



## Review Article

# Chaperonin GroEL uses asymmetric and symmetric reaction cycles in response to the concentration of non-native substrate proteins

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**The *Escherichia coli* chaperonin GroEL is an essential molecular chaperone that mediates protein folding in association with its cofactor, GroES. It is widely accepted that GroEL alternates the GroES-sealed folding-active rings during the reaction cycle. In other words, an asymmetric GroEL–GroES complex is formed during the cycle, whereas a symmetric GroEL–(GroES)<sub>2</sub> complex is not formed. However, this conventional view has been challenged by the recent reports indicating that such symmetric complexes can be formed in the GroEL–GroES reaction cycle. In this review, we discuss the studies of the symmetric GroEL–(GroES)<sub>2</sub> complex, focusing on the molecular mechanism underlying its formation. We also suggest that GroEL can be involved in two types of reaction cycles (asymmetric or symmetric) and the type of cycle used depends on the concentration of non-native substrate proteins.**

**Key words:** molecular chaperone, protein folding, protein–protein interaction

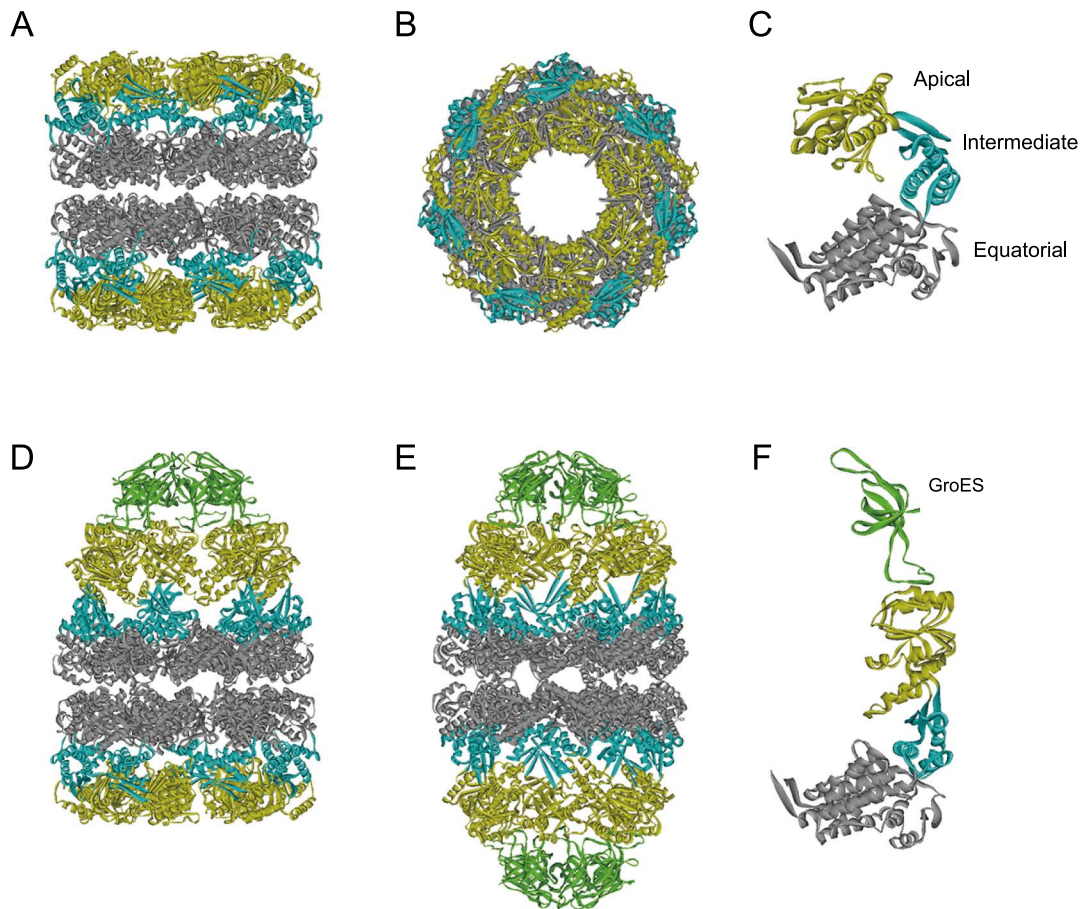
## Chaperonin GroEL

Protein folding is assisted by a number of molecular chaperones *in vivo* [1,2]. Chaperonins, a ubiquitous class of molecular chaperones, form double-ring complexes that

mediate the folding of nascent and denatured proteins (non-native substrate proteins) in an ATP-dependent manner. There are two distinct groups of chaperonins; group I chaperonins, found in bacteria and within mitochondria and chloroplasts, originating from endosymbiotic bacteria and group II chaperonins, in archaea and the eukaryotic cytoplasm [3–5]. The best characterized chaperonin is the *E. coli* chaperonin, GroEL, which is associated with its cofactor, GroES. The GroEL–GroES system is the only chaperone in *E. coli* that is indispensable for growth at all temperatures [6]. Proteomic studies demonstrate that the system is essential for the productive folding of ~80 *E. coli* proteins [7,8] and assists in the folding of an even larger number of proteins [9,10].

GroEL is a tetradecamer of 57 kDa subunits, arranged as two stacked, seven-member rings, each containing a large cavity (Fig. 1A and B). Each subunit is comprised of three domains: the apical, intermediate, and equatorial domain (Fig. 1C). The apical domain is involved in the binding to non-native substrate proteins and GroES (Fig. 1D and F). The equatorial domain contains the ATP-binding site and is involved in intra- and inter-ring interactions. The intermediate domain connects the equatorial and apical domains of each subunit and transfers the ATP-induced conformational changes from the equatorial to the apical domain [11,12]. GroES is arranged as a dome-shaped single heptameric ring composed of 10-kDa subunits. It caps one or both the ends of the GroEL cavities, forming chamber(s), in which non-native substrate proteins are encapsulated for folding (Fig. 1D–F). The chamber can accommodate proteins of up to 60 kDa [13].

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**Figure 1** Crystal structures of GroEL and GroEL-GroES complexes.

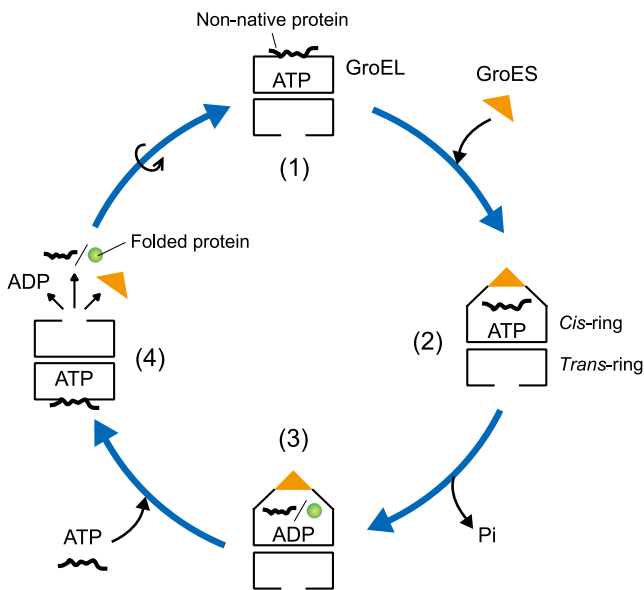
(A–C) Side (A) and top (B) views of GroEL tetradecamer and its subunit structure (C) [Protein Data Bank (PDB) code: 1GRL] [63]. The apical, intermediate, and equatorial domains are in yellow, blue, and gray, respectively. (D, E) Side views of an asymmetric GroEL-GroES complex (PDB code: 1AON) [12] and a symmetric GroEL-(GroES)<sub>2</sub> complex (PDB code: 3WVL) [50]. (F) Subunit structure of GroEL in the GroES-bound ring (PDB code: 1AON). The GroES subunit is shown in green.

### Accepted model for the GroEL-GroES reaction cycle

The widely accepted model for the GroEL-GroES reaction cycle is shown in Figure 2. First, one of the GroEL rings captures a non-native substrate protein via its hydrophobic sites, and GroES binds to the same ring (the *cis*-ring) in an ATP-dependent manner (Fig. 1D and F). GroES binding induces the displacement of the captured protein into the GroEL cavity, where the productive folding proceeds. Next, the hydrolysis of ATP in the *cis*-ring is followed by ATP binding to the opposite ring (the *trans*-ring). This results in the dissolution of the *cis*-ring, thereby releasing GroES and a (partially) folded protein. At the same time, the second GroES binds to the *trans*-ring to reorient a new *cis*-ring and starts the next ATPase cycle [14,15]. Because GroES binds alternatively to each ring of GroEL (two-stroke model), an asymmetric GroEL-GroES complex (also called the bullet-shaped complex; Fig. 1D), in which one GroES is bound to

one end of GroEL, exists throughout the reaction cycle. In contrast, a symmetric GroEL-(GroES)<sub>2</sub> complex (also known as the football-shaped complex; Fig. 1E), in which two GroES molecules simultaneously cap both ends of GroEL, is not formed. The origin of the GroEL-GroES interaction cycle has been explained by the conformational changes of GroEL. These changes are reflected in the binding and hydrolysis of ATP with positive intra-ring cooperativity and negative inter-ring cooperativity [16,17].

In contrast, the symmetric complex has been identified using electron microscopic examination [18–26], chemical cross-linking [27,28], analytical ultracentrifugation [29], and fluorescence-based detection [30,31]. Taguchi *et al.* [32] found that the symmetric complex is formed when the GroEL ATPase cycle is stopped by beryll fluoride (BeF<sub>x</sub>), a structural analog of inorganic phosphate. However, the symmetric complex has been considered as a non-significant complex formed under non-physiological conditions or an unproductive dead-end complex. The view that the func-



**Figure 2** Schematic model for the GroEL–GroES reaction cycle using an asymmetric complex.

tional chaperonin complex is asymmetric has been widely accepted [14,15,33].

### The symmetric complex can be formed during the GroEL–GroES reaction cycle

We previously examined the GroEL–GroES interaction in the reaction cycle and found that the cycle is significantly affected by the presence of non-native substrate proteins [34–36]. In the presence of these proteins, we then attempted to monitor the GroEL–GroES interaction using Förster resonance energy transfer, without stopping the reaction. As a result, we found that the symmetric and asymmetric complexes coexist in the presence of non-native substrate proteins and that the formation of the symmetric complex is promoted by increasing the concentration of these proteins [37,38]. On the other hand, in the absence of non-native substrate protein, the symmetric complex is not formed [38]. Our findings have been confirmed by other research groups in similar experimental systems [39–41]. The available results indicate that the symmetric complex appears as an intermediate in the presence of sufficient amounts of non-native substrate protein. It is likely that the symmetric complex has been overlooked because most of the previous studies have been performed either with or without small amounts of non-native substrate protein.

Based on a previous report [32], we assumed that the second GroES can associate with the *trans*-ring of the ATP-bound asymmetric complex. We then performed similar experiments using an ATPase-defective mutant (GroEL<sup>D398A</sup>). GroEL<sup>D398A</sup> undergoes a conformational change, in the manner of wild-type GroEL, to bind GroES upon the binding of

ATP, although the ATPase activity in this mutant is significantly reduced (~2% of the wild-type level) [14]. We found that GroEL<sup>D398A</sup> forms the symmetric complex when both rings are occupied with ATP [37]. At the same time, Koike-Takeshita *et al.* [42] also demonstrated that GroEL<sup>D398A</sup> forms a symmetric complex in the presence of ATP and GroES. These findings are surprising; the accepted model (Fig. 2) assumes that GroEL<sup>D398A</sup> forms an asymmetric complex in the presence of ATP and GroES, and the ATP-bound complex cannot bind non-native substrate protein and GroES to the *trans*-ring [15]. Thus, the accepted model has been challenged [4,5,43].

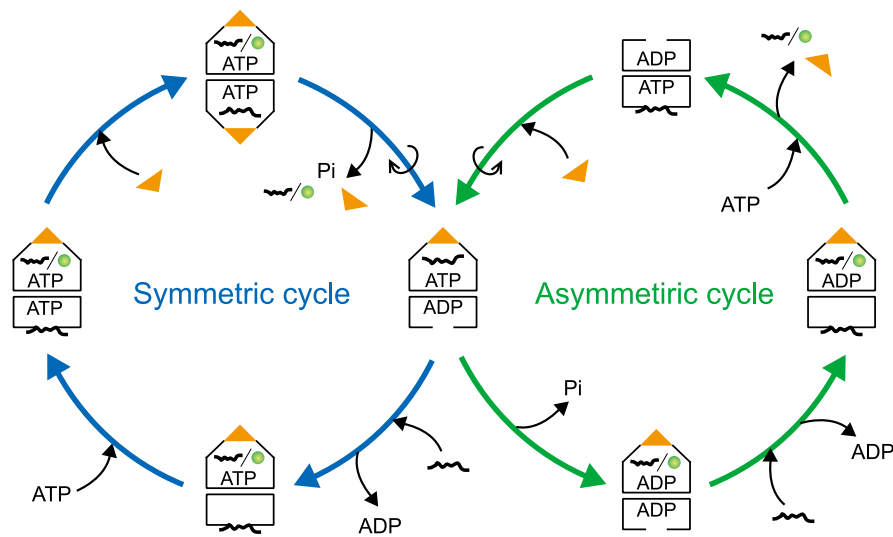
### The symmetric complex is a functional intermediate

We then attempted to probe the GroEL–GroES interaction cycle via the symmetric complex using a single-molecule assay [44]. The assay allows the direct observation and characterization of the symmetric complexes during the reaction cycle. We found that the first GroES that interacts with GroEL does not always dissociate from the symmetric complex before the dissociation of the second GroES molecule, *i.e.* the dissociation of GroES molecules from this complex can occur in a random order. It is likely that GroES dissociates from the GroEL ring, in which ATP hydrolysis occurs. We also found that GroEL exited in three different states: as GroEL alone, as the asymmetric complex, and as the symmetric complex. This finding indicates the existence of two reaction cycles in the GroEL–GroES interaction: an asymmetric cycle (GroEL ↔ asymmetric complex) and a symmetric cycle (asymmetric complex ↔ symmetric complex) (Fig. 3; the details will be discussed below).

Several studies have suggested that non-native substrate proteins can be encapsulated and folded in both rings of GroEL at the same time [24,25,32,42]. To understand how the protein folding proceeds in the symmetric complex, we previously visualized protein folding in this complex, employing a single-molecule assay [45]. We demonstrated that both rings in the symmetric complex actively assist in the refolding of GFP molecules. Furthermore, the kinetics of GFP refolding in each ring is in excellent agreement with that in the asymmetric complex. In other words, the same reactions occur in both rings of the symmetric complex. As the dissociation of GroES molecules from the symmetric complex can occur in a random order [44], the two rings might operate as parallel processing machines, indicating the lack of inter-ring communication in this complex.

### Structure of the symmetric complex

The symmetric complex can be formed when both rings of GroEL are occupied with ATP [32,37,42]. Nojima and Yoshida [46] found that GroEL can adopt a conformation in which the two rings open in the presence of ATP. This finding indicates that the binding of ATP to both rings of



**Figure 3** Schematic model for the GroEL–GroES reaction cycle using asymmetric and symmetric complexes.

GroEL mainly goes through the asymmetric cycle in the presence of a low concentration of non-native substrate protein. At high concentrations of non-native substrate protein, GroEL passes through the symmetric cycle.

GroEL results in the formation of the symmetric complex. Koike-Takeshita *et al.* [47] indicated that ATP $\gamma$ S, a non-hydrolyzable ATP analog, was relatively efficient, whereas AMP-PNP, another non-hydrolyzable ATP analog, was not effective in the formation of the symmetric complex. It is considered that ATP plays a unique role in the induction of structural rearrangements of GroEL, as has been suggested previously [14,48].

Recently, three groups determined the structure of the symmetric complex at atomic resolution [49–51]. As expected, the structure has an American football-like shape, showing that two GroES molecules bind to the two rings of GroEL (Fig. 1E). Fei *et al.* [49] determined the crystal structures of the symmetric complexes formed in the presence of ATP and BeF $_x$ . These complexes contain 14 ATP-analog (ADP-BeF $_x$ ) molecules at the nucleotide-binding sites, with no significant negative cooperativity between the two rings. Koike-Takeshita *et al.* [50] determined the structure of the symmetric complex using an ATPase-deficient GroEL mutant (GroEL<sup>D52A/D398A</sup>, with the activity <0.01% of the wild type). This mutant protein forms an extremely stable symmetric complex with a half-life of ~6 days [47]. In the crystal structure, the 14 nucleotide-binding sites are occupied by ATP. Importantly, in both symmetric complexes, the interactions between the two GroEL rings are reduced from those in the asymmetric complex. This reduction in the interactions can be attributed to the impaired inter-ring negative cooperativity in the symmetric complex. The importance of inter-ring interactions in the allosteric mechanism of GroEL has been confirmed using site-specific mutagenesis [52–56]. It has been also reported that a mutant GroEL (GroEL<sup>E461K</sup>), with rearranged inter-ring electrostatic contacts and decreased negative cooperativity between the rings, forms the symmetric

complex more easily than the wild-type molecule forms [56]. Nisemblat *et al.* [51] reported the crystal structure of the symmetric complex of human mitochondrial chaperonin, with a mutation (mHsp60<sup>E321K</sup>), stabilizing the complex [57]. As mHsp60<sup>E321K</sup> and cochaperonin (mHsp10) were mixed together with ATP and subjected to crystallization, mHsp60<sup>E321K</sup> hydrolyzed the ATP in the crystallization drop, resulting in the structure containing 14 ADP molecules. On the basis of these results, it is likely that group I chaperonins share the inherent “functional symmetry.”

### Mechanism of the formation of the symmetric complex

What is the mechanism by which non-native substrate protein promotes the formation of the symmetric complex? The most probable explanation is that non-native substrate protein facilitates the dissociation of ADP from the *trans*-ring of GroEL, leading to the association of ATP and the second GroES with this ring.

Kinetic studies have revealed that ADP remains in the GroEL ring even after GroES has been detached [58,59]. We have shown that ADP prevents the association of ATP with the *trans*-ring of GroEL and strongly inhibits the association of the second GroES [37,38]. These findings indicate that GroES cannot associate with the *trans*-ring of GroEL until ADP dissociates from this ring. Lorimer *et al.* demonstrated that the association of non-native substrate protein with the *trans*-ring promotes ADP/ATP exchange [39,59,60]. We also found that non-native substrate protein accelerates the association of the second GroES with the *trans*-ring of GroEL in the presence of ADP; this association is significantly reduced in the absence of non-native substrate protein [38]. On the



basis of the available data, we propose a mechanism by which the non-native substrate protein promotes the symmetric complex formation (Fig. 3). ATP hydrolysis in one of the rings of GroEL results in the dissociation of GroES because the loss of the  $\gamma$ -phosphate decreases the affinity between GroEL and GroES [61] (Fig. 3, *Symmetric cycle, right*). Subsequently, the association of non-native substrate protein dissociates ADP from the *trans*-ring of GroEL (Fig. 3, *Symmetric cycle, lower*), leading to the association of ATP and the second GroES with this ring (Fig. 3, *Symmetric cycle, left and upper*). This series reaction can be enhanced by increasing the concentration of non-native substrate protein (the concentration depends on its affinity for GroEL) [38,40]. In the resultant GroEL–GroES complex (Fig. 3, *Symmetric cycle, upper*), the *cis*-ring and the *trans*-ring become indistinguishable. The previously published model of inter-ring negative cooperativity is correct but incomplete in that it does not apply to high concentrations of non-native substrate protein.

### Model for the GroEL–GroES reaction cycle with the symmetric complex and the physiological significance of this complex

Based on our findings [37,38,44,45], we propose a schematic model of the GroEL–GroES reaction cycle, shown in Figure 3. The model consists of two cycles: the “asymmetric cycle” and the “symmetric cycle.” In the presence of non-native substrate protein at a low concentration, GroEL mainly goes through the asymmetric cycle due to the inhibitory effect of ADP in the *trans*-ring. However, a high concentration of non-native substrate protein causes a switch to the symmetric cycle as the protein weakens the inhibitory effect of ADP and facilitates the formation of the symmetric complex. Our model does not contradict the accepted model (Fig. 2), but rather shows that the GroEL–GroES system can work in a different mode in the presence of high concentrations of non-native substrate protein.

As indicated in previous reports [23,24,27], the symmetric complex is the advantageous form for protein folding because both cavities in the complex actively assist the process. Therefore, we expect the symmetric complex to be active when the amount of non-native substrate protein increases, and GroEL prevents the accumulation of these proteins in *E. coli*, e.g., under stress conditions. Interestingly, at elevated temperatures, the negative cooperativity between the two GroEL rings appears to be decreased [62]. This would also facilitate the formation of the symmetric complex. We also found that the ATPase activity of GroEL is higher when the levels of the symmetric complex increase [38]. GroEL does not have to employ the maximum ATPase activity in the presence of a small amount of non-native substrate protein. In other words, GroEL does not form symmetric complexes at low levels of non-native substrate protein, thus preventing unnecessary ATP consumption. These

might be the reasons why GroEL functions as a double-ring structure. The next challenge is to find the direct evidence that the symmetric complexes are formed in *E. coli*, leading to the understanding their physiological significance.

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### Conflicts of Interest

The authors declare no conflicts of interest associated with this manuscript.

### Ethics Standard

The authors declare that this manuscript is original and unpublished and is not currently being considered for publication elsewhere. This manuscript has been read and approved by the authors.

### Author Contribution

R. I. and T. F. co-wrote the manuscript.

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