 46% similar, but the former is an obligate substrate, whereas the latter is not a client. It is clear, however, that GroEL does not recognize the native states of substrates (117), but rather, that native states contain information about folding pathways and intermediates whose properties do affect the dependence on GroEL for efficient folding. Contact order, for example, which is calculated from the native structure (92), correlates with folding rate, and high contact order was found to increase the dependence on GroEL of GFP folding (7). Another example is frustration, i.e., when not all interactions in the native state are optimized energetically (28), which was also found in computational (82) and experimental (6) studies to increase the folding dependence on GroEL. Both high contact order and frustration slow folding and shift the kinetic partitioning between folding to the native state and misfolding in favor of the latter, thereby increasing GroEL dependence in vivo and in vitro. Consequently, substrate proteins are also more aggregation prone than are nonclients, as has been found

experimentally (85) and computationally (115). Folding rates in vivo are, however, determined not only by global and local protein structural features, such as contact order and frustration, respectively, but also by cellular factors such as those that affect translation rates. Computational and experimental studies indeed found that faster translation rates inferred from codon usage increase GroEL (86) and DnaK (as well as GroEL, but not significantly) (95) dependence, respectively. Despite the above-mentioned recent progress in identifying features that determine GroEL dependence, the relevance of these features for other systems remains to be demonstrated. It is clear that additional features (e.g., chain length, properties of the unfolded state) will need to be tested and incorporated to correctly predict a protein's folding dependence on GroEL from its sequence and/or structure.

2.2. The CCT Interactome

Several studies have been carried out to determine the CCT interactome (22, 36, 99, 134). Although these studies have shown that CCT interacts with a large set of proteins, it is clear that it does not assist in the folding of all of them. In many cases, CCT associates with folded proteins for other purposes, such as control of quaternary interactions, regulation, and protection from protein degradation. It has also become evident that, unlike GroEL, CCT is helped in all of these functions by other chaperones or cochaperones, with which it interacts transiently.

The major role of CCT seems to be to assist in the final steps of folding of cytoskeletal proteins, which have an important function in processes such as morphogenesis, cell polarity, and mitosis (99). Actin and tubulin are the major CCT clients (18, 126), but the eukaryotic chaperonin also interacts with other cytoskeletal and cytoskeletal-associated proteins like the actin-related proteins (ARPs) (78), myosin II heavy chain (HMM) (106), cofilin (77), and gelsolin, although in the latter case, CCT does not have a folding assistance role (11, 111). It is important to point out that, through its action upon these proteins, CCT has been shown to control centriole orientation and polarization of the tubulin dynamics induced by the T cell receptor in T lymphocytes that form an immune synapse (75).

The above-mentioned clients are all involved in cytoskeleton formation and control but have no structural similarities. There is, however, a group of CCT substrates that have a similar structure, and these substrates make up a large subset of WD40-repeat proteins (122, 131). Proteins in this group typically contain seven copies of an approximately 40-residue, degenerate motif that usually starts with Gly-His and ends with Trp-Asp (and is thus termed WD) (104). These WD40 repeats fold into four β-stranded domains, and each of these forms one of the blades of the propeller structure (**Figure 3*a***). These propellers usually have a scaffolding function, and the proteins that they form are involved in important cellular processes such as cell cycle control and protein degradation. Not all WD40 proteins require CCT for their folding, which clearly indicates the existence of a specific interaction mechanism between CCT and its clients. Among the WD40 CCT-interacting proteins are (*a*) Cdc20 and Cdh1, both of which are involved in cell cycle control through activation of the anaphase-promoting complex (12, 90); (*b*) the Gβ subunit of the transducing complex (70) (**Figure 4*a***); and (*c*) mLST8 (**Figure 4*b***) and Raptor, which are core subunits of the mTOR complexes (19). Other WD40 proteins shown to interact with CCT include TLE2 (90) and Cdc55, Cdc4, Tad5, and Vid27 (131).

It is also important to point out that a fair number of the WD40 CCT-associating proteins and other CCT clients have molecular masses that exceed the estimated mass that can be enclosed in the CCT cavity (60–80 kDa); examples include Cdc20, Cdh1 (12), and gelsolin (11). This suggests that the cytosolic chaperonin has roles other than folding assistance, such as regulating the activities of clients by controlling their post-translational modifications (121) and liberation from or association with other proteins.

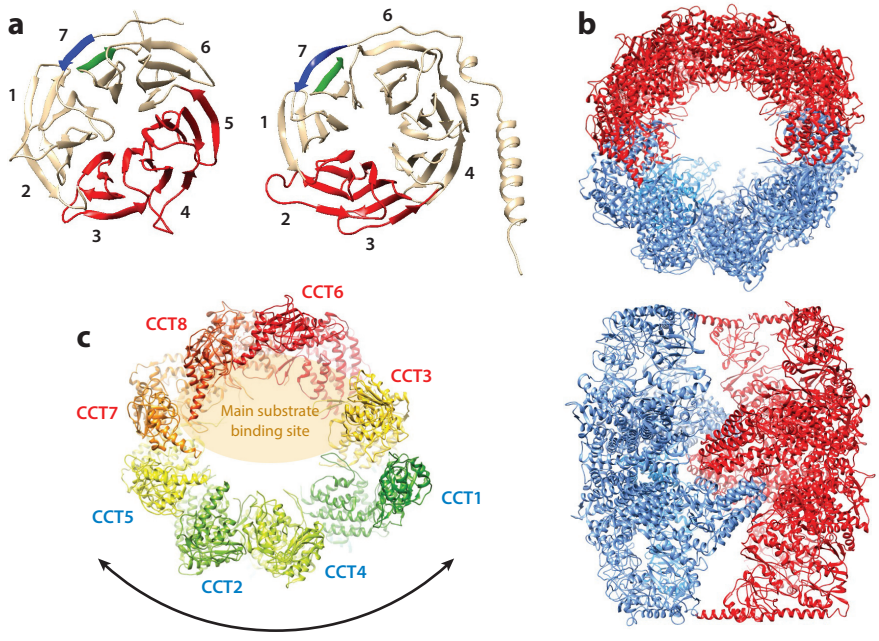

Figure 3

Structural features of chaperonin-containing t-complex polypeptide 1 (CCT)–client interactions. (*a*) Atomic structures of Cdc20 [*left*; Protein Data Bank (PDB) ID 4GGA] and Gβ-transducin (*right*; PDB ID 1TBG). Blade 7 is the one in which the propeller is circularized and stabilized, which occurs because of the interaction of the last β-strand of the C terminus (*blue strand*) with the first of the N terminus (*green*). These two WD40 proteins are assisted in their folding by CCT, and the red propellers are the CCT-interacting regions [Cdc20 (12) and Gβ (73)]. The Gβ protein interacts with CCT3 and CCT6 (93). (*b*) Two orthogonal views of the atomic structure of CCT (PDB ID 6QB8) showing the subunits belonging to the CCT5/2/4/1 pole (*blue*) and the CCT3/6/8/7 pole (*red*). (*c*) A model of mechanical CCT-assisted folding in which the main role of the CCT3/6/8/7 side is client protein recognition and binding, and the conformational changes that take place in the CCT5/2/4/1 side after ATP binding and hydrolysis act upon the client protein.

Proteins with other structures that are assisted in their folding by CCT are viral proteins such as the Epstein-Barr virus-encoded nuclear protein (EBNA-3) (56), the type D retrovirus Gag polyprotein (44), the hepatitis B virus capsid protein (66), and the reovirus capsid protein (60). All of these viral proteins are opportunistic proteins that have parasitized the functional cycle of CCT to solve their specific folding problems.

A unique feature of CCT among the chaperonins is the large number of chaperones and cochaperones with which it collaborates in its various functions [the type II thermosome also has a partner, the cochaperone prefoldin (PFD)]. Several of these cochaperones function by transferring their client proteins to CCT (pre-CCT cochaperones), while others interact with CCT after it has bound its client (post-CCT chaperones). Among the first type are Hsp70, an important chaperone on its own, whose collaboration with CCT increases folding efficiency (20) (**Figure 4*c***), and the cochaperone PFD, which delivers proteins like actin, tubulin, and VHL to CCT (3, 27, 74) (**Figure 4*e***). The post-CCT cochaperones include phosducin-like proteins (PhLPs), which assist CCT in the folding of Gβ-transducin (in the case of PhLP1) (73, 76) (**Figure 4*d***), as well as actin and tubulin (by PhLP2/3) (108) and TBCB, which, together with the other tubulin cofactors (TBCA-E), assists in the formation of the αβ-tubulin heterodimer (13).

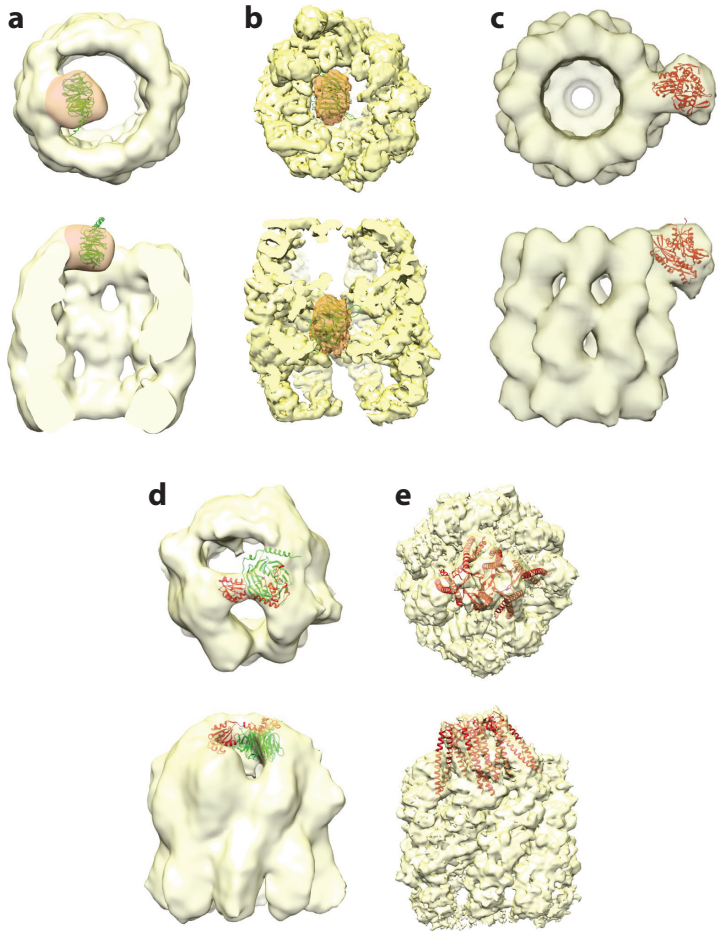

Figure 4

Cryo-electron microscopy (cryo-EM) 3D reconstructions of complexes between chaperonin-containing t-complex polypeptide 1 (CCT) and different client proteins and cochaperones. Shown are two orthogonal views (*top*, *end-on view* and *bottom*, *side view*) of (*a*) CCT and the client protein Gβ-transducin (93), (*b*) CCT and the client protein mLST8 (19), (*c*) CCT and the pre-CCT cochaperone Hsp70 (20), (*d*) CCT and the post-CCT cochaperone PhLP1 (*red*) and the client protein Gβ-transducin (*green*) (93), and (*e*) CCT and the pre-CCT cochaperone prefoldin (33). The higher resolution of the 3D reconstructions shown in panels *b* and *e* have to do with the fact that they were generated after the resolution revolution that has taken place in cryo-EM.

Despite the increasing number of structural and biochemical studies in which CCT clients have been identified, our understanding of the CCT interactome lags far behind that of the GroEL interactome. It remains unclear which CCT interactors are obligate substrates and what are the biophysical factors that render certain proteins dependent on CCT for folding and/or function.

3. GroEL MECHANISM OF ACTION

Numerous studies during the past three decades have been devoted to deciphering GroEL's mechanism of action, but there is no consensus yet with regard to several of its key aspects. One reason

for this impasse is the complexity of this machine, i.e., that GroEL-mediated folding can involve many parallel and sequential kinetic steps during which GroEL can cycle between multiple conformational and ligand-bound states. A second reason is that aspects of the reaction mechanism can vary depending on the substrate identity and experimental conditions. Our goal in this section is, therefore, not to present a definitive mechanism, which would be premature, but rather to discuss some key issues that need to be resolved.

3.1. Bullets Versus Footballs

GroES can bind to GroEL either at one or at both ends, thereby forming bullet- and football-shaped complexes, respectively (**Figures 1** and **2**). Both types of complexes have been observed in structural studies since the early 1990s (112), and their existence is, therefore, not in dispute. The main controversy has centered on which of these species is the main functional form in the reaction cycle. This is an important issue because it has bearing on the residence time of substrates in the GroEL cavity, which is approximately 10–15 s for the bullets (129) and approximately 1 s (137) for the footballs. A 10-fold change in residence time can have a big impact on the substrate folding reaction. The difference in residence times is due to the fact that the rate of GroES departure from the substrate-containing so-called *cis* ring in the bullet form is determined by the slow rate of ADP release from the *trans* ring, whereas, in the case of the substrate-containing football form, it is determined by the rate of ATP hydrolysis (137) (**Figure 2**). This change in the rate-determining step for GroES release occurs because substrate proteins accelerate ADP release from the *trans* ring, which is an effect that seems to be mimicked by the Glu257→Ala mutation in the apical domain (21).

A corollary of the substrate-promoted change in the rate-determining step for GroES release is that football formation is favored in the presence of substrates, thereby suggesting that this species is the main functional form (136). It is also clear that high [ATP]/[ADP] ratios, which are employed in most experiments and are thought to exist in vivo, also favor football formation. The in vivo ATP concentration can, however, vary greatly even within a single population of *E. coli* cells, as indicated by a study where it was found to be 1.54 ± 1.22 mM (133). Moreover, the [ATP]/[ADP] ratio of *E. coli* cells can decrease dramatically from a value of about 10 to 0.3 under some stress conditions (52). Such a change in the [ATP]/[ADP] ratio would favor formation of bullets over footballs. In addition to the above-mentioned kinetic effects, bullet formation is also favored at relatively low ATP concentrations owing to inter-ring negative cooperativity with respect to ATP binding (141), which is conserved in many group I and II chaperonins. Taken together, all of these considerations suggest that both bullets and footballs can serve as functional forms depending on the conditions and substrates (**Figure 2**).

Given the different residence times provided by bullets and footballs, it was speculated that these two species may cater to substrates with different folding properties. In particular, Bigman & Horovitz (10) hypothesized that slow folders might benefit more from the 10-fold-longer encapsulation time in the bullet form than in the football form. The folding rates of all of the obligate substrates (31, 57) were, therefore, calculated based on their secondary structures and lengths (51). Strikingly, a bimodal distribution of folding times was observed with two maxima that match very closely the residence times in bullets and footballs (10). It is important to note that all substrates, regardless of their folding rates, can benefit from the annealing function of GroEL (118), which is discussed below, regardless of the residence time. It should also be mentioned that the folding of substrates too large to be encapsulated in the cavity can also be assisted by bullets via a mechanism that involves GroES binding to the *trans* ring (17, 49).

3.2. Impact of GroEL on Folding Kinetics and Thermodynamics

Another major focus of controversy in the field has concerned whether GroEL is just a passive container that prevents protein substrate aggregation but does not affect the folding process itself (43, 45, 120) or an active device that alters folding in some manner. Two seemingly opposite kinetic effects have been attributed to GroEL in support of its active mechanism: unfolding (or unfoldase) activity and acceleration of folding. One obvious reason for GroEL's unfolding activity is that its apo state has a higher affinity for nonfolded over folded states of substrate proteins, since the cavity walls in the apo state are hydrophobic (132). Consequently, the equilibrium of substrates, in the presence of apo GroEL, will be shifted in favor of their nonfolded states (65, 94, 142). This process can account for the chaperoning effects of GroEL minichaperones (16), which lack a cavity and ATPase activity. Unfoldase activity implies, however, that this action of GroEL is enzyme-like and involves the lowering of an activation barrier. A shift in equilibrium toward nonfolded states owing to conformational selection of nonfolded states by apo GroEL does not indicate, however, a change in the activation barrier. The terminology of unfoldase activity in connection with the action of apo GroEL is, therefore, misleading if unfolding by GroEL takes place only via conformational selection.

Substrate proteins can also undergo unfolding when in complex with GroEL due to a stretching force applied to them upon ATP and GroES binding–promoted conformational changes (127). This mechanism, which is somewhat more akin to induced-fit as opposed to conformational selection, can involve a change in barrier height, although this has not been reported. ATP- and GroES-promoted forced unfolding is a key aspect of the iterative annealing mechanism of action proposed for GroEL (117, 118). According to this mechanism, each reaction cycle of GroEL involves kinetic partitioning of protein substrates between productive folding to the native state and misfolding. The misfolded protein substrates that remain are then rebound to GroEL and (partially) unfolded again, thereby giving them further opportunity to fold correctly. The iterative annealing mechanism can lead to enhanced folding yields and rates (117).

Possible acceleration of protein folding has also been attributed in experimental and theoretical studies to different features of the cavity, such as its confining nature (9, 114), its negatively charged walls (39, 114), and the presence of C-terminal tails with the Gly-Gly-Met motif (114, 128). It has been difficult, however, to determine whether the origin of the acceleration effect is due to prevention of aggregation (96, 120), iterative annealing (127), encapsulation in the cavity, or some combination of these factors. This difficulty has been due, in part, to the fact that substrate proteins can escape into bulk solution during the course of experiments owing to GroES cycling, or even when GroES is bound (80). Given that GroES release from the bullet form is triggered by ATP binding to the opposite (*trans*) ring after ATP hydrolysis in the *cis* ring (100), strategies employed to ensure that the substrate does not escape have included encapsulation in the cavity formed by GroES in complex with single-ring GroEL (43) or the ATP hydrolysis–defective GroEL mutant Asp398→Ala (138). Recent experiments (62) showed that encapsulated GFP, for example, escapes from the cage formed by single-ring GroEL in complex with GroES but not from the cavities of the ADP·BeF_x-stabilized football complex (113).

Encapsulation of a protein substrate in the ADP·BeF_x-stabilized football cage was recently shown to reduce the substrate's thermodynamic stability dramatically by more than 5 kcal/mol compared to that in bulk solution, even though the protein is not interacting with the cavity walls, as is indicated by its unhindered mobility (62). It was also recently shown that folding of rhodanese is retarded to the same extent upon encapsulation in the ADP·AlF_x-stabilized complex of single-ring GroEL with GroES and in the presence of the cycling GroE system (61). These results are in accord with the iterative annealing mechanism but are otherwise surprising given that

confinement and the negatively charged cavity walls are expected to be stabilizing. One possible explanation for these findings is that the cavity-confined water is ordered, and the hydrophobic effect is, therefore, diminished. Evidence for increased ordering of water near the bottom (72) but not the top (30) of the GroEL cavity has, in fact, been obtained recently. Regardless, it is important to point out that the magnitude (and possibly direction) of the effect of encapsulation on stability may vary depending on the protein substrate's size and other properties.

4. CCT MECHANISM OF RECOGNITION AND ACTION

The fact that CCT rings are composed of eight different subunits (compared to one in the case of GroEL or 1–3 in the case of the thermosomes) suggests a more complex mechanism governing how clients are recognized and acted upon. The eight CCT subunits bear charged and hydrophobic residues in their apical domains, the canonical substrate binding sites (35, 105). This observation, together with biochemical and structural studies (5, 93, 119), suggests that CCT–client interactions are mediated by contacts between particular apical domain residues in specific CCT subunits and specific domains of the client protein that have acquired a certain conformation before interacting with CCT (89) (**Figure 4*a***). However, other evidence indicates that the equatorial domains are also involved in client interactions. In the case of tubulin, for example, the crystal structure of its complex with CCT shows that it interacts with the so-called sensor loop, a region of the equatorial domain near the ATP binding pocket (81). This sensor loop is near the disordered N and C termini, which have been suggested to be involved in client interactions of chaperonins other than CCT (8, 42, 110). Indeed, a high-resolution cryo-electron microscopy (cryo-EM) reconstruction of the complex between CCT and the WD40 protein mLST8 shows that this client protein interacts with the N and C termini of specific CCT subunits (19). It is not known at this stage if different proteins use different CCT-interacting regions or if these correspond to different stages of the interaction between CCT and the client protein.

Several studies have determined that each CCT ring is divided into two poles (1, 14, 32, 97) (**Figure 3*b***). One pole corresponds to a group of adjacent subunits (CCT5/2/4/1) that are less tolerant to mutations in their ATP binding sites and have an apparent high affinity for ATP, and the second pole (CCT3/6/8/7) corresponds to subunits with less mutational sensitivity and a lower affinity for ATP. Identification of the bipolar nature of CCT rings has left open the question regarding the pathway(s) of the allosteric switch. It remains unclear whether the pathway has a defined starting point and direction or if it starts in different subunits and then proceeds in both clockwise and counterclockwise directions around the ring (**Figure 3*c***). It also remains unclear whether the pathways of conformational change triggered by ATP binding and hydrolysis are the same and whether they can be influenced by bound polypeptide substrates and/or cofactors. An Arrhenius analysis of CCT's ATPase activity, together with analysis of its subunit interface areas, indicated (38) that the conformational wave associated with hydrolysis starts at CCT3/6/8 and proceeds in both clockwise and counterclockwise directions, a suggestion that seems to be supported by recent single-particle cryo-EM data (53). An application of elastic network models to cryo-EM density maps also revealed parallel pathways but suggested that they originate at the other pole (143). Clearly, more work will be needed to further test these models and determine whether they also describe the pathways of ATP-promoted conformational waves in the presence of various other ligands.

Regardless of the pathway, most of the results obtained to date indicate that the CCT folding mechanism is an active, mechanical one in which the sequential conformational changes induced in the CCT subunits after ATP binding and hydrolysis act on the client protein and promote its correct folding (67, 109, 139). This model can be elaborated to take into account the bipolar structure

of CCT, according to which the more passive CCT3/6/8/7 side is involved mostly in the recognition and binding of client proteins, whereas the movements that take place at the CCT5/2/4/1 side act upon the bound proteins to help them reach their native conformation (**Figure 3*c***). A CCT active-folding model has been proposed for actin and tubulin (5, 67), and the progress in folding of the CCT-bound protein during the CCT functional cycle has been confirmed in fluorescence resonance energy transfer experiments using fluorophore-labeled actin bound to CCT (124, 125). This mechanical model of CCT-assisted folding could also be extended to the WD40 proteins. It can be observed that these β-propeller structures are stabilized by the formation of the last blade (usually the seventh) with three strands from the C terminus plus a fourth from the N terminus (**Figure 3*a***). In the case of the WD40 proteins, this stabilization (the closure of the velcro; 131) could be achieved by the sequential movements generated in the CCT cavity after ATP binding and hydrolysis. It is interesting to note that the two CCT-assisted WD40 proteins for which we have information on how they interact with CCT share a similar interaction pattern (**Figure 3*a***).

5. CONCLUDING REMARKS

Nature has devised folding nanomachines formed by oligomeric rings with cavities in which assisted folding can take place under shielding conditions. These oligomers have been termed chaperonins, and they usually work as two-ring structures that provide an extra element of control through allosteric signals. Although the overall general structure of chaperonins has been maintained during evolution, there has been diversification through two main pathways, which are exemplified in the two chaperonins discussed here, the group I chaperonin GroEL from *E. coli* and the group II CCT (or TRiC) found in the eukaryotic cytosol. Given the complexity of these machines, many questions pertaining to their different reaction mechanisms and substrate selectivities remain open, as we discuss above. There are additional issues, however, such as the mechanism by which these chaperonin complexes assemble, that we do not discuss, since they are not directly related to the folding function of the complexes. For example, it has been reported that not all CCT subunits are expressed in precisely the same stoichiometric amounts (29), but it is unclear whether this imbalance is related to CCT's assembly mechanism (102) or to possible folding-unrelated moonlighting roles of individual subunits (54). In the case of group I chaperonins, there is already mounting evidence regarding their moonlighting functions (41). These functions may be reflected in the coexistence in some bacteria of different chaperonins, some of which have structures other than an oligomeric ring. An example is GroEL1 from *Mycobacterium tuberculosis*, which can exist in different oligomeric forms, such as dimers, and appears to be involved in copper homeostasis (2). Therefore, in addition to the many questions concerning the mechanisms of client recognition and action of chaperonins, there is also much interesting and surprising biology with potential medical relevance waiting to be discovered.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Guillermo Montoya, Dave Thirumalai, and Keith Willison for critical reading and helpful comments. We also thank Sergio Pipaón for his help with preparing **Figure 1**. This work was supported by grants PID2019-105872GB-I00/AEI/10.13039/501100011033 (AEI/FEDER,

UE) from the Ministry of Science and Innovation and P2018/NMT-4389 from the Comunidad de Madrid (to J.M.V.). It was also supported by an Israel Science Foundation (842/20) grant and by the Minerva Foundation with funding from the Federal German Ministry for Education and Research (to A.H.). A.H. is an incumbent of the Carl and Dorothy Bennett Professorial Chair in Biochemistry. The Centro Nacional de Biotecnología (CNB) is a Severo Ochoa Center of Excellence (MINECO award SEV 2017-0712).

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