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Chaperonin GroEL Meets the Substrate Protein as a "Load" of the Rings

Hideki Taguchi*
Graduate School of Frontier Sciences, University of Tokyo and PRESTO, Japan Science and Technology Agency, FSB401, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562

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Chaperonin GroEL is an essential molecular chaperone that assists protein folding in the cell. With the aid of cochaperonin GroES and ATP, double ring-shaped GroEL encapsulates non-native substrate proteins inside the cavity of the GroEL-ES complex. Although extensive studies have revealed the outline of GroEL mechanism over the past decade, central questions remain: What are the *in vivo* substrate proteins? How does GroEL encapsulate the substrates inside the cavity in spite of an apparent entropic difficulty? Is the folding inside the GroEL-ES cavity the same as bulk spontaneous folding? In this review I summarize the recent progress on *in vivo* and *in vitro* aspects of GroEL. In particular, emerging evidence shows that the substrate protein itself influences the chaperonin GroEL structure and reaction cycle. Finally I propose the mechanistic similarity between GroEL and kinesin, a molecular motor that moves along a microtubule in an ATP-dependent manner.

Key words: chaperone, chaperonin, folding, GroEL, GroES, kinesin.

Chaperonins are a ubiquitous class of molecular chaperones that promote protein folding in the cell (*1*, *2*). Chaperonins are essential proteins that form large ring-shaped complexes and found in eubacteria, chloroplasts, mitochondria, archaea, and the eukaryotic cytosol. The best-characterized is the *Escherichia coli* GroEL and its partner GroES, which function together as a complex molecular machine (*3*–*6*). The double-ring GroEL tetradecamer encapsulates non-native substrate protein in the central cavity when capped by GroES heptamer in an ATP-dependent manner. How GroEL/GroES assist protein folding by using input of energy is one of the challenges in the field of molecular chaperones. In addition, an ultimate understanding of chaperonin function might answer the longstanding question of whether the chaperonin-assisted protein folding is the same as spontaneous folding in the bulk solution, possibly resulting in a verification of the Anfinsen's dogma, which states that the tertiary structure of a protein is solely determined by its amino acid sequence, without the input of energy (*7*).

Chaperonin performs two exclusive functions (Fig. 1A): (i) binding of non-native substrate proteins to prevent irreversible aggregation (the *holder* function), and (ii) release of the arrested protein to complete folding (the *folder* function). The chaperonin GroEL is a proteinous nano-machine that successfully balances the two competing functions for the binding and the release by using ATP and GroES.

As a "*holder*", GroEL captures a large spectrum of non-native proteins (*8*). To see the *holder* function directly, I have generated a simple, impressive demonstration using hen egg white and a GroEL-ES complex from *Thermus thermophilus* (Fig. 1B). After incubation at 70°C, the diluted egg white became opaque due to heat denaturation, whereas in the presence of the thermophilic GroEL-ES the egg white remained clear, indicating that the chaperonin prevented its irreversible aggregation.

The "*folder*" function involves the release of the bound substrate protein from GroEL, which is accompanied by an ATP-induced large conformational change of GroEL. The release of the substrate protein into the GroEL-ES cavity, in which folding to the native state can proceed without aggregation, is the most productive mechanism for the folding. An *in vitro* experiment showed that protein of up to ~57 kDa can be accommodated in the GroEL-ES cavity, and that green fluorescent protein (GFP) dimer (~54 kDa) can complete the folding (*9*) (Fig. 1C).

In this review, I summarize the recent progress on the molecular mechanism of the chaperonin GroEL from *in vivo* and *in vitro* studies. In particular, the influence of non-native substrate protein on the function and structure of GroEL are introduced.

I. GroEL-GroES *in vivo*

Although the study of chaperonin began with *in vivo* approaches (*10*), mechanistic investigation *in vitro* has been obviously developed over the past decade. In this section, I summarized recent knowledge on GroEL and GroES *in vivo*.

Structure of the native GroEL-ES complex accommodating substrate proteins. Structural information on the GroEL and GroEL-ES complex has only been obtained for reconstituted forms (*11*, *12*). In the *T. thermophilus* (*T.th*) chaperonin, the GroEL-ES complex can be purified without dissociation of GroES throughout the purification (*13*, *14*). This means that the chaperonin complex holds the *in vivo* substrate proteins inside the cavity (see below for the list of the substrate proteins). Structural comparison of the native *T.th* GroEL-ES complex with the *E. coli* "empty" complex reveals remarkable

*For correspondence: Phone: +81-4-7136-3644, Fax: +81-4-7136-3644, E-mail: taguchi@k.u-tokyo.ac.jp

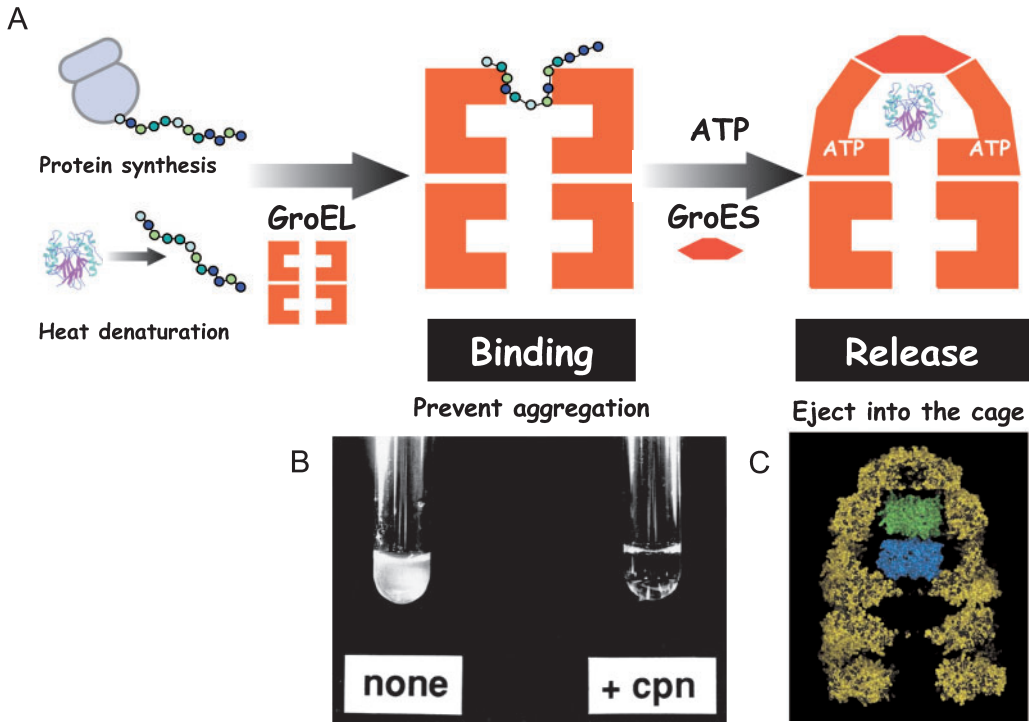

Fig. 1. **(A) Schematical drawing of chaperonin function. (B) Incubation of hen egg white without (left) or with (right) thermostable chaperonin.** Egg white was diluted 50-fold by adding buffer (25 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$) in the absence (left) or presence (right) of purified chaperonin from *T. thermophilus* (cpn, 6 mg protein/ml). Both solutions were incubated at 70°C for 10 min. (C) Simulated packing of two GFP molecules (GFP-BFP fusion protein) in the *cis* GroEL-ES cavity.

differences, including the fact that the apical domain around the *cis*-cavity of the *T.th* complex exhibits a large deviation from the 7-fold symmetry (*15*). Apical domain motions in *E. coli* GroEL upon binding of a strongly binding peptide are also reported (*16*).

Post-translational *vs.* co-translational involvement. In *E. coli*, three major chaperone systems are considered to contribute to the folding of newly synthesized polypeptides. Trigger factor (TF), a ribosome-tethered chaperone, and DnaK are known to exhibit overlapping co-translational roles, whereas GroEL is believed to be implicated in folding only after the polypeptides are released from the ribosome (post-translational role) (*2*, *17*). The overlapping role of TF and DnaK in the co-translational manner was suggested by the finding that their simultaneous deletion caused synthetic lethality (*18*, *19*). However, recent genetic analyses reveal that the lethality is abrogated either by growth at low temperature or by overproduction of GroEL/GroES (*20*, *21*). The latter strongly suggests that GroEL substitutes for TF and DnaK by interaction with newly translated peptides co-translationally. Recently, puromycin-sensitive association of GroEL/GroES with translating ribosomes *in vivo* has been reported (*22*). Further experiments *in vitro*, using a chaperone-free reconstituted cell-free translation system (*22*), clearly demonstrate that GroEL associates with the translating ribosome complex and accomplishes proper folding by encapsulating the newly translated polypeptides in the chaperonin cavity. Therefore, it has been proposed that GroEL is a versatile chaperone, which participates in the folding pathway co-translationally and also achieves correct folding post-translationally.

***In vivo* substrate proteins.** Overexpression of GroEL/ES or the conditional deletion of GroEL affect the fate of a broad spectrum of proteins in *E. coli* (*23*, *24*). *In vivo* observation of protein flux through the GroEL system estimates that ~10% of newly translated polypeptides are assisted by the GroEL system (*25*).

Recent development of proteomic analysis enables us to survey the GroEL substrate proteins *in vivo*. Houry *et al.* identified ~50 *in vivo* substrate proteins interacting with GroEL in *E. coli* (*26*). They suggest that the GroEL substrates consist preferentially of two or more domains with an αβ-fold, which contain an α-helix and buried β-sheets.

It is of great interest to know what substrates are encapsulated inside the GroEL-ES cavity. As mentioned above, the native GroEL-ES complex from *T. thermophilus* can be purified without the dissociation of GroES during the purification. After the removal of the substrates bound to the *trans* GroEL ring, 24 of the most abundant substrates caged in the *cis*-cavity were identified from the crystal of the native GroEL-ES complex (*15*) (Table 1). These substrates show no similarity in sequence, motif or isoelectric point (pI). Only three (Upp, ThiD, and RpoA) have so far been found to be *E.coli* GroEL-interacting substrates and *T. th* chaperonin substrates. Structually, all of the identified proteins (including homologues) contain αβ-folds, as suggested for *E. coli* GroEL-interacting substrates.

Table 1. ***In vivo* substrate proteins inside the native *T.th* GroEL-ES complex.**

Mw (kDa)	pI	Name	Category	PDB (1)	fold
12.8	10.5	50S ribosomal protein L22	Protein synthesis / Ribosomal proteins: synthesis and modification	1BXE	αβ–fold
20.8	5.1	adenylate kinase	Purines, pyrimidines, nucleosides, and nucleotides	(4AKE)	αβ–fold
22.4	7.4	4-diphosphocytidyl-2C-methyl-D-erythritol synthase (IspD)	Biosynthesis of cofactors, prosthetic groups, and carriers / Isoprenoid	(1I52)	αβ–fold
22.8	6.7	uracil phosphoribosyltransferase (Upp)	Purines, pyrimidines, nucleosides, and nucleotides	(1I5E)	αβ–fold
23.4	5.9	conserved hypothetical protein	Hypothetical / Conserved		
23.8	9.2	thiamin-phosphate pyrophosphorylase (ThiE)	Biosynthesis of cofactors, prosthetic groups, and carriers / Thiamine	(1G4E)	αβ–fold
24.8	10.3	probable RecO protein	DNA metabolism / DNA replication, recombination, and repair		
25.2	5.6	HisA	Amino acid biosynthesis / Histidine family	(1QO2)	αβ–fold
26.2	5.5	probable haloacid dehalogenase	Unknown / General		
26.8	9.0	phosphomethylpyrimidine kinase (ThiD)	Biosynthesis of cofactors, prosthetic groups, and carriers / Thiamine	1UB0	αβ–fold
27.4	5.5	indole-3-glycerol phosphate synthase (TrpC)	Amino acid biosynthesis / Aromatic amino acid family	(1IGS)	αβ–fold
29.6	7.6	probable methyltransferase	Unknown / General		
33.3	9.2	methionyl-tRNA formyltransferase	Protein synthesis / tRNA aminoacylation	(1FMT)	αβ–fold
35.0	4.8	DNA-directed RNA polymerase alpha chain (RpoA)	Transcription / DNA-dependent RNA polymerase		
36.4	6.8	conserved hypothetical protein	Hypothetical / Conserved		
36.9	5.9	rod shape-determining protein (MreB)	Cellular processes / Cell division	(1JCE)	αβ–fold
40.9	8.3	UDP-*N*-acetylglucosamine 2-epimerase	Cell envelope / Biosynthesis of surface polysaccarides	(1F6D)	αβ–fold
41.1	9.8	putative glycosyltransferase	Cell envelope / Biosynthesis of surface polysaccarides		
41.1	5.9	conserved hypothetical protein	Hypothetical / Conserved		
42.2	8.6	probable glycosyltransferase	Unknown / General		
42.9	7.2	putative acyl-CoA dehydrogenase	Fatty acid and phospholipid metabolism / Degradation		
44.6	9.5	probable tRNA/rRNA methyltransferase	Protein synthesis / tRNA and rRNA base modification		
45.3	8.2	probable glycosyltransferase	Unknown / General		
46.7	6.2	UDP-acetylmuramoylalanyl-glutamyl-diaminopimelate-alanyl ligase	Cell envelope / Biosynthesis of murein sacculus and peptidoglycan	(1GG4)	αβ–fold

PDB files listed are either those of the protein itself (no brackets) or of a close homologue (in brackets).

II. Reaction cycle of GroEL-GroES in the presence of substrate protein

Understanding the reaction cycle of GroEL is a central task. This section describes how GroEL assists the substrate protein folding.

Timer mechanism for GroEL-GroES cycling. The prevailing GroEL reaction cycle is as follows (*4*, *27*, *28*) (Fig. 2A). (i) Non-native substrate protein binds to GroEL near the inner rim of the central cavity. (ii) Binding of ATP to the polypeptide-containing ring of GroEL permits the binding of GroES to that ring, accompanied by release of polypeptide into a cavity (*cis*-ATP complex). (iii) Bound ATP is hydrolyzed (*cis*-ADP complex) and GroES is released upon subsequent ATP binding to the *trans*-ring of GroEL, permitting native or partially folded proteins to leave GroEL. The final ATP-triggered dissociation of the *cis*-ADP complex is accelerated by the presence of substrate proteins bound to the *trans*-ring.

Single-molecule analysis of GroEL function. The cycle model outlined above predicts that the lifetime of the GroEL-ES complex is governed by a single rate-limiting step, ATP hydrolysis, in the presence of saturating substrate proteins. However, direct observation of GroEL-GroES cycling at a single-molecule level has revealed more complicated kinetics for the GroEL-ES dynamics (*29*, *30*). After the GroES binding to GroEL, GroES remains for ~3 s (lag period), and departs GroEL over ~5 s (*29*). The single-molecule technique has been extended to the single-molecule GFP folding inside the GroEL-ES cavity, which shows the arrest of GFP folding for the first ~3 s in the cavity (*30*). Further bulk-phase kinetics including fluorescent resonance energy transfer (FRET) between substrate protein and GroEL, several assays for ATP hydrolysis, also support the presence of two rate-limiting steps in the GroEL functional cycle (*30*). Taken together, the successive two timers of ~ 3 s and ~5 s duration has been proposed (*30*) (Fig. 2B).

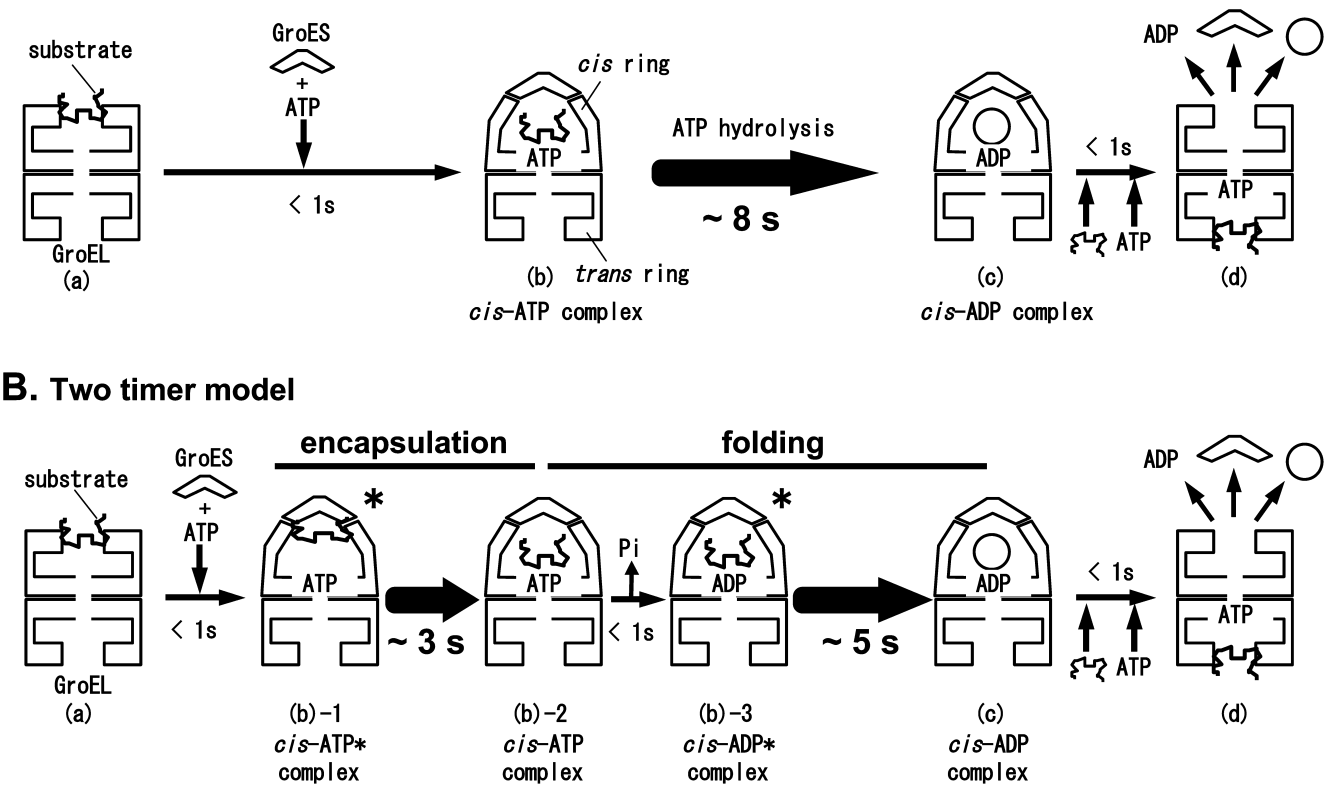

Fig. 2. **(A) Single-timer model.** Only a single rate constant governs all events of the functional GroEL cycle in the presence of saturating amounts of ATP, GroES and substrate proteins. In this model, neither the release of GroES from GroEL nor folding inside the cavity show a lag period. **(B) Two-timer model.** The functional GroEL cycle has two rate-limiting steps in the presence of saturating amounts of ATP, GroES and substrate proteins. Binding of GroES to the complex of GroEL-ATP-substrate protein produces the *cis*-ATP* complex, in which substrate protein is not fully released into the cavity. The first timer (lifetime, ~3 s) is the "ATP-transition," which is used to encapsulate non-native substrate protein into the cavity. ATP hydrolysis and P_i release of the *cis*-ATP complex occur rapidly to produce the *cis*-ADP* complex. The second timer (lifetime, ~5 s) is the "ADP-transition."

Two-timer mechanism. In the two-timer model, the GroEL-substrate protein complex binds ATP and GroES to generate a *cis*-ATP* complex in which, unlike the *cis*-ATP complex in the single timer model, folding of substrate protein is arrested. The *cis*-ATP* complex is transformed to the *cis*-ATP complex with a lifetime of ~3 s. As soon as this transition has taken place, the substrate protein becomes folding-competent in the *cis*-cavity, ATP is hydrolyzed, and phosphate is released. The lifetime of the *cis*-ATP complex is very short, but folding continues in the resulting *cis*-ADP* complex. The *cis*-ADP* complex is further transformed to the *cis*-ADP complex with a lifetime of ~5 s. Like the *cis*-ADP complex in the single timer model, the *cis*-ADP complex in the two-timer model can accept ATP and substrate protein to its *trans*-ring, which immediately induces the decay of the *cis*-ternary complex.

The essence of GroEL function is encapsulation of the substrate protein into a narrow cage in a protected compartment. The *cis*-ATP* complex can solve the apparent difficulty of how GroEL coordinates the binding and release of both GroES and substrate protein. If GroES and denatured protein compete for the same binding site in a mutually exclusive manner, substrate protein must leave GroEL before GroES binding. Then, most of the released substrate proteins should diffuse away in the bulk solution before GroES caps the cavity, and this would result in the mere displacement of the non-native protein by GroES. Therefore, efficient encapsulation of the non-native protein into the *cis*-cavity necessitates an intermediate state in which GroES caps the cavity but non-native protein is still bound to GroEL. This would ensure the efficient encapsulation of polypeptide into the *cis*-cavity of GroEL and hence a productive folding.

The Load of the rings. Binding of either ATP or ADP promotes the formation of GroEL-ES complex. However, only ATP can trigger the productive folding inside the GroEL-ES cavity. What is the difference between ATP and ADP? Recent mechanistic studies open the way to solve the conundrum and introduce a new concept that the substrate protein acts as a load of GroEL.

Although an argument had been made for the requirement of ADP to promote the folding of stringent substrates such as rhodanese (*31*), complete elimination of contaminating ATP by hexokinase clearly showd the exclusive role of ATP for such substrates (*32*). Since ADP plus a metalfluoride such as ADP-AlF (*33*) or ADP-BeF (*34*) also supports the productive folding of stringent substrates, the role of gamma phosphate in the nucleotide is critical to the GroEL function.

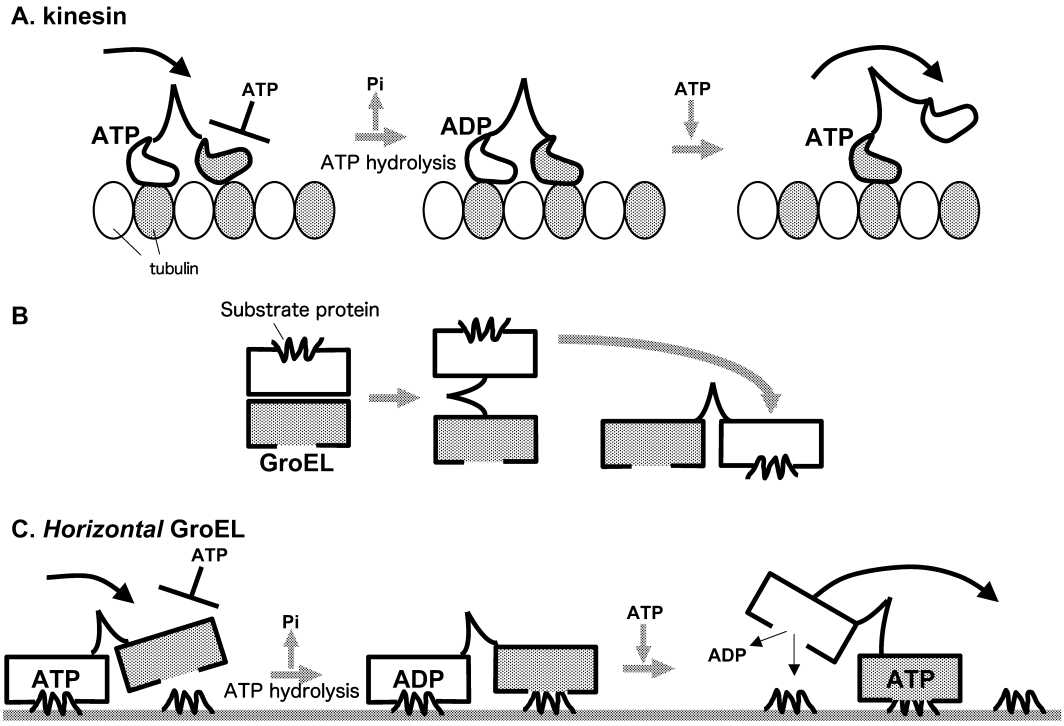

Fig. 3. **(A) Simplified hand-over-hand model of conventional double-head kinesin. (B) Virtual topological change of GroEL double ring.** Ring-ring interface in GroEL is peeled apart and the rings are connected with a linker. Then, the vertical topology of GroEL is changed to a horizontal arrangement. **(C) Virtual functional cycle model of the "horizontal" GroEL.**

With ADP, GroES bound quickly to GroEL without substrate protein (k_{on} ~10^7 M^{-1} s^{-1}), but bound very slowly to the GroEL loaded with a substrate such as rhodanese (k_{on} ~10^3 M^{-1} s^{-1}) or malate dehydrogenase (k_{on} ~10^5 M^{-1} s^{-1}) (*32*). In contrast, ATP promoted rapid binding of GroES to GroEL (k_{on} ~10^7 M^{-1} s^{-1}) regardless of the presence of a stringent substrate (*32*). A further important finding is that the load of the GroEL rings affects the rate of nucleotide-induced conformational change of GroEL (*35*). Direct monitoring of apical domain movement in GroEL by *intra*molecular FRET showed that the rate of conformational change in GroEL loaded with stringent substrates with ADP/GroES slowed by >100-fold compared with that with ATP/GroES (*35*). It has been proposed that the binding of ATP, not ADP, is required for driving the forceful excursion of the apical domains, which is necessary to encapsulate the substrate load into the cage (*35*).

Active role of GroEL in the assisted folding? How do GroEL and GroES assist the folding of so-called *stringent* proteins? Rubisco from *Rhodospirillum rubrum* is one of the most popular stringent proteins (*36–39*) employed at the cutting-edge of research at the chaperonin frontier.

Brinker *et al.* have reported that GroEL-assisted folding of Rubisco is ~ 4-fold faster than spontaneous folding (*40*). It has been proposed that the confinement into the narrow cage itself speeds the folding in the cage (*40*). Molecular simulation also supports the acceleration of the folding in the cage (*41*).

Finally, use of intramolecular FRET to monitor the folding of Rubisco reveals a series of GroEL-induced structural rearrangements of Rubisco (*42*). Binding of Rubisco to GroEL causes stretching of the misfolded Rubisco (*42*). Subsequent addition of ATP and GroES to a Rubisco-GroEL binary complex induces compaction of the encapsulated Rubisco (*42*).

Taken together, these experimental data strongly suggests that GroEL and GroES do not function as a passive folding cage. Rather, ATP- and GroES-induced transition of GroEL and the confinement into the cavity play an active role in the folding of substrate proteins, particularly stringent proteins like Rubisco.

III. GroEL and kinesin: similar double-unit coordination

The dependency of GroEL function on ATP-induced massive conformational change suggests that GroEL may act as a molecular motor, such as myosin, kinesin and F_0F_1-ATP synthase (*43*, *44*). For example, double-ring GroEL and double-head (conventional) kinesin seem to share some similarities in their molecular mechanism. Examination of the similarities provides insights into the function of the two proteins.

Conventional double-head kinesin. Kinesin is a molecular walking machine that moves along microtubules in an ATP-dependent manner (*45*, *46*). So-called conventional kinesin consists of two identical head domains connected to a coiled-coil tail (*45*, *46*). Figure 3A shows how kinesin walks on a microtubule filament as a track (hand-over-hand model) (*46*, *47*). When we start the cycle at the ATP-bound trailing head (left, white) and the nucleotide-free leading head (right, gray), binding of ATP to leading head is inhibited by the rear ATP-bound form. Subsequent ATP hydrolysis at the rear head primes the ATP binding to the leading head. The ATP binding to the

leading head triggers the conformational change in the neck linker, which then throws the rear head to the next position.

Chaperonin GroEL does not "walk" or "move" like kinesin. Suppose that the double rings of GroEL are peeled apart and then connected with a linker (Fig. 3B), and that topological change of the two rings results in their horizontal alignment. Further, non-native substrate proteins are laid on a rail just like tubulin. Then the GroEL reaction cycle is applied to the horizontal GroEL. The coordination of the two rings of GroEL is indistinguishable from that of kinesin (Fig. 3C).

There are other intriguing similarities between GroEL and kinesin.

(i) Binding of ATP, not hydrolysis, is sufficient to cause the conformational change for the function (*45*, *46*).
(ii) Stimulation of ATPase by the corresponding substrate proteins. The presence of tubulin and the non-native protein accelerates the ATPases of kinesin and GroEL by 500- and 5-fold, respectively (*27*, *34*, *46*).
(iii) The possible importance of the monomeric form [single-head kinesin (*48*, *49*) or single ring GroEL (*50*, *51*)].

This kind of comparison provides insight into the mechanism of both molecular machines. For example, kinesin is known for its processive movement, namely traveling long distances without detachment from the track. Applying the concept of the processivity to GroEL might be of interest.

Future perspectives

The substrate proteins need GroEL and, conversely, GroEL needs the substrate proteins to achieve the fully functional state. However, the influence of the substrate proteins on the GroEL function is not fully understood. For example, why do the substrate proteins accelerate the ATP-triggered dissociation of the *cis*-ADP complex? What is the structure of the *cis*-ATP* ternary complex? Future research will focus on understanding how GroEL and the substrate protein mutually influence each other. In addition, the precise role of the GroEL system *in vivo* is also an urgent topic to be solved. Further proteomic analysis, application of a reconstituted cell-free translation system, and a genetic approach will allow us to compile a complete list of *in vivo* GroEL substrate proteins, addressing the fundamental question of why chaperonins (GroEL and GroES) are essential for the cell growth.

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