Review

Chaperonins: two rings for folding

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Chaperonins are ubiquitous chaperones found in Eubacteria, eukaryotic organelles (group I), Archaea and the eukaryotic cytosol (group II). They all share a common structure and a basic functional mechanism. Although a large amount of information has been gathered for the simpler group I, much less is known about group II chaperonins. Recent crystallographic and electron microscopy structures have provided new insights into the mechanism of these chaperonins and revealed important differences between group I and II chaperonins, mainly in the molecular rearrangements that take place during the functional cycle. These differences are evident for the most complex chaperonin, the eukaryotic cytosolic CCT, which highlights the uniqueness of this important molecular machine.

Chaperonins: a common structure with important functional differences

Chaperonins (see Glossary) are the most universal molecular chaperones because they are present in all kingdoms. They are formed by 60-kDa subunits [heat shock protein (Hsp)60 chaperones] that oligomerize (800–1000 kDa) in two rings placed back-to-back (Figure 1) [1]. Group I chaperonins – mainly found in eubacteria, mitochondria and chloroplasts – are built of two heptameric rings, but the structure is more complex for group II members, which include the archaeal chaperonins (named thermosomes) composed of octameric or nonameric rings built by one, two or three different subunits. The most complex group II chaperonin is however the eukaryotic cytosolic chaperonin containing TCP1 [CCT, also termed TRiC (TCP1 ring complex)] which is composed of eight different subunits (CCTα, β, γ, δ, ε, ζ, η and θ in mammals, CCT1–8 in yeast) organized in a unique intra- and inter-ring arrangement that is still a matter of debate [2–5]. The chaperonin monomers share a common domain structure (Figures 1 and 2a) [6,7]: an equatorial domain, which contains all the inter-ring contacts, most of the intra-ring contacts and the ATP-binding site, binding and hydrolysis of which trigger the conformational changes that take place during the functional cycle; an apical domain, which contains the substrate-binding region; and the intermediate domain, which links the other two domains. All chaperonins have an open substrate-receptive conformation, in which the unfolded protein is recognized and trapped, and a closed conformation, in which the substrate is isolated from the bulk of the intracellular environment. The transition between the open and closed states is induced on ATP binding and hydrolysis, which trigger a complex set of intra- and inter-ring allosteric signals (Figures 2 and 3) [8].

However, important differences, both structural and functional, exist between the two groups of chaperonins. In contrast to group I chaperonins, whose closure mechanism involves the formation of a transient complex with a co-chaperonin (Hsp10) that caps the central cavity (Figures 2c and 3a; Box 1), group II chaperonins have an extra helix (helical protrusion) located at the tip of the apical domain to close their central folding chamber (Figures 1 and 2a) [7,9]. Another important difference is the manner in which the two rings interact with each other (staggered in the case of group I chaperonins and in phase for group II chaperonins), which suggests different intra- and inter-ring signaling mechanisms for the two chaperonin groups (Figure 1). Although the past two decades have witnessed accumulation of structural and functional information for group I chaperonins, this has not been the case for the more complex group II. Current research leading to the solution of several structures has shed light on the structural rearrangements that group II chaperonins undergo during

Glossary

Allostery: in general terms, the conformational change in a protein as a consequence of binding of a compound to a place different from the active site. Three models have been applied to chaperonins. The MWC model is a concerted model of allosterism proposed in 1965 by Jacques Monod, Jeffries Wyman and Jean Pierre Changeux. It postulates that the subunits of a complex that follows this model are connected in such a manner that a conformational change in one subunit is necessarily conferred to all other subunits, forcing all the subunits to be in the same conformation. The KNF model was proposed by Daniel Koshland, George Némethy and David Filmer and holds that subunits are connected in such a manner that a conformational change in one does not necessarily induce a similar change in the others. The nested allosteric cooperativity model combines both the MWC and KNF models to explain the positive intra-ring and negative inter-ring cooperativity in chaperonins. This concept is particularly useful in the case of large oligomeric proteins with a hierarchical structure. The model supports the existence of an established order in the allosteric interactions among oligomers due to the hierarchical structural arrangement of the assembly.

Anfinsen cage: concept proposed by John Ellis whereby the closed conformation of a chaperonin cavity offers an appropriate environment for folding of a denatured protein according to the information encoded in its amino acid sequence.

Chaperonin: also termed Hsp60 chaperone. The family of molecular chaperones constituted by ~60-kDa monomers that form double-ring oligomers. Each ring encloses a cavity in which protein folding takes place.

Molecular chaperone: protein involved in assisting the folding of other proteins. A large percentage of these proteins are heat shock proteins (Hsp) and therefore are usually classified according to the molecular mass of their constituents (e.g. Hsp10, Hsp25, Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, and Hsp110).

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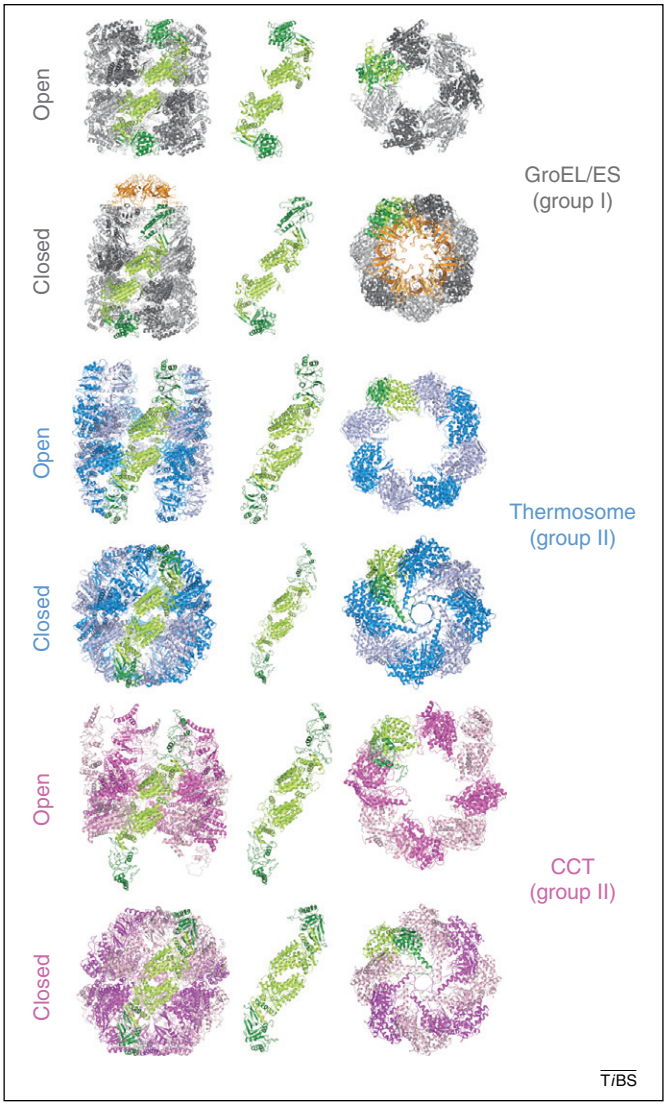

Figure 1. Cartoon representation of chaperonin structures. The two different views show the alternative conformations adopted by each chaperonin while open and closed, along with their general structural differences. The first column presents a side view of the structures, highlighting the geometry of a pair of subunits, one in each ring. A detailed view of this pair is depicted in the middle column. The equatorial and intermediate domains are in lime green and the apical domains in dark green. The third column depicts an end-on view illustrating tight packing when the chaperonin is in the closed state, thus trapping the substrate. The figures were generated with PyMol software (http://www.pymol.org/) from coordinates in PDB (GroEL/ES closed 1AON [9], GroEL open 3E76 [59], thermosome closed 1A6D [7], thermosome open (Cpn-Δlid) 3KFK [11], CCT closed 3IYG [3] and CCT open 2XSM [13]).

their functional cycle [3,10–14]. In this review we discuss these recent developments in an attempt to convey a comprehensive picture of the similarities and differences between these molecular machines. We address in detail the structure and function of the eukaryotic chaperonin CCT, a key complex that assists in the folding of diverse proteins, including the cytoskeletal building blocks actin and tubulin, among others.

Conformational changes in the chaperonin monomer during the functional cycle

Most structural studies focusing on group I chaperonins have been carried out using the *Escherichia coli* chaperonin GroEL (Figures 1 and 2). In the open nucleotide-free conformation, the tip of each apical domain faces the entrance of the cavity. On ATP binding, the intermediate domains rotate 25° toward the equatorial domains, which closes the ATP-binding pockets. In addition, the apical domains undergo a 30° counterclockwise rotation and elevation of 10° [15]. This movement facilitates docking with the co-chaperonin GroES [16], which induces additional elevation of 50° and a 120° clockwise rotation of the apical domain. These movements lead to enlargement of the chamber, which is sealed by GroES (Figures 2 and 3a) [9,17].

Until recently, only the atomic structures of the closed conformation of the *Thermoplasma acidophilum* and *Thermococcus* KS-1 thermosomes (Figure 1) [7,18] and some cryo-electron microscopy (cryoEM) models of several group II chaperonins were known [19–22]. The recent publication of several cryoEM and X-ray diffraction studies of the open and closed conformational states of *Bos taurus* CCT [3,13] and of the *Methanococcus maripaludis* and *Acidianus tengchongensis* thermosomes [10–12] has provided a more precise snapshot of the movements performed by the monomer during closure of the cavity. These comprise rocking motion of the monomer during transition from the open to the closed state (Figure 2), including a 35° rotation perpendicular to the longitudinal axis of the chaperonin and a 30° counterclockwise rotation parallel to the same axis, although in the case of the *A. tengchongensis* thermosome, with rings comprising nine monomers, the latter movement is restricted to the apical and intermediate domains [10].

Intra- and inter-ring communication

Chaperonins undergo a complex set of intra- and inter-ring allosteric signals, mostly associated with ATP binding and hydrolysis, that generally follow intra-ring positive cooperativity and inter-ring negative cooperativity [8]. These

Box 1. Chaperonin co-chaperones

Chaperonins require the assistance of other chaperones to enable them to function. For instance, the so-called co-chaperonins (Hsp10s) form small heptamers that assist group I chaperonins in the folding of their substrates (Figures 2a and 3a) [1]. Group II chaperonins are not assisted by co-chaperonins, but they work in conjunction with other co-chaperones. Prefoldin (PFD; also termed GIM), for example, is a heterohexamer that assists group II chaperonins (both archaeal and eukaryotic) by transferring unfolded proteins into the chaperonin cavity [4]. CCT interacts not only with PFD, but also with a host of other chaperones, both upstream and downstream of the folding pathway [35]. For example, CCT interacts with Hsp70 chaperones, the largest family of chaperones, which thus links the eukaryotic chaperonin with other chaperones such as Hsp40 and Hsp90. Hsp70 seems to collaborate with CCT in a similar way as PFD, by delivering some substrates into the chaperonin cavity [62]. Other upstream CCT co-chaperones include the phosducin-like proteins (PhLPs), which assist the eukaryotic chaperonin in the folding of Gβ-transducin (PhLP1) [63], as well as actin and tubulin (PhLP2/3) [64]. Hsp70-Hsp90 organizing protein (HOP), a tetratricopeptide (TPR) domain-containing protein that acts as a co-chaperone that links together the Hsp70 and Hsp90 systems, also interacts with CCT; its precise role, however, remains unclear [65]. CCT is a chaperone required for tubulin folding, but the folding pathway of this cytoskeletal protein does not end with the eukaryotic chaperonin; other downstream tubulin chaperones (tubulin cofactors A–E) are needed for proper formation of α- and β-tubulin monomers and the α,β-dimer [66].

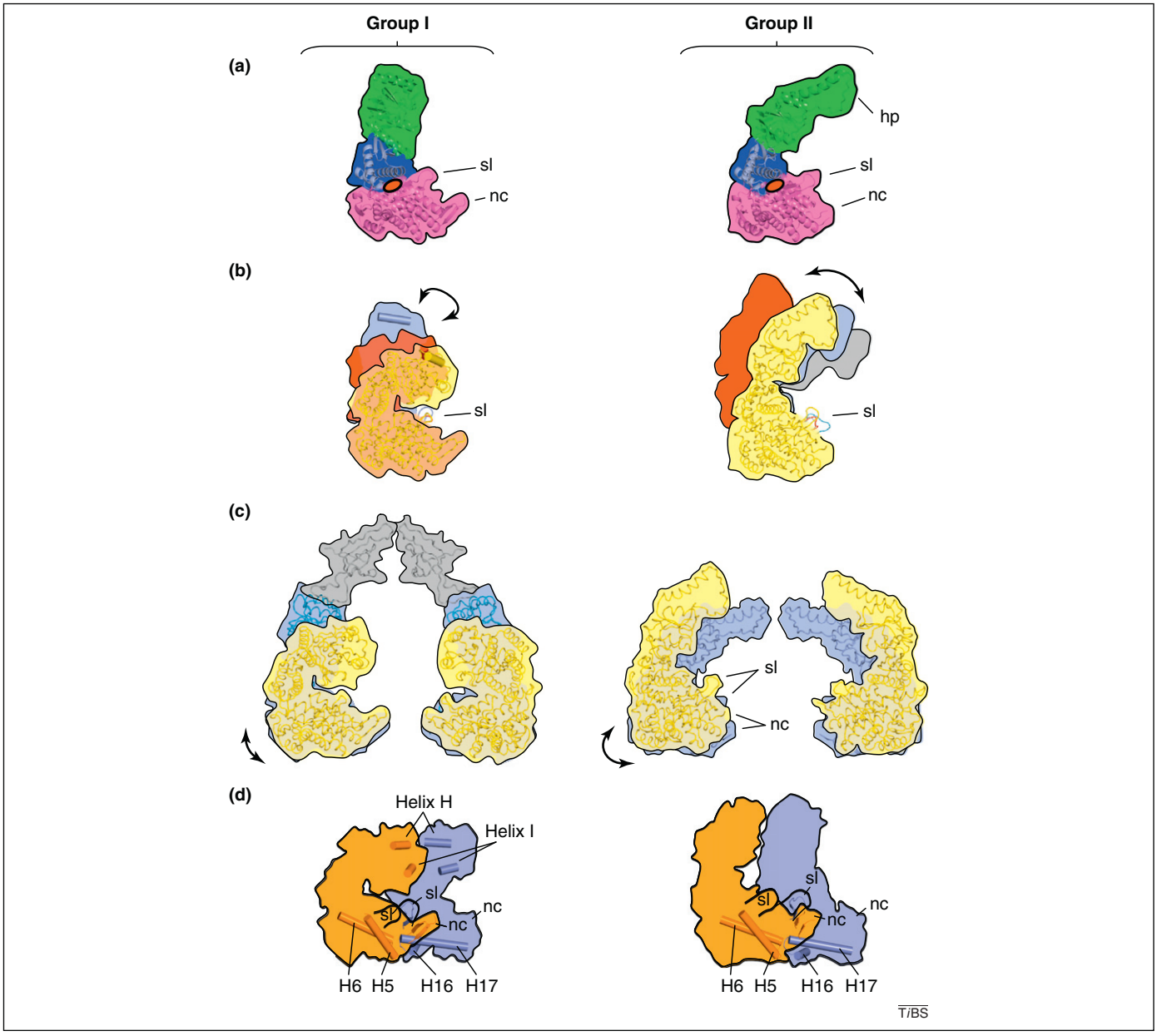

Figure 2. Conformational flexibility of the chaperonins. Chaperonins exhibit two types of plasticity, one related to the relative arrangement of the three domains in each subunit and the other corresponding to the orientation of every single subunit inside the chaperonin ring. **(a)** Domain organization of chaperonins from group I (left panel; GroEL structure from PDB 1PCQ [60]) and group II (right panel; thermosome structure from PDB 1A6E [7]). The apical, intermediate and equatorial domains are shown in green, blue and pink, respectively. The sensor loop (sl), the N and C termini (nc) and the helical protrusion (hp; only present in group II) are also indicated. The nucleotide binding sites, constituted by residues from both equatorial and intermediate domains, are depicted with red ovals. **(b)** Superposition of the equatorial domains of different chaperonin structures highlights the flexibility of the connection between intermediate and apical domains, which is essential for the dynamic behavior of these proteins and for ring closure. Left-hand sketch: three GroEL representative structures were aligned with their equatorial domains as reference: GroEL nucleotide-free complex (PDB 1OEL [61]; yellow), GroEL–ATP (PDB 2C7E [15]; red) and GroEL–GroES–ADP–AlFx (PDB 1PCQ; blue, GroES not shown for clarity). Helix H of the GroEL apical domain is depicted as a cylinder to highlight the large movements that this domain undergoes on ATP binding and hydrolysis (Figure 3a). Right-hand sketch: the apical domains of several CCT subunits (red, yellow, blue and gray representations) arrange as an open fan when superimposed using their equatorial domains (PDB 2XSM [13]). Comparison of different subunit structures of the CCT open conformation reveals that the equatorial domain behaves as a rigid body and the presence of variations in the sensor loop conformations. This feature could be interpreted as another type of conformational flexibility, which is also observed in GroEL. **(c)** Superposition of the open (yellow) and closed (blue) ring structures of chaperonins reveals that *en bloc* movements of the subunits are involved in closure of the complex, accompanied by adjustments of the different domains. The left-hand sketch shows the alignment of rings from GroEL structures (only two subunits are shown; the color code and associated structures are the same as in panel b; GroES is represented in gray). The right-hand sketch depicts the same type of representation for group II chaperonins: the CCT open complex (PDB 2XSM, yellow) and the thermosome closed complex (PDB 1A6E, blue). The thermosome open complex subunits (PDB 3KFK, not depicted for clarity) show basically the same orientation inside the ring as in the CCT open complex. Notably, important changes can be observed in the relative position of the sensor loops and the N and C termini inside the cavity of CCT. However, the β-strands of the sensor loop of one subunit remain associated with the N- and C-terminal β-strands of the adjacent subunit (see next panel). **(d)** Intra-ring contact regions. The right-hand drawing depicts two adjacent thermosome subunits (open complex PDB 3KFK [11]; orange and blue) that are in contact exclusively through two equatorial regions, a first that shows the interaction of the β-strands of the sensor loop and the β-strands of the N- and C- termini (β-strands representation), and a second showing contacts between the H5–H6 loop and the H16–H17 loop (cylinder and loop representation). The left-hand drawing shows the same depiction for GroEL (open complex PDB 1OEL), although in this case the apical and intermediate domains are also involved in intra-ring contacts. GroEL helix H and helix I, which are involved in substrate binding, are also shown.

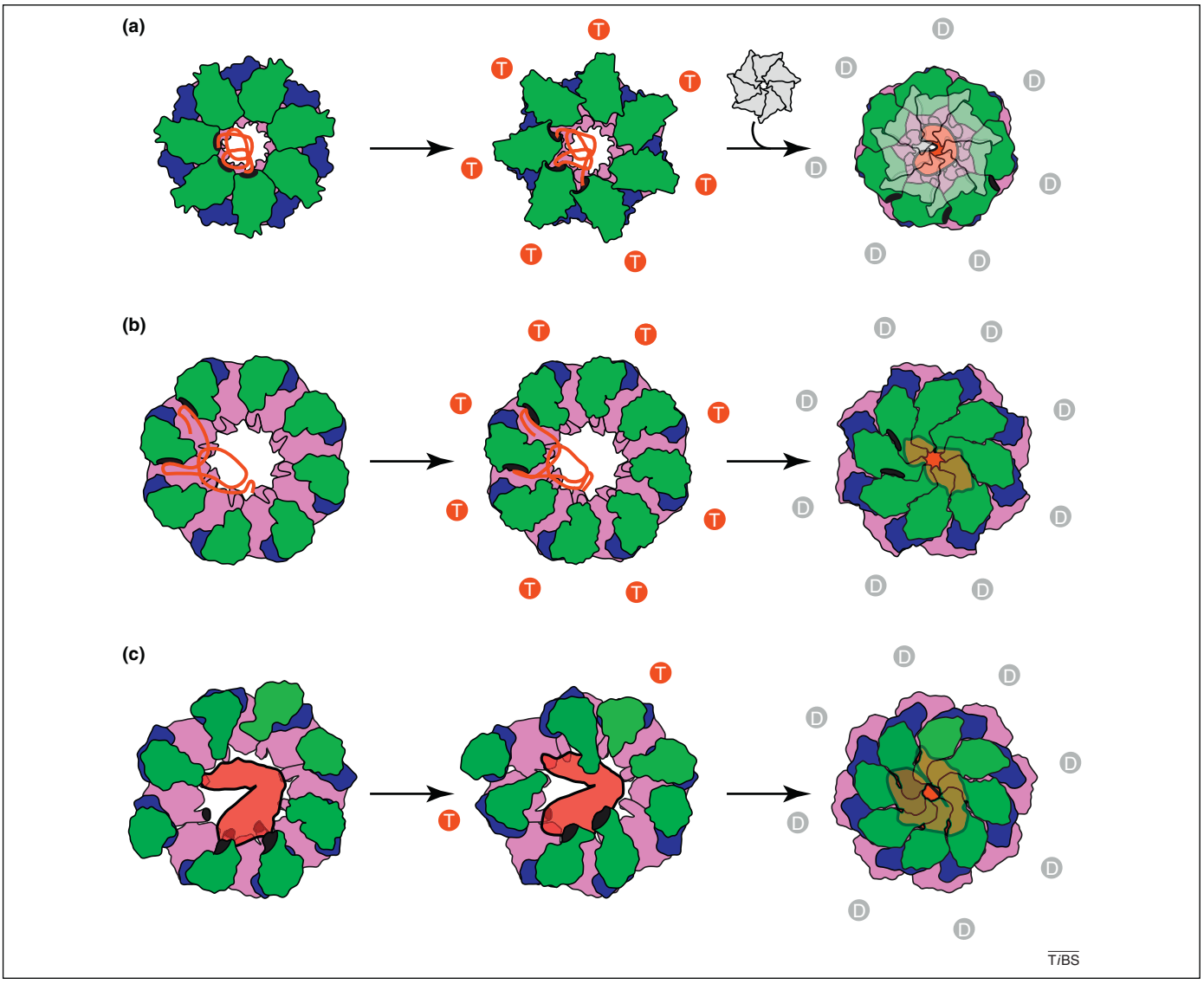

Figure 3. Models of ATP hydrolysis and folding cycles for the chaperonins. Overall end-on views of the different types of chaperonins show the three domains of each subunit (apical domain, green; intermediate domain, blue; equatorial domain, pink), the substrate-binding sites (black patches), the substrates (red) and a schematic representation of the bound nucleotide (red circles for ATP and gray circles for ADP). **(a)** GroEL (group I chaperonin) has an open, substrate-receptive conformation that recognizes and traps unfolded polypeptides through hydrophobic residues located in the apical domain at the entrance of the cavity. Concerted ATP binding in all seven subunits (centre) induces conformational changes that facilitate binding of a small heptamer, a co-chaperonin (GroES, transparent gray disc; Box 1) that caps the cavity and induces enlargement of the cavity (Figure 2c) and favors release of the unfolded polypeptide into the chamber, where it attempts to fold according to the information encoded in its amino acidic sequence. The substrate-binding sites are no longer exposed to the central cavity. **(b)** The thermosome (group II chaperonin) also has an open conformation that recognizes and traps unfolded polypeptides using a hydrophobic mechanism similar to that of GroEL. ATP binding and hydrolysis (centre) induces closure of the cavity, which is executed in group II chaperonins by a helical lid inserted in the apical domain (Figure 2a). Closure of the cavity (right) would release the substrate into the chamber, where it could fold. The folded substrate released in the cavity is represented as a shadow, covered by the apical lids that close the cavity. **(c)** CCT, the eukaryotic group II chaperonin, shows a more complex mechanism, probably arising from its own complexity (it comprises eight different subunits, depicted by different shades of green). Specific proteins interact through defined regions with specific CCT subunits in the open substrate-receptive conformation (left). The crystal structure 2XSM (Figures 1 and 2) [13] shows two regions involved in substrate binding, the apical protrusion and the sensor loop (in the equatorial domain). It also reveals partial nucleotide occupancy (here, two nucleotide-binding sites containing ATP). ATP binding to certain subunits triggers a non-concerted ATP hydrolysis mechanism (centre), which results in closure of the cavity (right) without release of the substrate.

allosteric signals are associated with the series of conformational changes that define chaperonin function [1].

Intra-ring communication

Intra-ring conformational changes in group I chaperonins have been described as being of a concerted nature and obeying the MWC model for allostery [8]. The molecular basis for this mechanism resides in specific electrostatic interactions between charged residues of the apical and intermediate domains belonging to adjacent subunits [23–25]. Normal mode analysis of the conformational dynamics in GroEL has shown that upward rotation and displacement of the apical domains of each single monomer during the co-chaperonin (GroES) binding stage can be reasonably accommodated only when these movements are concerted around the heptameric ring [24].

Whether the group II chaperonins undergo a concerted intra-ring conformational change remains an open question. However, increasing experimental evidence supports a non-concerted mechanism, at least for CCT [13,26–29]. In contrast to group I chaperonins, the structures for the open conformation of group II reveal no steric impediments

preventing conformational changes in individual subunits, given that no lateral contacts are formed between the intermediate or apical domains [10,11,13]. Thus, the area responsible for allosteric communication in group II chaperonins lies in the only intra-ring contact regions, entirely located within the equatorial domains. A first interaction is observed between two external loops of adjacent subunits (loop H5–H6 in one subunit and loop H16–H17 in the neighboring one; Figure 2d) [11,12]. It has been proposed that this area is the hinge point for the conformational change leading to closure of the cavity, which suggests that this is a key region for allosteric regulation [11]. The second interacting region occurs through a β-sheet formed by the N and C termini of one subunit and the β-hairpin (where the stem [12] or sensor loop [13] is located) of the neighboring subunit. Despite the conformational changes between the open and closed states, these regions maintain the interaction (Figure 2c,d), which suggests that the handshake between these two regions would be a key element in intra-ring subunit communication (Figure 4a) [12].

Inter-ring communication

ATP binding and hydrolysis within one ring trigger not only intra-ring movements, but also signals to the other ring (Figure 4a). Current models describe negative inter-ring cooperativity for both chaperonin groups that follows the sequential KNF cooperativity model [30]. Nevertheless, kinetic data suggest some differences, probably related to different inter-ring interactions between the two groups. For instance, in the case of GroEL it seems that saturation of both rings with ATP results in a lower rate of ATP hydrolysis [23]. The simultaneous conformational change in both rings on ATP hydrolysis leads to steric clashes between residues on both rings. This would hinder their concurrent conformational change [24] and explains the lower ATPase activity of GroEL when both rings hydrolyze the nucleotide [8].

By contrast, group II chaperonins, which do not have the same structural arrangement, do not show similar kinetic behavior. Saturation of both rings leads to the highest possible rate of ATP hydrolysis without any indication of inter-ring interference [27,29,31–33]. However, there are some discrepancies on this matter [34].

Analysis of the molecular basis of inter-ring communication in group I chaperonins using GroEL as a model indicates that the inter-ring interface is not strictly maintained during the conformational cycle (Figure 2c). ATP binding weakens the interface due to slight tilting of ATP-bound equatorial domains over unbound domains in the other ring [15]. The physical connection for allosteric communication between both rings is helix D (H5 in group II chaperonins, Figure 2d) [17].

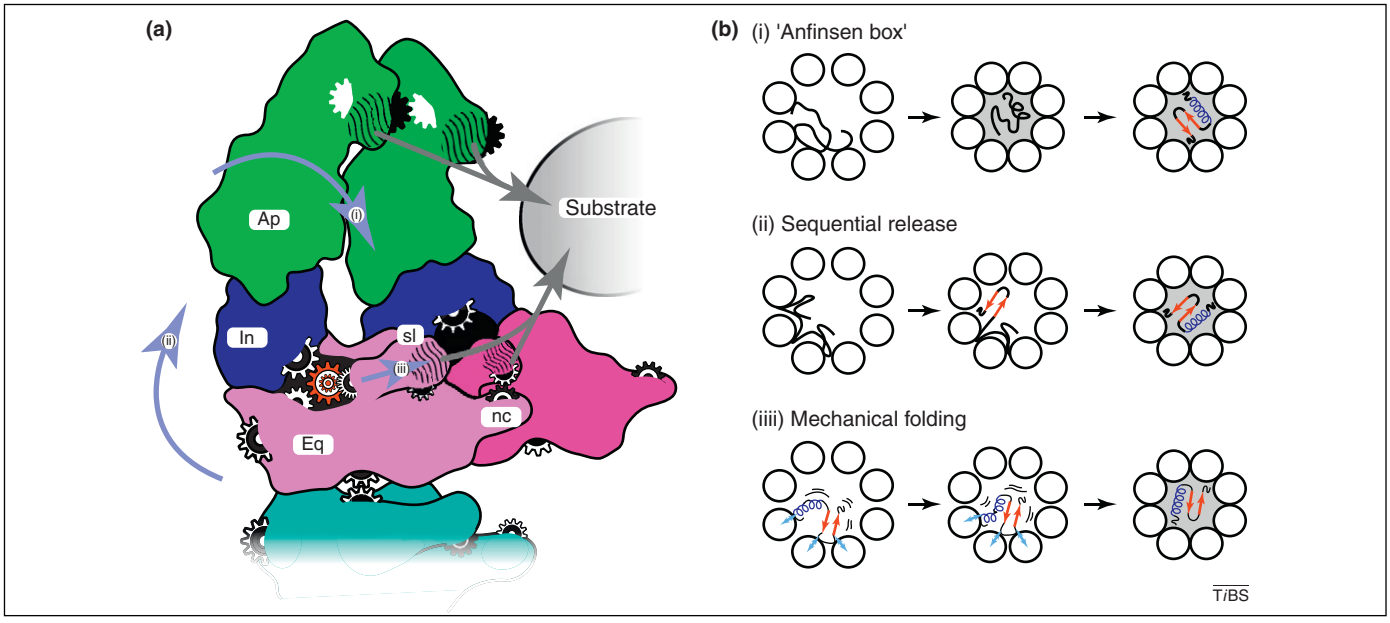

Figure 4. CCT molecular clockwork. **(a)** Schematic drawing of a CCT monomer and two neighboring subunits. The structural heterogeneity exhibited by CCT (Figure 2) suggests potential movements that the chaperonin experiences throughout the folding cycle. Three of these movements (blue arrows) are depicted as: **(i)** flexibility of the apical and intermediate domains; **(ii)** rigid body movement of the subunits inside the ring; and **(iii)** flexibility of the sensor loop that facilitates its interaction with the substrate. Each subunit forms intra-ring lateral contacts through the equatorial domain when the ring is open, and also through the apical domains when the ring is closed. In addition, each subunit establishes inter-ring contacts with neighboring monomers on the opposite ring. Different gears represent all these contacts. The substrate interacts with specific CCT subunits through two regions, one located in the apical domain and the other in the so-called sensor loop, located in the equatorial domain (substrate contacts depicted as gray arrows). All transitions between the open and the closed state are coordinated with local rearrangements in the interacting regions of the subunits (the gears). ATP (red gear) is not just a cog in the machine, but also connects different parts of the equatorial domain with the intermediate–apical domains, and the nucleotide-binding site with the sensor loop and with the adjacent subunits, which lead to the timing and actions needed for closure of the chamber and folding of the substrate and its final release. Abbreviations: In, intermediate domain; Eq, equatorial domain; Ap, apical domain; sl, sensor loop; nc, N and C termini. **(b)** General folding mechanisms for the chaperonins. In the schematic design, each circle depicts one subunit and the gray background represents the closed state of the ring. **(i)** The Anfinsen box theory implies protection of the unfolded polypeptide in the confined environment of the closed cavity, where it is released and given the opportunity to fold. **(ii)** Sequential release uses the same passive mechanism, but provides a means to direct sequential folding of the protein domains. **(iii)** Mechanical folding also uses a sequential mechanism, but the changes experienced during closure of the cavity are used to physically act on the unfolded protein. The three potential movements depicted in panel **(a)** are summarized here with blue arrows. If a substrate remains bound to several subunits at the same time, then closure of the ring drastically changes the distance of the contact regions and the accumulated tension can be used to force substrate folding. Once the substrate is folded, it is not able to interact with the chaperonin. The three folding mechanisms might not be mutually exclusive.

The inter-ring communication mechanism in group II chaperonins is not well characterized. Moreover, the different inter-ring arrangements for the two chaperonin groups (1:1 subunit pairing in group II vs 1:2 in group I; Figure 1) indicates that the signaling mechanism should be different. Nonetheless, the recently solved atomic structures of the open conformation of *M. maripaludis* and *A. tengchongensis* thermosomes [10,11] have shown that the structural integrity of the inter-ring interface in the open state relies on hydrophobic interactions and at least one preserved salt bridge. During the conformational change, the equatorial domain undergoes a 30–40° tilt (Figure 2c), a rearrangement that is wider than in GroEL and resulting in the formation of new salt bridges that stabilize the closed conformation. All structures of the closed conformation of group II chaperonins determined so far show that the two rings are closed [3,7,11,18]. However, this may not be a physiologically relevant conformation: the nested allosteric negative cooperativity model supports the notion that the two rings are not likely to be in the same conformational state [35]. Finally, recent results point to a surprising role for the helical protrusion located at the tip of the apical domain (Figure 2a) in the regulation of inter-ring communication [34,36]. Interactions between the helical protrusions of one ring could stabilize the closed conformation, inducing folding of the substrate and impeding its premature release. Thus, the helical protrusions could serve as signaling sensors that could regulate inter-ring communication and control timing for the substrate encapsulation phase in each ring (Figure 4a) [14,34].

The non-concerted conformational changes in CCT

As described above, the question of whether the intra-ring mechanism is concerted or non-concerted for group II chaperonins remains open. However, significant experimental evidence on genetic, kinetic and structural grounds supports the existence of a non-concerted mechanism for the eukaryotic cytosolic chaperonin CCT (Figures 3 and 4). Genetic studies carried out in *Saccharomyces cerevisiae* revealed different severity in phenotypes obtained by amino acid substitutions in the ATP-binding sites of several CCT subunits [37], which argues in favor of a non-concerted sequential mechanism of ATP binding and hydrolysis [26]. The sequential changes could take place through adjacent subunits, as observed in cryoEM analysis of CCT under low ATP conditions, which caused a wave of conformational changes transmitted around the CCT ring [28]. However, the sequential changes might not necessarily occur through contiguous subunits. The recent crystal structure of an open conformation of CCT in complex with tubulin revealed not only an asymmetry within the ring, in which each subunit has different conformational states (Figure 2b), but also that saturation with nucleotide leads to occupancy of only two sites in non-adjacent subunits per ring [13], which reinforces the hypothesis of a non-concerted mechanism. This finding also suggests that ATP hydrolysis could start independently at two different points of the ring (Figure 3c), which would support previous kinetic analyses that favor the sequential model. Moreover, it supports the proposal that the CCT intra-ring sequential conformational change might proceed through two parallel and simultaneous pathways [29].

Evolution of the concerted functional mechanism found in GroEL, and its difference compared to the non-concerted mechanism in eukaryotic CCT, might be linked to the challenges involved in folding of different and more complex proteins present in the eukaryotic cytosol. Simulations carried out relating single- or multi-domain protein chaperonin folding to a concerted or sequential model for chaperonin conformational changes have shown that whereas single-domain substrate folding benefits more from a concerted conformational change, the non-concerted change is more useful for folding of multi-domain proteins, which are more abundant in eukaryotic cells [38]. This rationale could also be applied to archaeal group II chaperonins, which fold substrates similar to those of bacteria.

The substrate recognition mechanism in chaperonins

The substrate recognition mechanism of all chaperonins seems to be based on hydrophobic interactions (Box 2), although an electrostatic contribution has been suggested for CCT [39,40]. It has been reported that the main binding determinant for unfolded proteins in group I chaperonins is the hydrophobic patch located between helices H and I in

Box 2. Comparison of group I and II chaperonin substrates

Over the past decade, entire bacterial, archaeal and eukaryotic proteomes have been searched for chaperonin substrates [43,67–70]. The cellular fraction of substrates is notably similar among all chaperonins, with different estimates ranging between 5% and 15% of the cell total protein content. Interestingly, whereas GroEL and thermosome substrates are relatively low in abundance, some CCT substrates, such as actin and tubulin, are among the most highly expressed proteins [43], even though CCT is present at very low levels in the cell [71]. These results also reveal that chaperonin substrate selectivity for chemically denatured substrates (whole cell extracts) differs considerably from the substrate selectivity determined under *in vivo* conditions [43,70], a discrepancy that has been related to the presence of upstream chaperones that modulate substrate presentation to the chaperonin. Chaperonin substrate recognition depends mainly on exposed hydrophobic residues [1], and also involves polar and charged residues in the case of CCT [40,45]. In most cases it seems to be directed towards proteins with complex topologies prone to populate kinetically trapped folding intermediates [43,68,70], including many that form multimeric complexes. Several characteristics differentiate group I and II chaperonin substrates. The former are, on average, relatively large (30–50 kDa), are more hydrophobic, have lower net charge and have complex αβ domain topologies. By contrast, group II chaperonin substrates have a wider molecular size distribution, and are less hydrophobic and more negatively charged proteins with higher topology variation, including many β-rich, all-α and other structural domains that are not usually found among group I chaperonin substrates (e.g. the RNase H-like family domain) [43,70].

CCT is probably the best-characterized chaperonin with regard to its substrate specificity. Although it was originally believed to be specific for actins and tubulins, it is now clear that CCT acts on as many as 15% of newly synthesized proteins [70]; some of its substrates, however, do share structural motifs. For example, several CCT substrates are WD40 repeat-containing proteins [72], including Gβ-transducin [73], cell division control 20 (CDC20) and CDC20-related 1 (CDH1) [74]. CCT also functions in the control of quaternary interactions. For example, VHL requires its interaction with CCT to adopt a conformation that can be recognized by the elongin BC complex, part of the VCB–CUL2 E3 ubiquitin protein ligase complex [75].

the apical domain of each subunit (Figures 2d and 3a) [41]. In this region a ring of hydrophobic residues placed at the entrance of the cavity can recognize unfolded polypeptides with exposed hydrophobic residues. However, a minimum of three contiguous patches is needed for efficient polypeptide binding (Figure 3a) [42]. It was initially suggested that thermosomes use a similar, non-specific, hydrophobic-based recognition mechanism (Figure 3b) [40]. Although this seems to be essentially correct, some differences must exist. Indeed, a recent proteomic study of the archaeon *Methanosarcina mazei,* which contains both types of chaperonins, revealed significant structural selectivity between the two chaperonin groups [43].

The recent X-ray structures of the open conformation of *M. maripaludis* and *A. tengchongensis* thermosomes revealed two possible hydrophobic substrate-binding sites [10,11]. The first is found in the helical protrusion and comprises several hydrophobic residues, whereas the second is localized to the same hydrophobic region described for group I chaperonins. Deletion experiments on the first region of *Thermococcus* KS-1 and *M. maripaludis* thermosomes and CCT revealed that all of them retain the ability to bind substrate [14,34,44], which argues against this region having a stringent role in unfolded polypeptide interaction. However, it has been shown that the second region is important for correct recognition and interaction of CCT with the Von Hippel Lindau (VHL) tumor suppressor protein [45].

In contrast to the other chaperonins, CCT has a heterogeneous subunit composition. Moreover, the apical domains, and more specifically the regions putatively involved in substrate interaction, have greatly diverged, with some subunits containing mainly hydrophobic residues, whereas others also bear a number of charged and hydrophilic amino acids (Box 2) [40,45]. Indeed, it has been proposed that the interaction between CCT and its substrates takes place through specific CCT subunits with specific domains of the unfolded protein that have acquired a certain structural context before interacting with CCT (Figures 3c and 4). The recently solved crystal structure of the CCT–tubulin complex [13] not only reinforces these notions (one of the tubulin domains interacts with three adjacent subunits), but also reveals a novel and surprising region associated with the substrate. This region is located in the equatorial domain, in the sensor loop, at the tip of a β-hairpin that connects with the ATP-binding pocket. This β-hairpin is also adjacent to the N and C termini (Figures 2d and 4a) and it has been suggested that these participate in protein folding in both GroEL [46,47] and the thermosome [12,48].

One of the most interesting features of the CCT–tubulin complex structure is the presence of one tubulin molecule per ring [13]. It has already been shown that this double binding exists not only in CCT [49], but also in GroEL [50]. The fact that the CCT–tubulin complex was not reconstituted *in vitro* but purified from mammalian tissue, together with cryoEM observation of populations of CCT chaperonins with two bound substrates [13], strongly suggests that, at least under certain physiological conditions, two substrate molecules could interact with a CCT complex simultaneously, one per ring, although this might not necessarily mean that the two substrates are at the same stage in the folding process. Indeed, the crystal structure of the CCT–tubulin complex [13] reveals a different conformation of the two rings: one presents all the apical domains in a semi-open conformation and the other has only two of the apical domains visible (Figure 1). The arrangement of the substrate inside the cavities also disrupts the chaperonin octagonal symmetry, as observed for the positioning of the nucleotide-binding sites [13].

The folding mechanism in chaperonins

It has been proposed that the role of GroEL in substrate folding is either active, with the confined environment and negatively charged surface of the closed chamber playing a dynamic role in remodeling the folding energy landscape of the substrates (Figure 3a) [46,51,52], or passive with the closed chamber providing an adequate environment for denatured proteins to fold by themselves (Anfinsen cage; Figures 3a and 4b) [53], which thus avoids multimeric aggregation states [41]. Regardless of the nature of the folding mechanism (a mixture of both mechanisms cannot be ruled out), recent cryoEM data localize the substrate in the closed conformation floating inside the chamber, which reinforces the notion that the unfolded substrate is released into the cavity (Figure 4b) [50,54].

The working mechanism for group II chaperonins, however, remains a matter of debate. One of the current models proposes a folding mechanism similar to that assigned to GroEL (Figures 3 and 4) [14,45] whereby the substrate would be released into the chamber, with its folding pathway modulated by the electrochemical environment of the closed cavity. Similarly, the existence of positively charged ring patterns on the inner surface of *M. maripaludis* thermosomes has been noted [11], but the lack of conservation in these patterns, not only between the two chaperonin groups but even among group II chaperonins, seems to weaken the likelihood of any specific pattern-dependent effect, which highlights substrate confinement and the highly preserved hydrophilic nature of the cavity as the main driving forces behind this folding model. Support for this view comes from a thorough biochemical and biophysical study in the *M. maripaludis* thermosome that suggested that the substrate, previously bound to a hydrophobic patch located between helices H10 and H11, is released into the folding chamber after its closure [14]. However, there is still no direct structural information on the thermosome–substrate interaction. Hence, the current functional models rely solely on biochemical and mutational data.

In considering the mechanisms underlying the function of the more complex CCT, a mechanical role has been suggested in which conformational changes arising from ATP binding and hydrolysis in the different subunits would physically act on the substrate and promote its correct folding (Figure 3c) [40,55–58]. The crystal structure of the CCT–tubulin complex in an open conformation showed that the different subunits are highly flexible and can adopt multiple different conformations within the ring (Figures 1, 2b and 3c). These different conformations and the identification of the subunit regions that contact the substrate have provided the basis for a new

active model (Figures 3c and 4a) [13]. In this model, the sensor loop [14] would act as a signaling module that senses interaction of a substrate with a particular CCT subunit and could influence ATP binding or vice versa. Moreover, the loop could act as a lever that extends and retracts during changes triggered by ATP hydrolysis [11–13], actively helping the substrate to overcome local energetic minima that might lead to incorrect folding intermediates. These conformational changes are reflected in the asymmetric layout of the ATP binding sites in the open and substrate-bound conformation [13] compared to the closed and substrate unbound structure [3]. The importance of the sensor loop was confirmed by RNA interference experiments that revealed a decrease in cell proliferation for mutations in the sensor loop and those affecting ATP hydrolysis [13]. Moreover, fluorescence resonance energy transfer experiments carried out on CCT–actin complexes using actin labeled in two different regions revealed a change in the donor–acceptor distance on ATP hydrolysis, thus supporting the notion that the substrate undergoes substantial conformational changes after closure of the CCT cavity. Such conformational changes do not take place when labeled actin is incubated in the presence of GroEL [57,58].

Notably, the currently proposed functional models for group II chaperonins are not mutually exclusive and the final mechanism might indeed be a combination of both. Regardless of the folding mechanism eventually identified for CCT, it is very reasonable to suggest the existence of a more complex mechanism for the eukaryotic chaperonin, given its unique subunit composition and the large number of specific substrates (Box 2). Therefore, the different apical domains could identify different types of substrates; however, once they are trapped, the chaperonin might fold them through a common mechanism after they are confined inside the chamber.

Concluding remarks

The avalanche of new data has raised new questions that need to be answered to fully unveil the working mechanism of group II chaperonins. Unlike the thermosome structures, the open crystal structure of CCT shows that not all ATP-binding sites are occupied in both rings on substrate binding, which leads to disruption of the octagonal symmetry (Figure 3c) [13]. This observation raises key questions about the intra- and inter-ring mechanisms that could control nucleotide binding in the other subunits and chamber closure. Therefore, more evidence is needed to determine whether all the ATPase domains in one ring are sequentially (consecutively or not) filled or whether the nucleotide is hydrolyzed in a concerted or sequential (consecutive or alternate) mode, as well as how this information is transmitted so that one of the rings is closed and the substrate is folded whereas the other is in either an open substrate-bound or a substrate-receptive conformation. These points need to be addressed to understand the working mechanisms of these complex molecular machines.

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