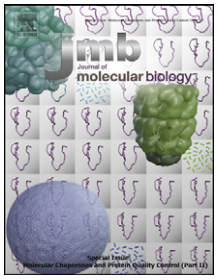
Review



The Mechanism and Function of Group II Chaperonins

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Abstract

Protein folding in the cell requires the assistance of enzymes collectively called chaperones. Among these, the chaperonins are 1-MDa ring-shaped oligomeric complexes that bind unfolded polypeptides and promote their folding within an isolated chamber in an ATP-dependent manner. Group II chaperonins, found in archaea and eukaryotes, contain a built-in lid that opens and closes over the central chamber. In eukaryotes, the chaperonin TRiC/CCT is hetero-oligomeric, consisting of two stacked rings of eight paralogous subunits each. TRiC facilitates folding of approximately 10% of the eukaryotic proteome, including many cytoskeletal components and cell cycle regulators. Folding of many cellular substrates of TRiC cannot be assisted by any other chaperone. A complete structural and mechanistic understanding of this highly conserved and essential chaperonin remains elusive. However, recent work is beginning to shed light on key aspects of chaperonin function and how their unique properties underlie their contribution to maintaining cellular proteostasis.

Chaperonins: The Protein Folding Machines

Among the most striking aspects of protein biology is the manner in which polypeptide chains routinely and rapidly attain an active three-dimensional structure with high fidelity. This property, first presented in historic work by Christian Anfinsen [1], implies that both the native conformation and folding trajectory of a protein are encoded in its primary structure. As Levinthal famously argued, if the sequence of a peptide did not place some restrictions on the conformational landscape accessible at physiological temperatures, an exhaustive search over all conformational degrees of freedom would take an unreasonably long time [2]. These two observations, namely that small globular proteins attain their native conformations autonomously and that they do so on surprisingly short timescales, serve to frame the biophysical problem of protein folding. The combined weight of many folding studies supports the idea that small globular proteins can fold productively in isolation in a two-state fashion [3,4]. Nevertheless, the model of two-state folding does not encompass the breadth of the folding problem under physiological conditions. In particular, the cellular environment places folding polypeptides in an environment that disfavors folding and promotes aggregation and misfolding [5]. The vectorial nature of protein synthesis places a topological constraint upon folding, as N-terminal regions of polypeptides are available for folding before the polypeptide is completed [6,7]. In the cell, proteins also encounter stresses such as temperature, free radicals, and osmolytes that can damage and/or unfold proteins. Unchecked, these perturbations in conjunction with the cytosolic pool of nascent or unfolded polypeptides would lead to protein aggregation en masse in the concentrated cytosol [8]. All of these issues are compounded for the many proteins that cannot fold independently and instead become trapped in intermediate conformations.

To cope with environmental stresses and to facilitate the folding of troublesome or large proteins, cells have evolved a system of molecular chaperones and quality control machinery, often called the

"protein homeostasis" or "proteostasis" network. Chaperones are proteins themselves that bind to unfolded or misfolded polypeptides and induce their folding, sequester them, or facilitate their degradation [8]. Members of this cellular proteostasis network constitute the first interacting partners seen by nascent peptides upon departing the ribosome exit tunnel and can be found in both bacteria and eukaryotes [9,10]. They also commonly represent the final interacting partner of proteins destined for degradation. Among the most important of the molecular chaperones are the chaperonins, large 1-MDa oligomeric complexes comprising two stacked rings, each of which creates a central cavity for polypeptide folding [11,12]. Chaperonins are ATPases that harness the energy of nucleotide binding and hydrolysis in order to encapsulate misfolded proteins in their central cavity such that they may fold in isolation. The chaperonins are present in every kingdom of life and are essential in all sequenced organisms excepting some members of the genus *Mycoplasma* [13]. The chaperonins are subdivided into two families, termed the group I and group II chaperonins.

The group I chaperonins, of which GroE from *Escherichia coli* is the archetype, are present in the bacterial cytosol and in the eukaryotic organelles derived from endosymbiosis. Less frequently, group I chaperonins can be found in archaea [14]. The group I chaperonin system consists of two components, a tetradecameric Hsp60 and a heptameric co-chaperone Hsp10. Hsp60, known as GroEL in *E. coli*, consists of two 7-fold symmetric rings related by a 2-fold inter-ring symmetry axis. Each GroEL ring harbors a central cavity in which client proteins are encapsulated for folding. The co-chaperone Hsp10, called GroES in *E. coli*, binds to GroEL in an ATP-dependent manner acting as a "lid" to prevent substrate egress while greatly expanding the size of the folding chamber [15].

By contrast, group II chaperonins are found in archaea and the eukaryotic cytosol. They also consist of two stacked rings, each composed of eight 50- to 60-kDa subunits, but do not have an obligate co-chaperone in the same manner as the group I chaperonins. Rather, they contain a built-in lid that closes the folding chamber and are thus competent to fold substrates *in vitro* without the assistance of accessory proteins. This should not be taken to mean that the group II chaperonins function in isolation in the cell. On the contrary, the group II chaperonins appear to be at the heart of a complex network of co-chaperones [16–20]. Notable examples include the hexameric prefoldin complex that is often thought to bind to and prevent aggregation of unfolded substrates before handing them off to the chaperonin [21,22] and the phosducin-like proteins that have been shown to enhance TRiC-mediated folding of several substrates [20,23].

The eukaryotic group II chaperonin, which is known as TRiC/CCT (TRiC hereafter), differs from its simpler archaeal homologues in that it is composed of eight paralogous subunits. Most notably, TRiC is absolutely required for folding many essential proteins, including cytoskeletal proteins such as tubulin and actin, as well as cell cycle regulators such as CDC20 and CDH1 [24–26]. It has been estimated that as much as 10% of cytosolic proteins interact with the eukaryotic chaperonin TRiC along their folding trajectory [27].

Architecture of Group II Chaperonins

Like the group I chaperonins, group II chaperonins are composed of two oligomeric rings related by a 2-fold symmetry axis. While group I chaperonins have 7-fold symmetric rings [28,29], the group II chaperonins have 8-fold and occasionally 9-fold [30–34] symmetry within their rings. Unlike GroEL, most group II chaperonins are heteromeric. The extreme case is the eukaryotic chaperonin, TRiC/CCT in which each ring contains eight distinct, paralogous subunits occupying fixed positions in the complex [35,36].

The archetypal group II chaperonin that served as the first structural model for the family is the *Thermoplasma acidophilum* α/β-thermosome. The first atomic-resolution structure of a group II chaperonin was of an isolated apical domain from the thermosome α-subunit [37]. The apical domain, which is the domain that diverges most from the group I in terms of primary sequence, was shown to contain a helical protrusion [37,38] absent from the structures of *E. coli* GroEL. A comparison of the domain structures of group I *versus* group II chaperonins is presented in Fig. 1A highlighting the helical protrusion extending from the apical domain of the group II chaperonin MmCpn [39]. The equatorial domain of the chaperonins forms the ring interface and contains most of the residues involved in nucleotide binding. The intermediate domain forms the apical surface of the nucleotide binding pocket and contains the catalytic aspartate that activates water for nucleotide hydrolysis. The structure of the equatorial and intermediate domains is conserved between the group I and group II chaperonins (Fig. 1A). When the first structure of the full-length thermosome was solved [40], the significance of the apical helix could be appreciated for the first time. The thermosome structure demonstrated that the apical helices form an iris enclosing the folding chamber (Fig. 1B, inset) thereby allowing the group II chaperonins to function without a co-chaperone lid. The structure also revealed how the subunits of one ring are seated directly in register on a subunit in the second ring, in contrast to the staggered inter-ring registry of the group I chaperonins.

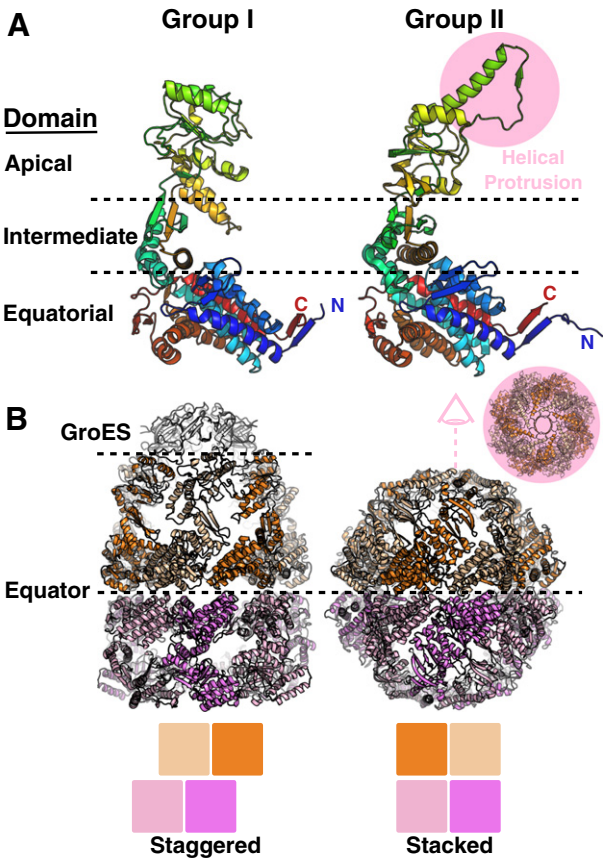

Fig. 1. Structural comparison of group I and group II chaperonins. (A) Domain architecture is conserved between group I and group II chaperonins. Left, chain A from the *cis*-cavity of a GroE crystal structure. Right, chain A from the MmCpn crystal structure. (B) Comparison of the GroEL (left) and MmCpn (right) complex architectures. The GroE and MmCpn crystal structures used were PDB ID: 1AON [15] and PDB ID: 3RUW [39], respectively.

The Structure of the Eukaryotic Chaperonin TRiC

Structures of the TRiC and several of its substrates [41–45] or co-factors [17,41,46–48] have been solved by cryoelectron microscopy [45,49–52] and small-angle X-ray scattering [53]. However, atomic-resolution electron density maps have long eluded the field. Owing to the structural similarity of the eight paralogous TRiC subunits, the field has struggled to assign an arrangement to the subunits in the TRiC rings [51,54,55]. Only recently has a hybrid approach utilizing X-ray crystallography and chemical cross-linking mass spectrometry finally yielded the definitive arrangement [56,57]. The subunit arrangement of TRiC leads to the spatial partitioning of subunits with different chemical properties. Specifically, subunits are segregated by their ATP binding affinities [58] (Fig. 2A) and by their net charge (Fig. 2b and c). Thus, the hetero-oligomeric nature of TRiC generates functional and chemical asymmetries absent from other chaperonin systems, which likely provide the basis for the unique ability TRiC possesses to fold specific substrates such as actin, which cannot be folded by the GroE chaperonin system [59].

Nucleotide-Driven Conformational Cycle of the Group II Chaperonins

Productive folding of proteins by the group II chaperonins is an ATP-dependent process [35,60]. The use of archaeal model systems for structural and biochemical work has greatly benefited the group II chaperonin field and contributed to our understanding of the nucleotide cycle of group II chaperonins [61]. Recently, the structures of the apostate, nucleotide-bound state, and closed states of archaeal group II chaperonin have been solved at atomic or near-atomic resolution leading to an improved understanding of the domain motions that occur upon nucleotide binding and hydrolysis [30,62–64]. Furthermore, the first atomic-resolution structure of a monomeric archaeal chaperonin was solved in the unliganded state [65], revealing a more spacious nucleotide binding face than what is observed in the closed-state structures. The more accessible pocket bears a striking resemblance to that of apo-GroEL.

The formation of the closed chaperonin complex begins with the binding of ATP. The lidless variant of the homo-oligomeric archaeal group II chaperonin from *Methanococcus maripaludis* (MmCpn hereafter) [66] was observed under different nucleotide conditions by cryoelectron microscopy. When compared to the apostate conformation, the apical domains of ATP-bound subunits are rotated approximately 45° [64]. This observation has been corroborated by directly monitoring movements resulting from ATP binding of the thermosome by diffracted X-ray tracking. The phase associated with ATP binding was observed to finish within 1 s after the freeing of caged nucleotide [67]. Substrate binding sites on the apical domain are still accessible in this transient state, though not to the extent of the fully open complex.

Transit of the complex along the reaction cycle requires that the state of the bound nucleotide be monitored and communicated across the domains of a single subunit and to neighbors within the complex. This role is fulfilled by a conserved lysine in the intermediate domain of the group II chaperonins [39]. The orientation of Lys161 is reliant upon the phosphate state of bound nucleotide, moving to interact with either the γ-phosphate or the ∝-phosphate of ATP and ADP, respectively. This results in

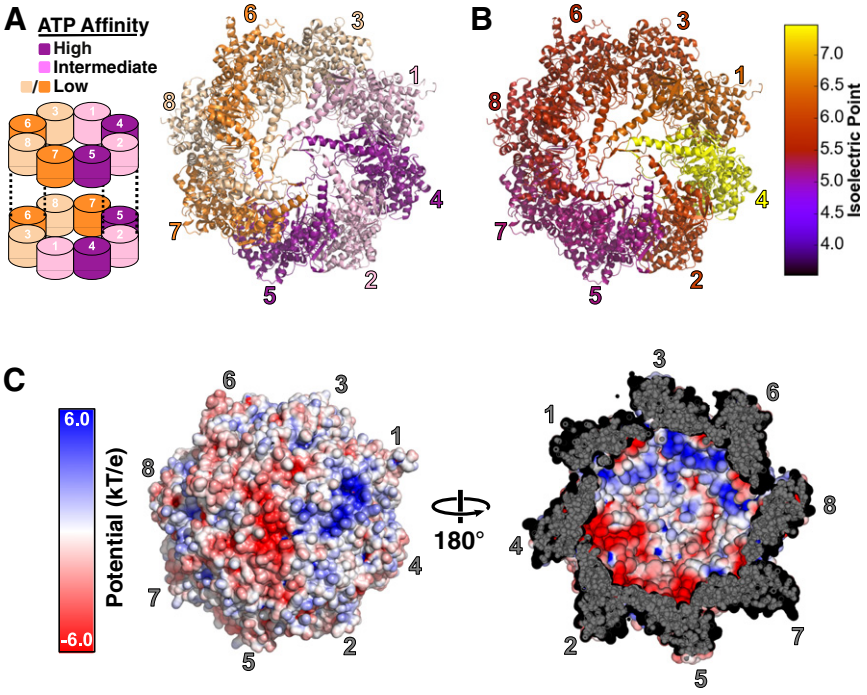

Fig. 2. The subunit arrangement of the hetero-oligomeric eukaryotic chaperonin TRiC. (A) Left, a schematic of the subunit arrangement of TRiC showing the inter-ring register. Right, the crystal structure of TRiC showing the subunit arrangement and the partitioning of ATP affinities. Subunits are colored by their ATP affinity. (B) Influence of subunit arrangement on charge distribution. Subunits are colored by their isoelectric points. Isoelectric points were estimated for the *S. cerevisiae* CCT subunits using the pepstats program from the EMBOSS suite [110]. (C) The surface charge characteristics inside the closed TRiC cavity. Left, a view of the outside of the chaperonin folding chamber colored by surface electrostatic potential. Right, a view of the lid of the folding chamber from the inside illustrating the polarized nature of the TRiC cavity. Surface electrostatics were rendered in PyMOL [111] using APBS Tools [112,113]. The TRiC structure is PDB ID: 4V94 [56] prepared by adding missing heavy atoms in PDBFixer [114].

significant rearrangement of the loop composed of residues 160–169, termed NSL (the *n*ucleotide *s*ensing *l*oop), altering intra-subunit and inter-subunit points of communication. Homologous residues in the CCT subunits can be seen to adopt similar conformations when crystallized with ADP-BeF_3 [56]. Interestingly, one notable difference between the archaeal and eukaryotic NSLs is the expected flexibility of the each sequence. In the archaeal chaperonin MmCpn, the NSL is relatively unencumbered, with glycines flanking Lys161. By comparison, the CCT sequences contain more sterically restrictive β-branched amino acids. Like other archaeal chaperonins, MmCpn has a much faster nucleotide hydrolysis rate than TRiC [66]. However, the "TRiC-like" mutant G160S of MmCpn was shown to have a drastically slower rate of ATP hydrolysis, roughly equivalent to the steady-state hydrolysis of TRiC [39]. The identity of this loop may represent the simplest determinant for controlling the overall speed of the nucleotide hydrolysis cycle, hence acting as a "timer" that determines the residence time of the polypeptide within the closed folding chamber. However, detailed analyses of the discrete steps of nucleotide hydrolysis need to be carried out for mutants of both archaeal and eukaryotic complexes in order to define the particular transitions that are being delayed [68]. Advances in expression and purification of recombinantly sourced TRiC are beginning to make necessary experiments such as these a possibility [69].

Hydrolysis is required to bring the lid helices into close proximity, forming the iris and parallel β-barrel responsible for capping the folding chamber [70]. Opening of the lid occurs in conjunction with releasing ADP from the active site. This slow reopening and ADP release represent the rate-limiting steps of the reaction cycle [68]. In MmCpn, this reopening has been shown to be strongly dependent upon the nature of a specific loop within the apical domain [71]. The release loop for substrate, RLS, is a five-residue stretch located opposite the substrate binding site and mutation to alanines causes the complex to stall in a doubly closed configuration (Figs. 3 and 4). The complex can exist in a symmetrically closed conformation even during ATP cycling conditions, raising questions about the mechanisms that lead to reopening of the lid and their coordination across the rings.

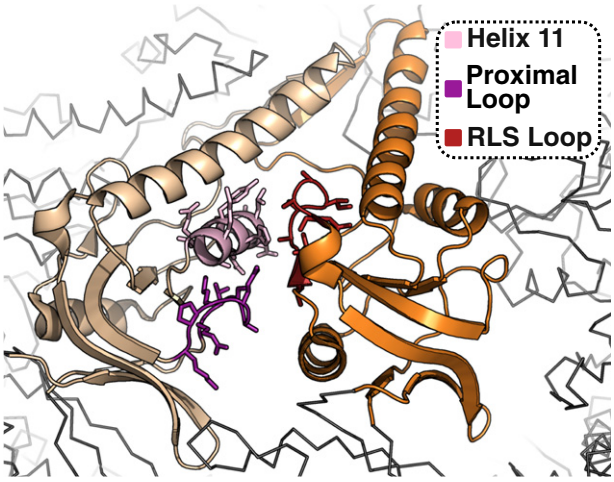

Fig. 3. Structural basis for substrate release during lid closure. The apical domains of the group II chaperonin MmCpn in the closed state, highlighting the substrate binding surface at the interface between two adjacent apical subunits, from PDB ID: 3RUW [39]. The substrate binding surface of the left subunit comprising helix 11 and the proximal loop is indicated, as well as the RLS loop of the right-hand subunit that is responsible for evicting bound substrate during closure.

For GroE, cycling is suggested to occur through a two-stroke mechanism [66,72]. Failure of a chaperonin to release GroES has been observed in the single-ring mutant of GroEL SR1. As a result, GroES stays permanently associated with the single-ring chaperonin [73], and this observation was viewed as evidence that proper cycling relies on the interaction between the two chambers. This mechanism is less clear in the case of the group II chaperonins, as the allosteric communication that occurs between the rings is less well understood.

The stoichiometry of ATP utilization by the subunits of the group II chaperonin rings is very different from the 1:1 ratio observed in GroEL [15,74]. It has been shown that the heteromeric α/β-thermosome behaves quite differently from the homomeric all α-complex [75]. TRiC provides the extreme example of differentiation of subunits leading to novel allosteric mechanisms. There, specialization has led to a mechanism wherein only four out of the eight CCT subunits appreciably bind ATP [58]. Studies performed in *Saccharomyces cerevisiae* demonstrated the *in vivo* significance of this observation. Mutations that removed the ability to bind or hydrolyze ATP had severe phenotypes in the four "high-affinity" TRiC/CCT subunits but showed no effect on growth or viability when made in the four low-affinity subunits [58]. The role that this decentralization of nucleotide usage plays in the context of productive folding remains an area of ongoing research.

The unusual stoichiometry of ATP utilization by the subunits raises the question of how many nucleotides the complex requires for cycling. Single-molecule experiments have been performed where fluorescently labeled ATP bound to individual complexes were counted by recording discrete photobleaching steps [76]. The observed distributions centered on eight bound nucleotides under both cycling and non-cycling conditions, meaning that half of the total nucleotide binding sites are occupied, consistent with the finding that only four subunits per ring bind ATP. Under these ATP concentrations, biochemical experiments demonstrated that both rings contained a closed lid. The doubly closed conformation has also been observed under cycling conditions by small-angle X-ray scattering [70] and in cases with stalled complexes by cryoelectron microscopy [63,71]. A GroEL-type inter-ring allosteric model, which predicts an asymmetrically closed conformation, does not explain these results [77–79]. Given that certain subunits never bind ATP under nearly identical conditions [58], it is likely that the ATP binding subunits of each ring are occupied simultaneously. The hydrolysis state of these bound nucleotides is unknown. It may be the case that the negative cooperativity observed in ATP hydrolysis [66] is a result of a distinct mode of allosteric regulation that remains to be examined.

Substrate Properties and Interaction

Knowledge on how TRiC recognizes and folds proteins has increased as the list of known substrates of the eukaryotic complex continues to grow. TRiC interacts with approximately 10% of the proteome and its function is absolutely essential for viability [27]. TRiC disfunction is associated with a growing number of diseases. Spontaneous and inherited mutations in subunits CCT5 and CCT4 of TRiC are linked to sensory neuropathy [80,81]. Tumor-associated mutations are found in the TRiC binding sites of the von Hippel-Lindau protein that lead to misfolding and loss of function [82–84]. Cancer-linked proteins p53 and STAT3 are known to be TRiC substrates [85,86]. TRiC has been shown to mediate the folding of a number of β-propeller-rich proteins, including telomerase co-factor TCAB1 [87], the cell cycle regulators CDC20 and CDH1, and G_{β}

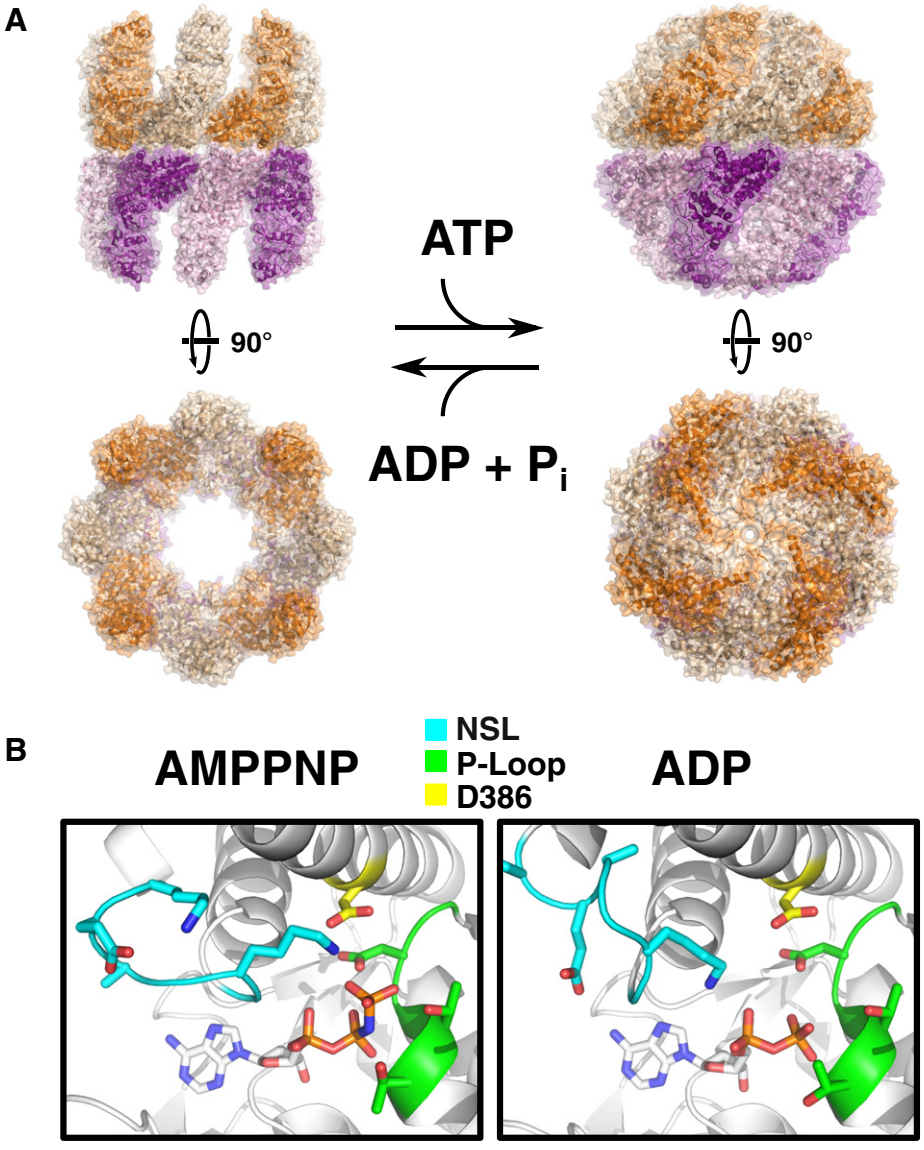

Fig. 4. The ATP-driven conformational cycle of the group II chaperonin. (A) Left, the open apostate of a group II chaperonin MmCpn determined by cryoelectron microscopy, PDB ID: 3IYF. Right, the cryoelectron microscopy structure of wild-type MmCpn in the ATP-induced closed state, PDB ID: 3LOS [63]. (B) The position of the NSL is dependent upon the presence of the γ-phosphate. Left, the crystal structure of the archaeal chaperonin MmCpn in a pre-hydrolysis state, complexed with the non-hydrolyzable ATP analogue AMPPNP, PDB ID: 3RUV. Right, the crystal structure of the same MmCpn complexed with ADP, PDB ID: 3RUW [39]. The NSL, P-loop, and the catalytic aspartate D386 are indicated in cyan, green, and yellow, respectively. The conformation of the NSL is altered by the scission of the gamma phosphate between the left and right panels.

subunits of signaling complexes containing WD40 domains [19,20,25,88–91]. TRiC also participates in suppressing aggregation and toxicity of Huntingtin in Huntington's disease [92–95] and has recently been linked to susceptibility to Alzheimer's disease [96]. Interestingly, several viral proteins have evolved to require TRiC for proper folding and processing [97–100]. As a result, downregulation of TRiC impairs replication of several important human pathogens, including HCV (*h*epatitis *C* *v*irus) and HIV (*h*uman *i*mmunodeficiency *v*irus) [97,100]. TRiC interacts with the HIV proteins Gag, Vif, and p6, and it is required for HIV replication [101,102]. The expanding list of processes that TRiC has been shown to be involved in highlights the global importance the group II chaperonin plays in maintenance of the proteome and proper cellular physiology.

A robust definition of the specific sequence or structural properties that define chaperonin dependence remains underdetermined. This difficulty is compounded by the fact that each subunit in the chaperonin recognizes diverse sequence determinants. While some motifs are frequently found in proteins that interact with TRiC, such as the WD40 β-propellers [88,89], they cannot be said to be clear identifiers. Consistent with the notion that there is no one feature that dictates chaperonin requirement, studies carried out in *in vitro* translation systems predict that approximately 10% of all cytosolic proteins transit through the TRiC complex [27]. This is in strong agreement with pulse-chase efforts that sought to quantitate the portion of the proteome that required the chaperonin for folding [103]. The proteins identified by Yam and colleagues lack clear sequence or fold conservation; however, they do have properties consistent with a greater potential for aggregation. Substrate proteins are often larger, have extended hydrophobic stretches, or are involved in multi-protein complexes [27]. The ability to assist such a breadth of proteins with diverse folds and sequence properties raises the potential for multiple mechanisms through which the complex can promote folding.

Recent *in vitro* work has illuminated the molecular determinants of substrate interaction with the apical domains of TRiC/CCT subunits. Using known subunit–substrate pairs, Joachimiak and colleagues have demonstrated that substrate motifs are recognized by a cleft formed between helix 11 (H11) and a proximal loop (PL) in the CCT apical domains (Fig. 3). While chemically distinct, this region bears structural semblance to the substrate binding site of GroEL [105]. They further demonstrate that the amino acid composition of the helix 11/proximal loop region in each subunit dictates the sequences that can be bound, whereby each apical domain presents a unique arrangement of charged and polar residues around a hydrophobic core. Kinetics of substrate binding for a panel of mutant apical CCT3 domains revealed that the k_{on} and k_{off} of binding were controlled by the flexible PL and H11, respectively. Interestingly, the PL of CCT3 is composed of a string of positively charged residues while H11 varies between polar and non-polar along the helix. In this model, the flanking hydrophilic residues define the nature of the substrate that a given apical domain recognizes, promoting association to a particular sequence. The hydrophobic patch then serves as an anchoring point for the substrate [106].

The Huntingtin protein contains a polyglutamine tract (polyQ) that promotes neuronal toxicity and aggregation when expanded beyond 35 consecutive glutamines, leading to Huntington's disease [92–95]. TRiC can modulate the aggregation of Huntingtin through interactions with specific subunits [92,95]. TRiC action is not due to direct binding of the expanded polyQ stretch. Rather, an amphipathic helix at the N-terminus of Htt-exon1 was found to be responsible for binding solely to the apical domain of CCT 1. Disruption of the hydrophobic face of this helix was shown to fully eliminate the interaction with both TRiC and the soluble apical domain of CCT 1 [104]. This clearly demonstrates what has come to be suspected about the nature of substrate binding within the group II chaperonins; the distinct features of the paralogous CCT subunits have evolved to recognize divergent sequence motifs.

While diversification at each subunit appears to have evolved to recognize a somewhat narrowly defined stretch of amino acids, the combinatorial nature of binding would afford the complex and large sequence space through which to interact with substrates. This suggests a model where subunit--specific contacts between TRiC and specific regions interspersed in the primary sequence of the unfolded polypeptides allow the hetero-oligomeric complex to bind stably to substrates through polyvalent interactions. For each subunit, the interaction relies on a recognition code integrating polar and hydrophobic contributions. As a result of the fixed arrangement of subunits in TRiC, the distribution of binding sites in the substrate will stipulate the global topology of the bound polypeptide, which in turn may direct substrate folding along a productive pathway. Indeed, instances of specific expansion within actin have been reported based on measurements that are based on fluorescence resonance energy transfer [107,108]. Such a mode of interaction demonstrates the capability the complex has to direct a folding trajectory.

One further challenge that the chaperonin must overcome is the fact that it has only a limited volume in which to protect proteins from the crowded cytoplasm. It has been estimated that the complex can encapsulate proteins up to ~ 60 kDa in size, given the observed chamber volume [40]. While many substrates of the chaperonin are smaller than 60 kDa [27,103], it has been shown that TRiC interacts with proteins that are larger than 70 kDa. The potential exists for proteins to be enclosed co-translationally or for specific domains to be sequestered within the chamber while the remainder of the protein sticks out into the cytosol. The latter has been demonstrated to occur with TRiC in *in vitro* translation systems using several fusions between actin and fluorescent proteins, as well as a natural substrate, hSnu114, which exceeds the chamber size [109]. There, encapsulation was monitored by the pattern of proteolytic fragments produced under different nucleotide conditions. In the case of actin constructs that were too large to fit within the chamber, fragments corresponding to the chaperonin-independent fluorescent proteins could be detected upon protease treatment of closed complexes. This was the case for all constructs observed when actin was terminal to the fused proteins, suggesting that the

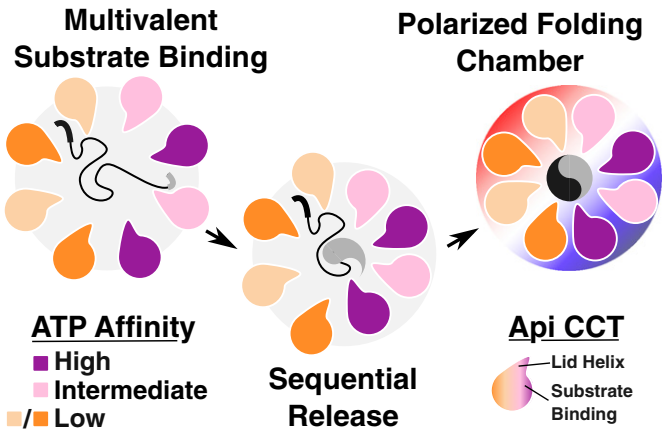

Fig. 5. A schematic view of factors promoting substrate folding by TRiC and other group II chaperonins. The substrate folding process involves multivalent binding of distinct substrate epitopes by the different TRiC apical domains. Release of substrate may proceed in a sequential fashion owing to the asymmetric usage of ATP by the eukaryotic group II chaperonin and the non-concerted nature of lid closure. Once encapsulated, substrates experience a polarized charge environment with one lobe of the complex demonstrating a positive electrostatic potential while the other one is negative.

remainder of the polypeptide was capable of being threaded out of the complex. However, no protection was observed when actin was flanked by the fluorescent proteins. Taken together, this suggests that, despite the walls of the chaperonin having space between subunits [62], threading of protein out of the chamber likely only occurs through the lid iris.

Combining this new appreciation for differential substrate recognition [106], the demonstration of substrate folding on a per-domain basis [109] and the knowledge that there is an asymmetric utilization of nucleotide among the CCT subunits [58] present an exciting picture of how a substrate may be released into the central chamber and folded upon ATP hydrolysis (Fig. 5). With the apical domains possessing specificity for a particular sequence, each substrate would be bound in an orientation defined by the presence and arrangement of different recognition motifs within its sequence. These would then be released in a defined, stepwise, progression as the complex closes upon ATP hydrolysis. A sequential release of substrate into the chamber could be therefore employed to control the folding trajectory of proteins with complex topologies. That these events occur, let alone serve as integral components to the chaperone mechanism, remains purely speculative. Demonstrating such time-resolved substrate release events or capturing the intermediates structurally represents some of the greatest opportunities for furthering the understanding of the group II chaperonin mechanism.

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