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Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets

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Abstract

Chaperonins are key components of the cellular chaperone machinery. These large, cylindrical complexes contain a central cavity that binds to unfolded polypeptides and sequesters them from the cellular environment. Substrate folding then occurs in this central cavity in an ATP-dependent manner. The eukaryotic chaperonin TCP-1 ring complex (TRiC, also called CCT) is indispensable for cell survival because the folding of an essential subset of cytosolic proteins requires TRiC, and this function cannot be substituted by other chaperones. This specificity indicates that TRiC has evolved structural and mechanistic features that distinguish it from other chaperones. Although knowledge of this unique complex is in its infancy, we review recent advances that open the way to understanding the secrets of its folding chamber.

Protein misfolding has been implicated in several human diseases that have both systemic and neurological implications, including ‘conformational diseases’ such as Huntington’s and Parkinson’s that are characterized by the accumulation of toxic protein aggregates [1]. Although small proteins with simple chain topologies can fold spontaneously, the vast majority of cellular proteins is unable to reach its native state without the assistance of elaborate cellular machinery composed of proteins known as molecular chaperones (for review, see Refs [1–4]). A complete understanding of chaperone-assisted protein folding in the cell would be an intellectual tour de force that might, eventually, lead to effective treatments for these diseases.

In the cell, protein folding faces additional challenges compared with the refolding of proteins in solution [1,2,4]. For example, *in vivo*, the linear polypeptide chain emerges vectorially into the cytosol during synthesis on ribosomes. Because the information for the native state is encoded by the entire amino acid sequence, the nascent polypeptide chain is unable to fold stably until fully synthesized, but it exposes hydrophobic sequences into the crowded cellular milieu [4]. Details of how newly translated proteins navigate toward a final, fully functional, folded structure *in vivo* are not entirely understood, but it is clear that exposed hydrophobic surfaces can contribute to misfolding and aggregation. Accordingly, all cellular compartments contain many structurally and functionally distinct classes of chaperones that vary in size and complexity, ranging from those that bind only to misfolded polypeptides and prevent their aggregation to those that recognize specific classes of proteins and facilitate their folding to the native state in an energy-dependent manner [1–3]. In cells, these different classes of chaperones work together to form elaborate, cooperative networks that ensure the correct folding of newly translated proteins; they also ensure that potentially damaging misfolded polypeptides are cleared from the cell [5].

The so-called ‘chaperonins’ comprise an intriguing class of oligomeric, high-molecular-weight chaperones that have the unique ability to fold some proteins that cannot be folded by simpler chaperone systems [2,3]. Chaperonins consist of two-ring assemblies that contain a central cavity to which unfolded polypeptides bind and where they reach the folded state [3,6]. Based on the ability to bind to unfolded polypeptides in their ring cavities, chaperonins prevent off-pathway reactions and facilitate productive protein folding to the native state in a highly cooperative, ATP-dependent manner.

There are two structurally distinct classes of chaperonins. Group I chaperonins are found in prokaryotic cells and endosymbiotic organelles, and group II chaperonins occur in Archaea and Eukarya [3,6]. Group I chaperonins, such as GroEL from *Escherichia coli*, consist of 14 identical subunits and require a ring-shaped cofactor, GroES, to function [7]. Following binding, GroES acts as a detachable lid for the cavity and creates a folding chamber that encloses polypeptide substrates [7]. By contrast, group II chaperonins, such as TCP-1 ring complex (TRiC, also named CCT for chaperonin-containing TCP1) in eukaryotic cells and the thermosome in archaea, are heterooligomeric complexes, with either eight or nine subunits per ring [6,8].

Both group I and group II chaperonin subunits share a similar basic structure (Figure 1a) that consists of three domains: an equatorial ATP-binding domain; an apical domain that is involved in substrate binding; and a central hinge domain that enables communication between the equatorial domain and the apical domain. The equatorial domain is relatively conserved among the paralog subunits, and most sequence divergence occurs in the apical domains, which are thought to contain the substrate-binding sites [9].

Unlike their bacterial counterparts, group II chaperonins seem to be fully functional in the absence of a GroES-like cofactor. The crystal structure of the thermosome (Figure 1b), a group II chaperonin from *Thermoplasma acidophilum*, provides a clue to the lack of a GroES cofactor because the complex seems to contain a ‘built-in’ lid [10] (Figure 1c). Each subunit of the thermosome complex can be superimposed onto a GroEL subunit, with the exception of an additional loop protruding from the tip of the thermosome apical domain. In the crystal, the apical protrusions form an iris-like lid structure that restricts access to the cavity [10] (Figure 1c). This crystal structure further indicates that the central chamber could encapsulate a polypeptide of up to 50 kDa in the closed state [10]. As described below, the apical protrusions have been shown recently to have a GroES-like function, acting as a built-in lid that opens and closes during the ATPase cycle of TRiC [11] (Figure 1d).

Recent studies indicate that there are significant mechanistic and functional differences between the eukaryotic chaperonin and its better-understood bacterial homolog GroEL. Here, we review the major advances in the understanding of the mechanism and substrate-binding properties of TRiC and highlight the questions raised by these studies. Better understanding of the folding cycle of TRiC might explain its unique ability to fold some eukaryotic proteins and reveal basic differences in the protein-folding machinery of prokaryotic cells.

Contribution of TRiC to cellular folding

The essential role of TRiC relates to its absolute requirement for folding of a subset of essential proteins. TRiC transiently interacts with ~10% of newly synthesized proteins [12]. Although initially proposed to fold only actin and tubulin, numerous non-cytoskeletal substrate proteins have been identified, including cyclin E, Cdc20 and the Von Hippel-Lindau tumor suppressor (VHL). Recently, the list of potential TRiC substrates has been expanded greatly by proteomic approaches and it includes many proteins that contain tryptophan-aspartic acid (WD) repeats, which form a β -propeller domain [13,14] (Table 1). No apparent sequence and structural motifs are shared by all TRiC substrates – even their sizes vary greatly and include some proteins with

a molecular weight > 100 kDa. As mentioned above, this exceeds the size that can be encapsulated in the central chamber [10] and raises the question of how large substrates are accommodated by the chaperonin. One possible scenario is that TRiC binds to and promotes folding of individual domains of these large substrates. Notably, many TRiC substrates cannot be folded by other prokaryotic and eukaryotic chaperones [15]. Because proteins with WD repeats, and other TRiC substrates such as cyclin E and VHL, are not found in bacterial genomes [16], it is tempting to speculate that TRiC and some of its substrates arose concurrently at the inception of eukaryotic cells and co-evolved [17]. Thus, understanding the substrate-binding determinants and mechanism of this chaperonin might have implications for understanding the evolution of many eukaryotic proteins.

An intriguing commonality among many of the TRiC substrates identified so far is that they are subunits of either homo- or hetero-oligomeric complexes (Table 1). For these proteins, folding and function is coupled to their incorporation into higher-order assemblies [18]. Furthermore, for some substrates, such as tubulin, VHL and CDC20, release from TRiC occurs only in the presence of their partner proteins and is coupled to substrate assembly into the oligomeric complex [19–21]. This mechanism might provide a quality-control strategy that prevents the premature release of an unassembled component that might function in a dominant-negative manner. For example, several substrates of TRiC function as substrate-recognition components of ubiquitin ligase complexes, including VHL in the VHL–elongin BC (VBC) ligase, and Cdc20 and Cdh1 in the anaphase-promoting complex [22]. If folded and released into the cytosol without assembly into the functional ligase complex, these catalytically inactive substrate receptors might function as dominant-negative proteins that impede the proteolytic elimination of key regulatory proteins. TRiC might prevent this by liberating the newly synthesized subunits only as part of the assembled ligase complex.

TRiC–substrate interactions

Because no clear structural and sequence features are shared by the known substrates of TRiC, it is unclear how these substrates are selected from all newly made polypeptides. Unlike the bacterial chaperonin GroEL, TRiC can bind co-translationally to nascent chains as they emerge from the ribosomes [23–26]. In the cell, binding to TRiC requires the assistance of upstream chaperones. The emerging polypeptide chain is transferred from the ribosome to TRiC via either the chaperone GimC [26] or the Hsp70 chaperone machinery [23,24]. Although GimC and Hsp70 have partially overlapping functions, GimC seems to be specific for actin and tubulin [26]. The cooperation between TRiC and other chaperone systems is well established but it is unclear whether these chaperones have a role in determining the classes of proteins that interact with TRiC in the cell.

How does TRiC recognize its substrates? By analogy to group I chaperonins, substrate recognition by TRiC is believed to occur at the apical domain but the exact location of the binding sites in this domain is not defined. Three hypotheses have been proposed (Figure 2a). First, substrate binding might be mediated by structural elements that are homologous to those defined in GroEL, namely two helices in the distal region of the apical domain [3,7]. Alternatively, it has been suggested that the helical protrusions might contain substrate-binding sites, based on their structural flexibility and partial hydrophobic character [6,27]. However, substrate binding is not impaired in a group II chaperonin in which the helical protrusion is deleted [28], which indicates that this region is not essential for binding. A third hypothesis [29], based on evolutionary analyses, proposes that the binding sites reside in the inner face of the closed cavity and consist mostly of charged and polar amino acids. In this view, binding sites for polar residues direct the chaperonin to recognize surface-exposed, charged and polar regions in substrate proteins, which stabilizes the substrate in a structured, quasi-native conformation [30]. Based on the available data, it is not possible to favor one model at present.

Given the hetero-oligomeric nature of the TRiC subunits and the ensuing sequence diversity in apical domains, an attractive hypothesis is that different subunits recognize different types of motifs, including both polar and hydrophobic-recognition sites. Stable interactions between an unfolded polypeptide and TRiC seem to result from a set of multivalent, weak interactions between defined motifs in the substrate and individual chaperonin subunits [25,31,32]. Thus, it is possible that the sequence divergence of TRiC subunits expands the range of possible motifs that are accepted in substrates beyond the simple hydrophobic sites offered in group I chaperonins. However, the search for TRiC-recognition determinants in substrate proteins has yielded conflicting data. Studies using actin indicate several possibilities, ranging from polar and charged sequences surface-exposed on the native protein [33] to delineated, hydrophobic sequences [34]. Recognition of native, surface-exposed, charged elements is novel for a molecular chaperone and raises questions of how binding interactions might be disrupted permanently to release the folded substrate and how TRiC might discriminate between native and non-native states. Clearly, defining the structural basis of TRiC–substrate interactions will help answer these questions.

The best-characterized TRiC-recognition determinants in a substrate are those of the tumor suppressor protein VHL [31] (Figure 2b). Recognition of VHL by TRiC involves combinatorial interaction with two linear motifs in the polypeptide (Figure 2b). Each motif is comprised of a short β strand, which is enriched in hydrophobic side-chains that are required for binding to TRiC [31]. Because the side-chains that contribute to TRiC binding are aligned on the same side of the β strand in native VHL it is possible that they provide a hydrophobic surface that interacts with the chaperonin. The identification of β strands as TRiC-recognition motifs resonates with the observation that many candidate chaperonin substrates contain WD β -propeller domains that are composed entirely of anti-parallel β strands [13,14]. Detailed studies of two substrates that contain a WD domain show that their TRiC-binding determinants also reside in the β strands of this domain [21] (Figure 2c). These results indicate a role for TRiC in facilitating folding of domains that contain β sheets. Folding of β -sheet-containing domains is notoriously difficult because it involves long-range contacts between β strands that are often hydrophobic and inherently aggregation-prone [35]. Domains that contain β strands seem to fold more slowly than α helices because of the longer search times that are needed to establish interactions between non-contiguous sequences. Localization of chaperonin-binding elements within β strands might prevent either strand-swapping between domains or the formation of kinetically-trapped, non-productive folding intermediates that can lead to aggregation. TRiC binding to individual strands could also stabilize a β -sheet-containing domain until it is synthesized fully, at which point concerted release into the protected environment of the chaperonin cavity would ensure productive folding.

Nucleotide-induced conformational changes in TRiC

A crucial question in understanding the TRiC folding mechanism is how ATP-binding and hydrolysis drive the conformational changes that result in productive folding. In the absence of nucleotide, the apical domains of TRiC are in an open conformation that exposes the substrate-binding sites [36–38] (Figure 1d and Figure 3a). Addition of ATP induces formation of the closed lid, which confines the substrate in the central cavity. Experiments with non-hydrolyzable analogs of ATP indicate that ATP binding alone does not promote closure of the lid [11,39]. Nevertheless, these non-hydrolyzable nucleotides do induce a conformational change in TRiC, which is observed by tryptophan fluorescence [40], which might, in turn, affect the conformation of the bound substrate (Figure 3b). However, neither ATP-binding nor ADP-binding can support actin folding, which requires hydrolysable ATP [11]. The requirement for hydrolysable ATP is linked to the dramatic conformational rearrangements that the γ -phosphate of ATP undergoes during the hydrolysis reaction. Thus, mimics of the trigonal–bipyramidal transition state of the ATPase reaction, such as ADP–AlF_x [41,42],

induce formation of the closed lid [11]. Lid formation then confines the bound substrate in the central cavity (Figure 3c) [11]. After release of the γ -phosphate, ADP-bound TRiC is again in an open state [43]. One striking feature of the ATPase cycle of TRiC is that it lasts ~4 min [43], which is much slower than that of GroEL and other chaperones. Biophysical experiments indicate that the chaperonin spends a large proportion of its kinetic cycle in the closed state, which indicates that opening the lid is the rate-limiting step (Figure 3d). This is, perhaps, not surprising, because lid opening involves breaking a highly ordered β -sheet ring. The event that triggers lid opening has not been determined precisely. TRiC adopts an open conformation in the presence of Mg-ADP, which argues for a role of either bond scission or P_i dissociation in opening the lid (Figure 3d). In principle, the conformational change induced by the transition state of ATP hydrolysis could persist after bond scission, provided that P_i is still in the nucleotide pocket, as suggested for the thermosome [44].

How are these conformational changes coordinated between TRiC subunits? Each subunit of one TRiC ring is thought to bind to and hydrolyze ATP during the conformational cycle. Therefore, allosteric communication between the subunits is likely to be required to organize the actions of subunits within and between the rings. Kinetic studies have shown that subunits of one ring bind to ATP in a positively cooperative manner [40,45], which indicates a concerted mode of action during lid closure. The subunits might change their conformation either simultaneously or sequentially around the ring. Genetic evidence indicates a sequential mode of action [46], but this remains to be addressed biochemically.

An important question for understanding the TRiC mechanism is whether it operates as a ‘two-stroke motor’, by alternating conformations in each ring as shown for group I chaperonins. In addition to positive, intra-ring cooperativity, biochemical experiments demonstrate negative inter-ring cooperativity [40,45]. This allostery results in a decrease in affinity for nucleotide binding in one ring when the other is already occupied. Biochemical and biophysical assays of TRiC [11] in the presence of the transition state analog ADP-AlF_x indicate the existence of an asymmetric conformation of TRiC in which the lid in only one ring is closed. Thus, it seems that TRiC possesses an intrinsic mechanism to generate an asymmetric conformation, which keeps both rings in different stages of the conformational cycle.

Mechanism of substrate folding

Although chaperone-assisted folding has been studied for nearly a decade, the exact role of chaperonins with respect to the substrate is still a mystery, even for the well-studied chaperonin GroEL [1,3]. When closed, the central folding cavity seems to provide an optimal chemical environment that minimizes entropy and maximizes folding potential of an unfolded polypeptide. One interesting aspect of TRiC function is its unique ability to fold proteins such as actin, which is not folded by any other chaperone [15]. This specificity indicates that TRiC operates by a fundamentally different mechanism of folding. In turn, the distinct structural and mechanistic features of TRiC might have enabled the evolution of some eukaryotic proteins. Although the precise mechanism by which TRiC mediates substrate folding remains to be determined, recent studies are beginning to shed light on basic aspects of this problem.

One controversial aspect of TRiC-mediated folding is whether substrates bind in either a quasi-native or unstructured conformation. Biochemical experiments indicate that TRiC-bound actin is highly protease-sensitive [11], which indicates a largely unstructured conformation. In a similar manner, the TRiC-binding sites of VHL are located in the core of the native structure, indicating that VHL is not folded significantly while bound to TRiC [31]. These observations indicate that substrates either bind to TRiC in an unfolded state or that they are induced to unfold on binding. By contrast, cryoelectron microscopy (cryoEM) images of denatured actin and tubulin in a complex with TRiC show the substrates binding in a native-like, structured

conformation [30]. Further work to integrate structural and biochemical analyses is required to reconcile these differences.

Another question concerns the role of the built-in lid in substrate folding. In group I chaperonins, GroES has an essential role in linking ATP-hydrolysis to productive folding of the substrate confined inside the GroEL cavity. The importance of confinement of the folding substrate inside the central cavity of TRiC has been addressed recently using a modified TRiC that is unable to form a lid [11]. This lid-less variant of TRiC still hydrolyzes ATP and binds to unfolded actin at wild-type levels but is unable to mediate actin folding. Thus, it seems that the built-in lid of TRiC has a GroES-like role in coupling the ATP hydrolysis reaction to productive folding of substrate. Lid formation also leads to substrate encapsulation, as shown by biochemical experiments [11] and cryoEM reconstructions that situate actin and tubulin inside the closed cavity [47]. As mentioned before, some TRiC substrates are too large to be accommodated in the chaperonin cavity, which is 50 Å in diameter [10]. These proteins might undergo domain-wise folding outside of the cavity by a mechanism that does not involve total encapsulation of the substrate.

Relevance of TRiC studies for health and disease

It is becoming increasingly clear that TRiC is linked to several pathological states. For example, a spontaneous, recessive mutation in subunit 4 of TRiC, which substitutes a highly conserved cysteine for tyrosine, results in sensory neuropathy [48]. In addition, tumor-causing mutations in the TRiC-binding sites of the VHL tumor suppressor lead to severe misfolding of VHL *in vivo*, despite the intrinsic ability of the mutant protein to reach the native state. This indicates that disease-causing mutations might inactivate protein function by interfering with chaperonin-mediated folding [31]. Experiments in *Caenorhabditis elegans* show that decreasing the concentration of TRiC subunits by RNA interference enhances the formation of polyglutamine aggregates, such as those found in Huntington's disease [49]. Because these aggregates consist of highly ordered β -sheet structures, it is tempting to speculate that the specificity of TRiC for substrates that contain β sheets helps the chaperonin suppress aggregation through a direct interaction with the polyglutamine protein.

In addition to folding nascent polypeptides, TRiC also seems to have roles in other cellular processes. In antigen presentation, TRiC is a cytosolic component that binds to post-proteasomal cleavage products, protects them from degradation and transfers them to the endoplasmic reticulum for antigen presentation on MHC class I molecules [50]. In addition, TRiC might assist the posttranslational translocation of proteins across the endoplasmic reticulum membrane by maintaining them in a state that is competent for transport [51]. These findings highlight the essential role of this chaperonin in different cellular processes and call for a better understanding of the structural motifs that direct polypeptides to specific, chaperon-mediated, folding pathways.

Future perspectives

The unique structural and mechanistic features of the eukaryotic chaperonin TRiC probably underlie its ability to fold an essential, diverse subset of eukaryotic proteins. Despite the biomedical and biotechnological implications of elucidating how TRiC folds its substrates, little is known about its mechanism and substrate-recognition determinants, particularly compared with chaperones such as Hsp70 and GroEL. As summarized in this article, recent progress in understanding the basic aspects of TRiC function raises many fundamental questions. Considering the relevance of TRiC to cellular folding, the coming years are likely to provide the answers to many of these fascinating problems.

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Figure 1.

General architecture of group II chaperonins. **(a)** Ribbon diagram of an α subunit of the thermosome from *Thermoplasma acidophilum*. The equatorial ATPase domain (red) is linked to the substrate-binding apical domain (yellow) by a flexible hinge or intermediate domain (blue). The helical protrusion, which is unique to group II chaperonins, is in green. **(b)** Side view of the closed conformation observed in the X-ray structure of the thermosome [10], with subunit domains colored as in (a). Viewed from the side, the oligomeric structure is formed by two octameric rings. In TCP-1 ring complex (TRiC), each ring is composed of eight subunits. **(c)** Top view of the closed thermosome structure [10] that highlights how the apical protrusions close into an iris-like lid. For clarity, only apical domains are shown, with one domain in red. **(d)** Bead models of the ATP-induced transition from the open to closed state for group II chaperonins. The model of the nucleotide-free, open state (left) is based on electron tomographic studies on the thermosome. The closed state is from the X-ray structure of the thermosome [10] and presumably reflects the ATP-induced state.

Figure 2.

Molecular determinants of TCP-1 ring complex (TRiC)–substrate interaction. **(a)** Proposed location of substrate-binding sites in the apical domains of TRiC. The individual regions of the three hypothetical substrate-binding sites are highlighted in red. (i) Based on analogy to GroEL. (ii) Based on structural flexibility and hydrophobic character. (iii) Based on amino acid conservation between ortholog but not paralog subunits. **(b)** Location of TRiC-binding sites in the Von Hippel-Lindau tumor suppressor (VHL) substrate. Folded VHL contains a β domain (cyan) that contains multiple, antiparallel β strands. Boxes 1 and 2 (red), which are required for association with TRiC, are located in adjacent β strands. **(c)** Location of TRiC-binding sites in the tryptophan-aspartic acid (WD) domain of substrate CDC20. The subset of antiparallel β sheets that is required for association with TRiC is highlighted in red. The WD domain of CDC20 is modeled on its structural homolog β -transducin [63].

Figure 3.

Model of the nucleotide cycle of the eukaryotic chaperonin. **(a)** In the absence of nucleotide, the open complex can bind to unfolded substrates (U-substrate) through binding sites in the central cavity (red lines). **(b)** ATP binding alone does not produce closure of the lid and significant folding of the substrate, at least in the case of actin. **(c)** Formation of the trigonal-bipyramidal transition state of the hydrolysis reaction triggers lid closure and confines the substrate in the central cavity. Folding probably occurs at either this stage of the cycle or following scission of the β - γ phosphate bond. **(d)** Bond scission or inorganic phosphate (P_i) dissociation is likely to trigger reopening of the lid and the release of folded substrate. The cycle is probably asymmetric but the mechanism that keeps both rings in different stages of the hydrolytic cycle is unclear. Consequently, changes in only one of the two rings are shown (blue). Reproduced, with permission, from Ref. [11].

Table 1

Substrates of TCP-1 ring complex

Protein	Molecular weight (kDa) ^a	Participates in oligomeric complex	WD repeat motif	Refs
α Actin, β actin	42.1, 41.7	Yes?	No	[37]
α Tubulin, β tubulin, γ tubulin	50.2, 49.8, 51.2	Yes	No	[52,53]
Myosin heavy chain	223.0	Yes	No	[54]
Luciferin	60.1	No	No	[23]
4-monooxygenase G α -transducin	40.0	Yes	No	[55]
Von Hippel-Lindau disease tumor suppressor	24.2	Yes	No	[20]
G1-S-specific cyclin E1	47.1	Yes	No	[56]
Cofilin	18.5	No	No	[53]
Actin-depolymerizing factor 1	16.1	No	No	[53]
Actin-related protein V (centractin)	42.6	Yes	No	[57]
Hepatitis B virus capsid protein	20.9	Yes	No	[58]
EBNA-3 nuclear protein	89.1	No?	No	[59]
Gag polyprotein of M-PMV	73.1	Yes	No	[60]
Histone deacetylase 3	48.8	Yes	No	[61]
SET domain protein 3	85.5	Yes	No	[62]
Probable histone deacetylase HOS2	51.5	Yes	No	[62]
Cell division control protein 20 (Cdc20p)	67.4	Yes	Yes	[21]
Cell division control protein 15 (Cdh1p)	110.4	Yes	Yes	[21]
Protein phosphatase PP2A regulatory subunit B (Cdc55)	59.6	Yes	Yes	[26]
Peroxisomal targeting signal 2 receptor (Pex7p)	42.3	No	Yes	[26]
Pre-mRNA splicing factor PRP46	50.7	Yes	Yes	[26]
Coatomer β' subunit (Sec27p)	99.4	Yes	Yes	[26]
Guanine-nucleotide-binding protein β subunit (Ste4p)	46.6	Yes	Yes	[26]

^aDeduced from amino acid sequences.