Chapter 17
GroEL and the GroEL-GroES Complex

Noriyuki Ishii

Abstract Chaperonin is categorized as a molecular chaperone and mediates the formation of the native conformation of proteins by first preventing folding during synthesis or membrane translocation and subsequently by mediating the step-wise ATP-dependent release that result in proper folding. In the GroEL-GroES complex, a single heptameric GroEL ring binds one GroES ring in the presence of ATP/ADP, in this vein, the double ring GroEL tetradecamer is present in two distinct types of GroEL-GroES complexes: asymmetric 1:1 "bullet"-shaped GroEL:GroES and symmetric 1:2 "football" (American football)-shaped GroEL:$GroES_2$. There have been debates as to which complex is critical to the productive protein folding mediated by the GroEL-GroES complex, and how GroES coordinates with GroEL in the chaperonin reaction cycle in association with regulation by adenine nucleotides and through the interplay of substrate proteins. A lot of knowledge on chaperonins has been accumulating as if expanding as ripples spread around the GroEL-GroES from *Escherichia coli*. In this article, an overview is presented on GroEL and the GroEL-GroES complex, with emphasis on their morphological variations, and some potential applications to the fabrication of nanocomposites using GroEL as a nano-block. In parallel, a guideline is presented that supports the recognition that the *E. coli* and its GroEL-GroES complex do not always receive in standard literature because the biochemical features of chaperonins derived from others special, such as mammals, are not always the same as those confirmed using GroEL-GroES derived from *E. coli*.

Keywords Chaperonin • Electron microscopy • Equatorial split • GroEL • GroES • Hsp60 • Nanocomposite • Self-assembly • Chaperone • Protein folding

N. Ishii (✉)
Biomedical Research Institute, Department of Life Science and Biotechnology, National Institute of Advanced Industrial Science and Technology (AIST),
Tsukuba Central-6, 1-1-1 Higashi Tsukuba Ibaraki, 305-8566 Tsukuba, Japan
e-mail: n.ishii@aist.go.jp

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17.1 Introduction

Synthesis of proteins in the cell is promoted by many ribosomes distributed in the cytoplasm surrounding the nucleus. The amino acids are linked sequentially by peptide bonds and form a chain of polypeptide according to the translation of genetic information, leading to the autonomous folding of protein into its own active three-dimensional structure. More strictly, protein folding into its native tertiary structure is achieved automatically by each protein's amino acid sequence. This is the well known Anfinsen's dogma in molecular biology which implies in this context that the full information for both the native conformation and folding pathway is encoded in its primary structure (Anfinsen 1973). Anfinsen shared the Nobel Prize for chemistry in 1972 for their historic work with ribonuclease A. Since then, many purified proteins, which once had been denatured to random coil-like structures, have been confirmed to refold spontaneously *in vitro*. The folding is explained as a process driven by small differences in the Gibbs free energy between the unfolded and native states. Many exotic and exciting results relevant to protein folding and manners of self-assembly have been accumulating on the basis of the dogma. Consequently, scholars and researchers have been accepting the dogma. It may be said that the study of protein folding especially *in vitro* folding from denaturation is indeed itself the history of protein science.

How polypeptide chains of amino acids routinely and correctly fold into an active form in three-dimension is one of the most outstanding questions in structural biology. The entropy driven collapse of random coil-like conformation converges towards the so-called pre-fold 'molten globule' state. Some specific regions in the string of the amino acid sequence defined by genetic translation achieve secondary structures such as α-helices or β-strands, which successively makes an acquisition of the peculiar tertiary structures in an intermediate structure of 'molten globule' and in some cases, further followed by assembling into quaternary structures. However, how each sequence finds and reaches the correct structure among the number of possible conformations available within surprisingly short timescales is still the large challenging subject. Protein folding research has been promoted as one of the important issues in life sciences requiring further clarification and understanding despite having been studied by many scholars and researchers worldwide for many years. Currently, genomic information of many organisms has been deciphered, and amino acid sequence information of tens of thousands of proteins is available, yet it remains difficult even now to predict their three-dimensional structures.

On the other hand, although it is not the direct purpose of this article, the author will only briefly mention that there are exceptions to the rule that sequence uniquely determines structure, and that counterexamples have been discovered in the early 1980s. Prions are proteins which can have the same sequence but different tertiary structures. For example, a prion protein can exist as an aberrant β-sheet form or the normal α-helix form; the former causes a prion disease while the latter is expressed in a healthy human body without harm. In some cases, native proteins refold into a

different stable conformation with inter-molecular interactions, which causes fatal amyloid formation. There are other structural states where the Anfinsen's dogma falls short. Proteins involved in Alzheimer's and Parkinson's diseases are counter-examples to the dogmas. It may be said that amyloid and related diseases are caused by changes in protein folding (misfolding). Furthermore, the existence of intrinsically disordered proteins is another interesting issue in connection with protein folding, however, the author shall not attempt to deeply cover this very important area at this time (Chouard 2011).

Getting back to the main subject, through billions of years of evolution, cells have developed the protein homeostasis (or proteostasis) network in order to cope with environmental stresses and to facilitate the correct folding for complicated larger protein molecules. Several proteinaceous components have been discovered that mediates the process. These components under the network form a family called "molecular chaperones". Ellis introduced the concept of molecular chaperone, and the current definition remains very similar to that given in 1987 (Ellis 1987). The component which differs from the original is associated with the final oligomeric structure, defined as a molecular chaperone. They are expressed ubiquitously in prokaryotes, eukaryotes, and in the cytosol as well as within organelles. In spite of the extremely crowded environment within the cell, thanks to the conserved systems of chaperones such as GroE (GroEL/GroES) or DnaK (DnaK/DnaJ/GrpE) systems (Ellis 1996; Horwich et al. 2006; Yamakoshi et al. 1998; Motohashi et al. 1994), newly synthesized proteins and possible refolding proteins, after having been post-translationally imported across biological membranes, are able to achieve their functionally active form by avoiding inter-molecular aggregation formation. Nearly 30% of *E. coli* proteins are reported to be aggregation prone without the assistance by chaperones (Niwa et al. 2009).

The most representative of the chaperone class of molecules is chaperonins, namely GroEL and GroES, which is the focus of this article. Chaperonins are distributed in all three kingdoms, and are classified into Group I and Group II according to the presence or absence of co-chaperonin, their amino acid sequences, and oligomeric structures. Group I chaperonins are found in the bacterial cytosol (GroEL) (Fig. 17.1), eukaryotic organelles (Hsp60) such as mitochondria (mtHsp60) and chloroplast (Rubisco binding protein), and some archaea cytosol (Klunker et al. 2003). Group II consists of the archaeal (thermosomes) (Trent et al. 1991) and eukaryotic cytosolic variants (TCP1, TCP1 ring complex (TRiC), or chaperonin containing TCP1 (CCT)) (Frydman et al. 1992; Kubota et al. 1994). Although some of those members can be heat-induced, chaperonins are essential in protein folding at almost all temperature ranges. Furthermore, chaperonins are constitutive proteins and are induced under a large variety of cellular stresses.

Proteins are the central building blocks to life. It is a polymer consisting of the amino acids which determine its three-dimensional shape. In a sense, it is the chaperonin that mediates the process by providing favorable conditions. Chaperonin assists in oligomeric assembly by preventing the formation of improper protein aggregates. The chaperonin-mediated folding reaction remains an interesting feature, for example, allosteric mechanism of the chaperonin coupled to ATP binding.

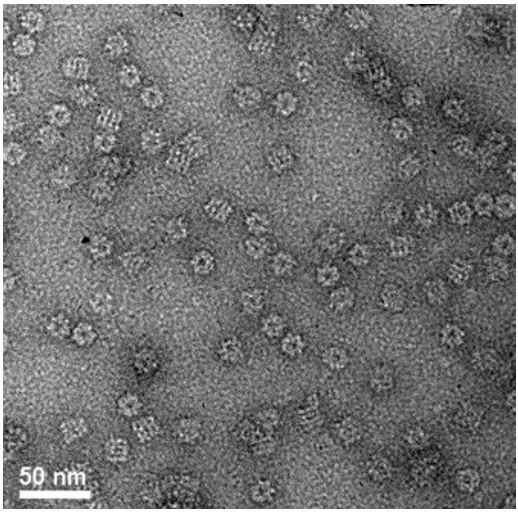

Fig. 17.1 An electron microscopic image of GroEL tetradecamer from *E. coli*. Seven-membered rings as viewed from top and rectangular (*square*) shapes with stripes from the side are seen

Although there are numerous superb publications reviewing chaperonins (Ellis et al. 1993; Ellis 1996; Lorimer and Todd 1996; Fink and Goto 1998; Dobson 2003; Horwich et al. 2006; Zanin-Zhorov and Cohen 2007; Richter et al. 2010; Hartl et al. 2011; Saibil et al. 2013), especially the GroEL-GroES complexes, one of the greatest unsolved problem thus far in the chaperonin field is how GroES coordinates with GroEL in the chaperonin function reactions regulated by ATP (both binding and hydrolysis reaction) and with the existence or nonexistence of substrate protein folding intermediates. In this article, the author will present an overview on chaperonins of biological macromolecular machines, GroEL and the GroEL-GroES complex, and will introduce some potential applications to the fabrication of stimuli-responsive, mechanical movable nanocomposites using GroEL as a nano-block.

17.2 Molecular Structures

It is now generally accepted that protein folding in the cell needs mediation by molecular chaperone components. Chaperonins are member of a subcategory under molecular chaperones. Although the term “GroEL” has been used synonymously with chaperonin, strictly speaking, GroEL is a product of *groEL* gene (growth essential large) from *E. coli* (Fig. 17.1). The *groEL* gene encodes a polypeptide of 548 amino acids, and is part of *groE* operon that also contains *groES* gene (growth essential small) for GroES. GroES is known as cochaperonin and consists of 97 amino acids (Chandrasekhar et al. 1986). The primary structures for both, GroEL and GroES, as well as their homologs from different organismic species are available in internet databases (Hill et al. 2004, http://www.cpndb.ca).

When talking about structure-function relationship, there always seems to be a compromise between the best achievable resolution of the analytical technique versus the depth we desire to know about a molecular functional mechanism on the basis of the protein structure. In the early stage of the investigation on GroEL and of course thus far, transmission electron microscopy (TEM) has played an important role providing precious morphological information on its structures to delineate as a molecular model (Hohn et al. 1979; Hendrix 1979). While X-ray crystallography needs three-dimensional single crystals with appropriate sizes in which each protein molecule in a unique conformation is regularly arranged, electron microscopy is suitable for structural analysis of huge macromolecules with larger dynamical structural changes. Such characteristics of TEM have been, in later years, combined with the cryogenic technology, which has brought a new horizon in protein structural biology (Fujiyoshi 1998; Glaeser 1971, 1985; Roseman et al. 1996). Due to the limitation on the resolution available at the early days of investigation, overspeculation regarding molecular model reconstruction based only on projection image data by electron microscopy under the limited resolution had frequently occurred. We have presented another model, correcting the previously prevailing model, as to the manner of subunit arrangements in the GroEL tetradecamer by using the electron microscopic projection image of GroEL homolog isolated from a thermophilic eubacteria, *Thermus thermophiles* (Ishii et al. 1992). We have reported the functionally active form as holo-chaperonin, which is now known to be an asymmetric 1:1 "bullet"-shaped GroEL:GroES complex, although the origin is different from *E. coli* (Ishii et al. 1992; Taguchi et al. 1991). Later, the structure of GroEL tetradecamer determined by X-ray crystallography has verified the correctness in the subunits' arrangement of our molecular structure model for GroEL (Braig et al. 1994).

GroEL generally exists as double layered homo-heptameric rings having totally about 800 kDa, in which two rings with seven 57 kDa subunits are stacked back to back (isologously), subsequently adopting cylindrical and hollow shaped appearance and enclosing two large but non-contiguous central cavities. The cochaperonin, GroES is a single layered heptameric ring consisting of seven 10 kDa subunits with mobile loops extending from the rim, which attaches coaxially to the ends of the GroEL cylinder. The X-ray structure of the cylindrical GroEL tetradecamer has been determined at 2.8 Å resolution (Braig et al. 1994) (PDB entry ID: 1GRL), and that of the asymmetric 1:1 bullet-shaped GroEL:GroES complex with ADP_7 has been determined at 3.0 Å resolution (Xu et al. 1997) (PDB entry ID: 1AON). According to the crystal structures determined by X-ray crystallography at atomic resolution, 18 α-helices and 19 β-strands are assigned for the secondary structural elements in one GroEL subunit. Meanwhile, 9 β-strands and no α-helices are assigned in GroES subunit. In GroEL, the subunit consists of three structurally distinguishable domains as follows (Fig. 17.2): the apical domain that plays an important role in binding to GroES or substrate proteins using its hydrophobic surface region, which constructs the end-on surface of GroEL; the equatorial domain that has an adenine nucleotide (ATP/ADP) binding pocket, is located at the middle region in the cylindrical GroEL tetradecamer so as to form an equatorial interface

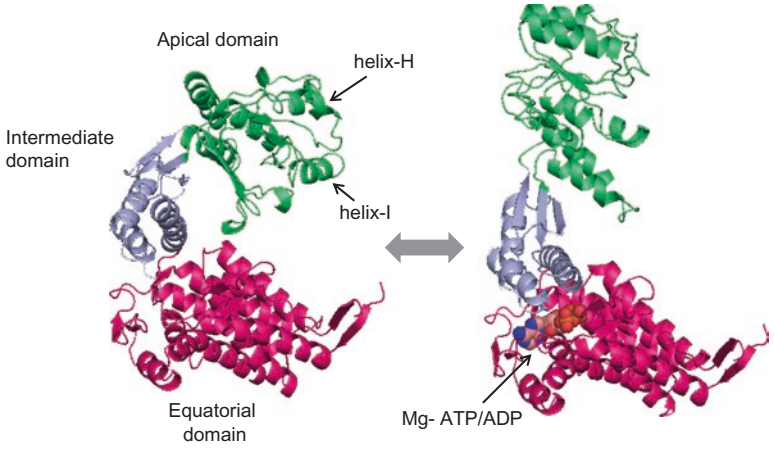

Fig. 17.2 The crystal structures of GroEL subunits, with and without a bound nucleotide. Helices H and I, are the binding site for the substrate protein. Upon binding of nucleotide, the upward movement of apical- and intermediate- domains creates a large expanded cavity on the *cis*-side, which facilitates the substrate protein encapsulation to the Anfinsen's cage

between the two heptameric GroEL rings (*cis*- and *trans*-rings); and the intermediate domain with a hinge region is located between the apical and the equatorial domains connecting both, and that also transmits allosteric signals between them. Upon binding of ATP to the GroEL ring, it induces intra-ring positive cooperative upward movement of the intermediate and apical domains, which prompts the formation of GroEL that binds GroES (*cis*-GroEL ring). The formation of a metastable GroEL-GroES complex with a folding intermediate appears to be taking place at the common site for substrates that interact with GroEL (Horowitz et al. 1999) and yet, there is still some debate regarding this.

17.3 Various Structural Forms of the GroEL-GroES Complex

GroEL from *E. coli* is usually isolated and purified as GroEL tetradecamer alone (without GroES bound), and its crystal structure has been determined by X-ray crystallography (Braig et al. 1994). Although the GroEL tetradecamer has been considered as a constituent unit, which is the general consensus nowadays among researchers, a single-ring form of GroEL heptamer has been detected in some Group I chaperonins such as mitochondrial Hsp60 (Viitanen et al. 1992), *Paracoccus denitrificans* GroEL (Ishii et al. 1992; Sumi et al. 1992) and *Thermoanaerobacter brockii* GroEL (Todd et al. 1995). The mammalian mitochondrial chaperonin, mtHsp60 has been reported to function predominantly as a single-ring rather than a double-ring complex (Viitanen et al. 1992; Nielsen and Cowan 1998). Chen et al. have reported on the reconstruction from a single particle analysis applied to the

cryogenic electron microscopic images of the single-ring GroEL-GropES complex using an ATPase-deficient single ring mutant (SR398) of GroEL and has a discussion on the dynamic conformational change (Chen et al. 2006). Interestingly, the C-terminal region of GroEL subunit that was unresolved in the crystal structure (Braig et al. 1994) emerged from the density map, and the truncated cone-shaped cavity appears to transform to an ellipsoidal cavity when encapsulating the larger substrate protein (Chen et al. 2006). The dynamic nature of conformational changes and its continuous structural variation would not be easily determined by crystallographic approach. There may be more conformational variability that has not yet been previously observed. It should be important to investigate further the aspect of single-ring chaperonin behavior.

Since the single GroEL ring consisting of seven 57 kDa subunits binds one GroES heptamer ring in the presence of ATP/ADP, in this vein, the double ring form of GroEL tetradecamer has allowed to take two distinct types of GroEL-GroES complex formation, that is, asymmetric 1:1 bullet-shaped GroEL:GroES and symmetric 1:2 "football" (American football) -shaped GroEL:$GroES_2$ complexes. The bullet-shaped GroEL-GroES complex is the form with GroES bound to only one end of the GroEL tetradecamer, and the football-shaped GroEL-$GroES_2$ complex is with two GroES bound to both ends of the GroEL tetradecamer (Fig. 17.3). The X-ray crystal structures have been determined for both, the bullet-shaped GroEL-GroES (Xu et al. 1997) and the football-shaped GroEL-$GroES_2$ complexes (Koike-Takeshita et al. 2014) (PDB entry ID: 3WVL).

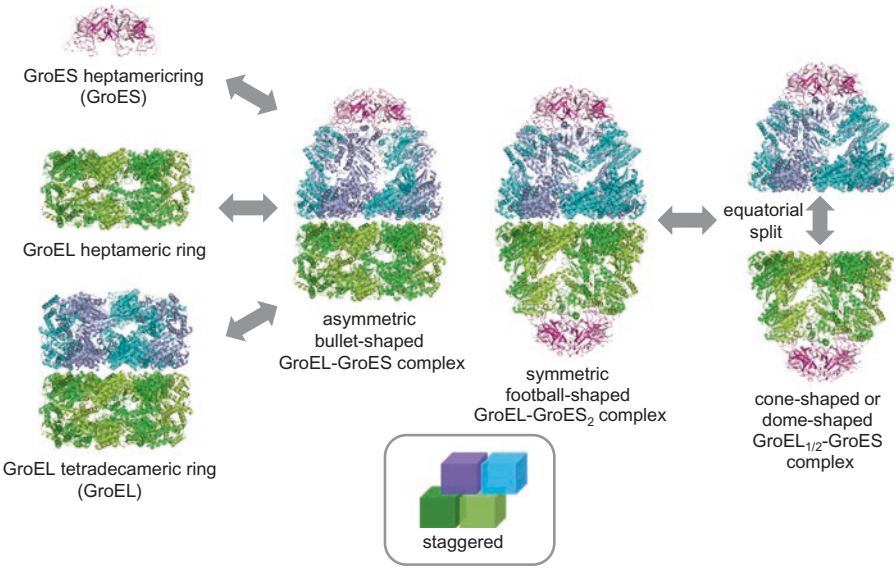

Fig. 17.3 Various structural forms of the GroEL-GroES complex affixed with the crystal structures. The staggered inter-ring registry of the subunits of one heptameric ring seated directly on subunits in the second ring is illustrated (PDB entry IDs: 1GRL, 1AON, 3WVL)

Recently the bullet-shaped complex formation is understood to occur only in the presence of ADP, meanwhile formation of both the bullet-shaped and the football-shaped complexes are reconciled with the presence of adenine nucleotides having ATP-configuration including the non-*hydrolyzable* ATP analogues. The football-shaped GroEL-$GroES_2$ complex formation appears not to be controlled by a single parameter, because the football-shaped complex form has been detected under a variety of combined conditions such as [ATP]/[ADP] ratio, [K^+] concentration, and further the presence or the absence of non-native substrate protein or folding intermediate (Azem et al. 1994; Schmidt et al. 1994).

Taking a closer look at the crystal structure of GroEL, the Group I chaperonin, it should be important to describe the manner in which the subunits of one heptameric ring are seated directly on a subunit in the second ring, which is the staggered inter-ring registry as illustrated in Fig. 17.3 (Lopez et al. 2015). This is an important feature in understanding the dynamic morphological transition of the GroEL-GroES complex. It is now believed that the symmetric football-shaped complex is a precursor to the equatorial split, which leads to a single-ring GroEL-GropES complex (cone-shaped or dome-shaped complex) formation (Ishii et al. 1995). The split at the equatorial plane of the symmetric GroEL-GroES complexes would be attributed to the weakened inter-ring interactions between the *cis*- and *trans*- heptamer rings in the complex, which has smaller contact interaction energy due to lack of a salt bridge at the inter-ring interface, as compared with the bullet-shaped complex (Ishii and Sato 2013; Taguchi 2015).

On the other hand, the filamentous complex (one-dimensional assembly) of*E. coli* GroEL and GroES has been reported to form when GroEL and GroES at relatively low molecular rate (1:1–1:3) are incubated at room temperature in the presence of ATP and magnesium (Harris et al. 1995). Such filamentous cytoskeleton-like assemblies consisting of chaperonin molecules has attracted keen interests from nano-biotechnology and nano-biomedical research fields (Biswas et al. 2009; Muramatsu et al. 2006). The author will introduce examples that may lead to further insight into bridging protein (bio-macromolecular) science with chemical nano-biotechnological research.

17.4 Function and Chaperonin Reaction ATPase Cycle

A cylindrical GroEL tetradecamer and its lid-like cofactor GroES heptamer form a nano-cage in which a single polypeptide chain is transiently enclosed and prompted to fold properly. Apparently during folding, the substrate proteins or polypeptides are isolated from the bulk phase of the solution (or cytosol in the cell). Interestingly, GroEL tetradecamer is also required for its own assembly (Cheng et al. 1990; Motojima and Yoshida 2010). Urea-denatured GroEL appears to first reassemble spontaneously into tetradecamer and then catalyze further assembly in Mg-ATP dependent chaperonin reaction (Lissin et al. 1990). On the other hand, GroES has been reported to self-assemble under appropriate condition *in vitro* (Mascagni et al. 1991).

The function and the GroEL-GroES chaperonin reaction mechanism by which there is general consensus is as follows (Horwich et al. 2006; Walter 2002); First, a non-native protein or a polypeptide chain binds as a molten globule-like folding intermediate to hydrophobic sites located on one of the apical domain of seven candidate subunits of GroEL and then followed by intra-ring positive cooperativity. Subsequently, upon binding of ATP to GroEL, *cis*-GroEL subunits change to the elongated conformation (Fig. 17.2) and GroES caps the GroEL ring that holds the substrate (termed *cis*-ring of GroEL), then the unfolded protein is encapsulated in the cavity underneath GroES. The enclosed chamber known as Anfinsen's cage is large enough to accommodate proteins up to ~60 kDa. The second GroES and another ATP cannot bind to the opposite GroEL ring (termed *trans*-ring) until ATP in the *cis*-ring is hydrolyzed, due to the negative cooperativity between the two rings of GroEL (Horovitz and Willison 2005). Thus the encapsulated protein is rendered to fold in the hydrophobic environment of the elongated cavity of GroEL caped with GroES. The grace time for the protein substrate to (re)fold is equal to a period required for the hydrolysis of the seven ATP molecules bound in the *cis*-ring. After ATP in the *cis*-ring is hydrolyzed, GroES, ADP, and the committed protein are released in a step-wise manner from the *cis*-ring by the binding of ATP and another unfolded protein to the *trans*-ring. GroES is thought to regulate the step-wise process. This occurs irrespective of whether the protein has fully folded, meaning most of proteins released are still in non-native form that has to be rebound by GroEL for a further trial at folding. The second GroES associates with the *trans*-ring of GroEL, forming another *cis*-ring to succeed the chaperonin reaction cycle (Hayer-Hartl et al. 1995; Engel et al. 1995). Therefore, which GroEL heptamer ring may be active at a time for binding to the substrate protein should be dependent on the regulation by GroES. The process can be interpreted as a two-stroke engine, and the current mechanistic model appears to well explain the relationship between the chaperonin-assisted protein folding and the molecular morphology of the asymmetric bullet-shaped GroEL-GroES complex.

Electron microscopy has often played a critical role in structural biology, and continues to open new horizons. Analyses by electron microscopy revealed the existence of football-shaped complex during the chaperonin reaction cycle (Grallert and Buchner 2001). There had been an agreement that football-shaped complex might occur as a transient intermediate state in the reaction cycle, when GroES bound to the *trans*-ring of GroEL before the release of GroES from *cis*-ring was completed. Koike-Takeshita et al. demonstrated the formation of a symmetric GroEL-$GroES_2$ complex using a slow ATP-hydrolyzing GroEL mutant (D398A) in the presence of ATP (Koike-Takeshita et al. 2008). The interaction between GroEL and GroES was investigated using fluorescence resonance energy transfer (FRET) by Sameshima et al., and they found that nearly equivalent amounts of the asymmetric GroEL-GroES and the symmetric GroEL-$GroES_2$ complexes coexist during the reaction cycle (Sameshima et al. 2008). They further characterized the emergence of the football-shaped complex and concluded that denatured proteins facilitate the dissociation of ADP from the *trans*-ring of GroEL and the concomitant association

of ATP and the second GroES, subsequently facilitating the formation of the symmetric football-shaped complex (Sameshima et al. 2010).

Recent reports suggest that the presence of non-native substrate protein affects the GroEL-GroES reaction by shifting its morphology from the asymmetric bullet-shaped to symmetric football-shaped complexes. Haldar et al. have reported on the relative population in the formation between the bullet-shaped and the football-shaped GroEL-GroES complexes recently (Haldar et al. 2015). They have characterized the symmetric GroEL-$GroES_2$ complex as substantially populated by the association of non-foldable proteins with asymmetric GroEL-GroES complex. Such non-foldable proteins like β-casein, α-lactalbumin, and so on appear to overstimulate the GroEL ATPase activity and uncouple the negative GroEL inter-ring allostery. It is also worthwhile to investigate the biochemical role of intrinsically disordered or unfolded proteins in the cell. Thus in the symmetric GroEL-$GroES_2$ complex mode, both GroEL rings bind to GroES simultaneously, realizing the folding active form. They also mention that the asymmetric GroEL-GroES complex is dominant both in the absence of substrate proteins and in the presence of foldable substrate proteins. Furthermore, uncoupling of the GroEL ring and formation of symmetric GroEL-$GroES_2$ complex is suppressed at physiological [ATP]/[ADP] concentration. The interaction with non-foldable substrate proteins appears to weaken the negative cooperativity between the two GroEL rings, extending the time to bind and hydrolyze ATP, but it does allow binding to GroES. However, their conclusion is that the asymmetric GroEL-GroES complex represents the main folding active form. As they suggested, whether the presence of non-foldable or foldable substrate proteins makes a difference on the GroEL-GroES complex formation to either asymmetric bullet-shaped or symmetric football-shaped complexes remains to be clarified.

Taguchi has recently studied the GroEL-GroES chaperonin reaction cycle from the viewpoint of the symmetric football-shaped GroEL-$GroES_2$ complex (Taguchi 2015). The ATPase kinetics of GroEL appears to change when substrate proteins exist, and the nucleotide exchange should be accelerated by the substrate protein, leading to the symmetric football-shaped formation. Although there have been debates as to which complex, either the asymmetric bullet-shaped or symmetric football-shaped, is critical to the productive folding assisted by GroEL-GroES chaperonin, both complexes appear not to be mutually exclusive. The substrate protein itself configures the shape of the folding cradle that suits each protein, switching GroEL-GroES chaperonin reaction cycling from the asymmetric bullet-shaped to the symmetric football-shaped complexes, and *vice versa*. Fundamentally different mechanisms might underlie the GroEL-GroES reaction cycle in the absence and in the presence of substrate protein, and depend on whether the substrate protein is foldable or non-foldable.

Our understanding of how the substrate protein interacts with the Anfinsen's cage of *cis*-GroEL ring capped with GroES has been undergoing revision. Although the polypeptide of unfolded substrate protein is completely contained in the Anfinsen's cage, which has become a current prevailing dogma described in textbooks, recent stimulating results reported by Motojima and Yoshida (2010) seem to

support that the polypeptide is not completely confined to the cavity, and that the folding proceeds while the elongated polypeptide partly protrudes out of the cavity (Motojima and Yoshida 2010; Ishii et al. 1994).

Furthermore, taking together the fact that the equatorial split happens at the equator plane of the complex, resulting in two-cone (or dome-shaped) $GroEL_{1/2}$-GroES complexes (here, $GroEL_{1/2}$ stands for GroEL heptameric ring), investigation and analysis to clarify mutual relationships between morphological transitions is the next challenge. In such a challenge, characteristic figures such as asymmetric bullet-shaped, symmetric football-shaped and cone- or dome-shaped GroEL-GroES complexes as well as single heptameric rings of GroEL and GroES are positioned in the chaperonin-mediate reaction cycles. Clarifying the connections and positioning of these complexes to appropriate sites in the reaction cycles should be a primary goal leading to better understanding of the chaperonin reaction cycle.

17.5 Applications as the Nanomolecular Machine

A protein has a peculiar higher-order structure, and each has a physiological function based on the original structure. The sophisticated structure and function are products of a long process of protein evolution. Some architecture possesses such sophisticated and minute functionality at the nanometer-scale which can hardly be achieved by human engineering (Ishii et al. 1991, 2010; Zahn et al. 1993; Ishii 2014). In this section, the author will introduce the development of exotic functional materials by utilizing the cylindrical structure of GroEL.

17.5.1 As a Carrier of the Artificial Substances

In order to utilize the GroEL macromolecule as a functional nano-material, one of the important criteria should be the possibility of introducing the different artificial substances from the original substrate of protein folding intermediates into the cavity of the molecular complex. Another criterion is whether it can be released by intra-molecular structural changes using energy from the hydrolysis of Mg-ATP, which is exactly the feature of GroEL. As the details have been reported by Ishii et al. (2003), here we briefly outline the procedures (Fig. 17.4). First, we examined experimental conditions for the preparation of the nanoparticle-GroEL conjugates, by introducing the semiconductor nanoparticles (CdS) with proper size (2.2 nm in a diameter), for the stabilization of the nanoparticles, and for the releasing of nanoparticles in response to the hydrolysis of Mg-ATP. The semiconductor nanoparticles such as CdS have fluorescent properties, which aid in detecting the uptake and the release of CdS by GroEL. The CdS nanoparticles prepared in the water-soluble solvent, dimethylformamide was mixed and gently stirred with GroEL in 25 mM Tris-HCl buffer (pH 7.5) containing 100 mM KCl for ~100 h at 4 °C, then purified

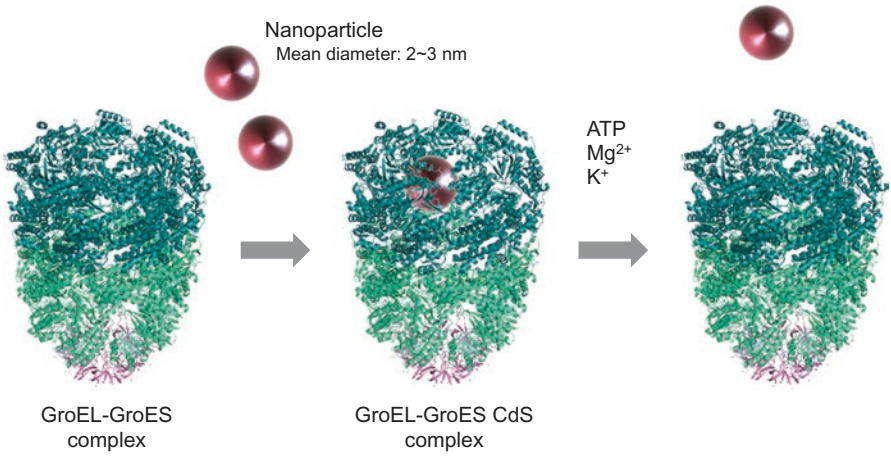

Fig. 17.4 Schematic representation of the formation of GroEL-GroES plus CdS nanoparticle complexes by inclusion of CdS nanoparticles into the cylindrical cavity of the asymmetric bullet-shaped GroEL-GroES, and its ATP-triggered guest release (Ishii et al. 2003)

and fractionated by gel filtration column chromatography. The formation of the GroEL, which encapsulated a CdS nanoparticle in the central cavity, was confirmed visually by TEM, as well as by fluorescence spectroscopy. The resultant complex was thermally stable against any denaturation, decomposition, and the release of nanoparticles up to 50 °C. The heat-resistant temperature of the complex appeared to be consistent with that of the species habitat of GroEL origin. Although the GroEL-CdS nanoparticle composite body appears stable, CdS nanoparticles which have been encapsulated is released immediately after the addition of ATP, MgCl, and KCl into the buffer solution. It is remarkable that even after the release of the nanoparticles, GroEL maintains its higher-order structure. The condition for such release is realized only when the three components of ATP, Mg^{2+}, K^{+} ions are present, and it is similar to the conditions required for functional expression of GroEL chaperonin *in vivo*. From the above, it has been found that GroEL chaperonin uptakes guest compounds even for the artificial substances employing the same molecular mechanism as the native protein folding intermediates, and then release them under the control of ATP binding and hydrolysis.

17.5.2 As an "AND" Logic Gate

As mentioned in the previous section, GroEL chaperonin can uptake (even artificial) substances and release under ATP control. When considering its use as a carrier for functional materials, it would be more advantageous to be controlled by another physical stimulus. In addition to the chemical control responding to ATP, Muramatsu et al. (2006) have reported on the creation of a photo-responsive functional GroEL

that would be controllable by light. Although there are three cysteine residues in the 57 kDa subunit constituting GroEL, all of them were replaced by genetic engineering to alanine, then, the mutant subunit was prepared so that lysine-231 which is located at the entrance of the central cavity was substituted with cysteine. The modified GroEL mutant introduces an azobenzene moiety as a photo-responsive gate around the entrance of the central cavity. This was prepared by chemical binding the azobenzene derivative having a maleimide site to K231C GroEL (Fig. 17.5). The modified GroEL chaperonin was confirmed to have similar architecture to wild-type GroEL, and is capable of incorporating the denatured protein (green fluorescent protein) into the cavity. It is well known that the azobenzene moiety can change its form from *trans*- to *cis*-form by ultraviolet irradiation, and from *cis*- to *trans*-form by visible light irradiation, meaning that its isomerization can be controlled reversibly by the wavelength of irradiation. In *trans*-form, the molecule takes the extended structure, and the transition has resulted in *cis*-form in a relatively short structure, therefore, it is possible to control the size of the opening of the gate by selecting irradiation as either ultraviolet or visible light (Fig. 17.5). The effect of ATP on modified GroEL appears to be similar to that of the wild-type GroEL, that is, the guest emission is very slow in the absence of ATP. In contrast, in the presence of ATP, when azobenzene site is isomerized to *cis*-form under the irradiation with ultraviolet light, the guest protein is released rapidly. On the other hand, when isomerized to *trans*-azobenzene form by the irradiation with visible light, the release rate has been significantly reduced. These results can be interpreted as follows; taking each of the ATP addition and the ultraviolet light irradiation as an independent input signal, only when both are loaded, the output (release of denatured protein or folding intermediate) to respond occurs, and this is considered as an "AND" logic circuit. The above is one example of the exotic nano-scaled applications using the protein function of GroEL in the construction of the logical operator in chemical biotechnology.

17.5.3 As a GroEL Nanotube

Thus far, even in the wild-type GroEL-GroES system, formation of the one-dimensional tubular-like assembly has been reported (Harris et al. 1995). However, its formation is difficult to control, and such tubular-like assemblies appear to be by-chance products associated with specimen grid preparation for electron microscopy.

The addition of ATP binding and light control to the functions of the GroEL chaperonin, the chemically modified mutant GroEL can form a tubular assembly aligned one-dimensionally under certain conditions. The author would like to introduce this achievement by Biswas et al. briefly (Biswas et al. 2009, 2013).

The spiropyran known as photochromic molecules of which polarity is greatly changed by light irradiation has been introduced site-specifically at around the entrance of the central cavity of GroEL. Thereby the spiropyran / merocyanine units

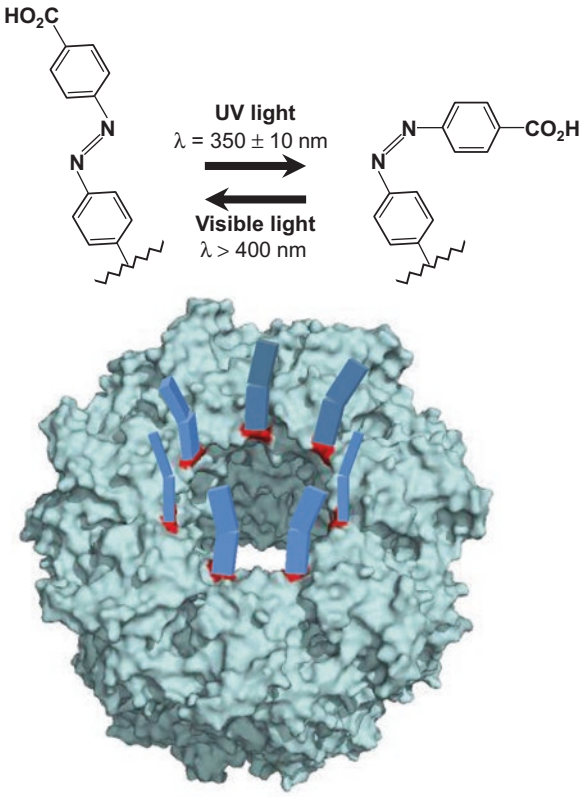

Fig. 17.5 Schematic representation of structural changes of the azobenzene-based photomechanical gates of azo-GroEL upon exposure to UV and visible lights (Muramatsu et al. 2006)

contribute to the gate switching between the open and the close motions of GroEL by ATP and also light. Construction of $GroEL_{SP/MC}$ was as follows; Briefly, all cysteine residues of each subunit of the native GroEL have been replaced with alanines, and then lysine-311 and leucine-314, and those residing on the end-on surface have been each substituted with a cysteine, respectively. Finally, the GroEL mutant having 14 cysteine residues in each entrance of the cavity was prepared, which means the resultant GroEL mutant has 28 cysteine residues that can be modified on the both end-on surfaces. The introduction of spiropyran to the cysteine sites has been achieved by applying a spiropyran derivative having a maleimide site to the GroEL mutant. After incubation, allowing the reaction of cysteines with spirobenzopyran-appended maleimide for 12 h at 4 °C, the color of the mixture turned light-purple indicating that the spontaneous reaction of partial isomerization of spiropyran to merocyanine had occurred. Therefore, spiropyran and merocyanine coexist in the buffer (Fig. 17.6). Using the gel filtration chromatography to remove the unreacted substances, the purified protein was used as $GroEL_{SP/MC}$. After treatment with the modification reaction, and when magnesium chloride was supplemented to the $GroEL_{SP/MC}$ mutant, the ratio of the polymeric regions of the elution profile from the gel permeation chromatography (GPC) significantly increased.

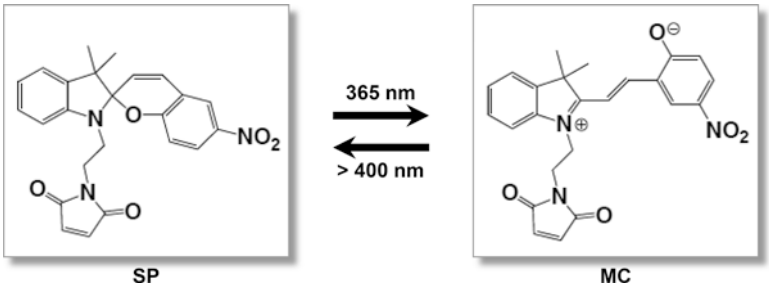

Fig. 17.6 Isomerizaton behavior of a spiropyran (SP)/melocyanine (MC) derivative (Biswas et al. 2013)

In the TEM observation of the eluate fraction corresponding to the polymeric region, unexpectedly, the modified GroEL$_{SP/MC}$ chaperonins self-assembled into a long tubular architecture (Fig. 17.7). The conditions for GroEL$_{SP/MC}$ to form self-linked one-dimensional assemblies, that is, GroEL$_{SP/MC}$ chaperonin nanotubes, were optimized assessing the buffer conditions, incubation temperature and periods and so on. Careful inspection confirmed that the chemical modification with spiropyran / merocyanine played critical role in contributing to its formation. The influence of coexistence of monovalent, or divalent cations, and with/without addition of nucleotides, on the nanotube formation was investigated by TEM (Ishii 2013). From a sequential study of the addition of metal salt, not only magnesium chloride but divalent metal ions such as calcium and zinc have been found to be effective in facilitating nanotube formation. From the above considerations, the locations of the spiropyran / merocyanine sites with the coordination of the metal ions are the most important factors to contribute the GroEL$_{SP/MC}$ nanotube formation. Moreover, the addition of ethylenediaminetetraacetic acid (EDTA) to the formed nanotubes resulted in the nanotube dissociation into the components of the building blocks. The divalent metal ions are chelated by EDTA and removed, therefore, it has been confirmed that the coordination plays an important role in the GroEL$_{SP/MC}$ nanotube forming.

Next, when the bovine α-lactalbumin, which had been denatured and fluorescently labeled, was applied to the GroEL$_{SP/MC}$ in the absence of any divalent metal ions, the unfolded α-lactalbumin was found to be incorporated by the GroEL$_{SP/MC}$ in the same manner as the wild-type GroEL. When magnesium chloride was added to this condition, it was found that the nanotube formation occurs while GroEL$_{SP/MC}$ encapsulates the modified α-lactalbumin in the cavities. Thus, by taking advantage of the chaperonin function of capturing the guest compound within the central cavity, Biswas et al. have succeeded in the GroEL$_{SP/MC}$ nanotube formation keeping the guest molecules within the nanotube (Biswas et al. 2013). Surprisingly, the chaperonin nanotube encapsulating the substances in the cavities can penetrate across the bio-membrane into the living cell, and then dissociate into compartments releasing the guest substances in the cellular ATP-responsive manner (Biswas et al. 2013). Furthermore, Sim et al. recently reported the construction of magnetic field

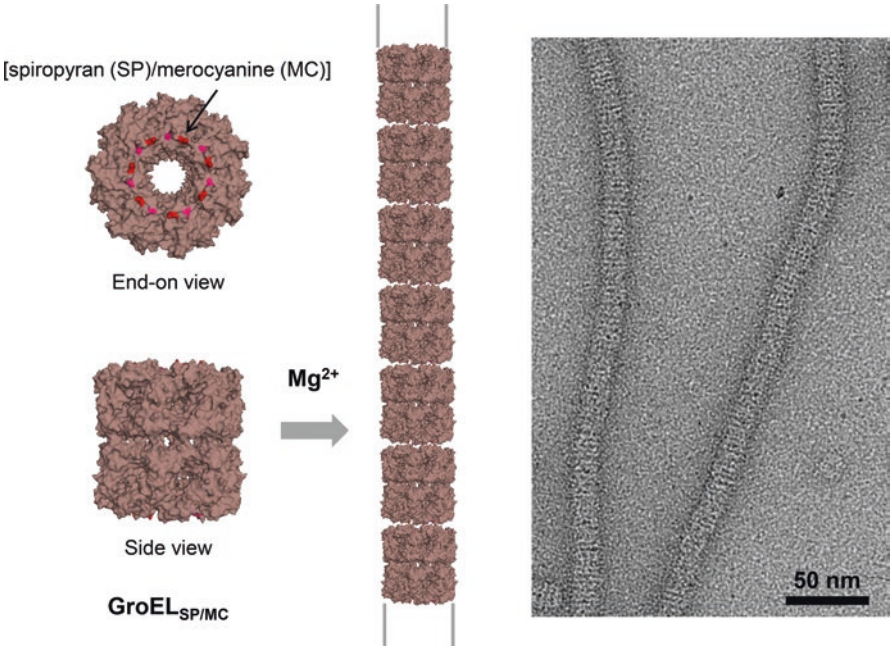

Fig. 17.7 Schematic representations of the GroEL$_{SP/MC}$ forming into a nanotube from mutant GroEL$_{SP/MC}$ and Mg^{2+}-mediated supramolecular polymerization, affixed with TEM image

sensitive GroEL nanotube (Sim et al. 2015). These nano-machines are potential components towards the fabrication of intelligent nano-devices.

17.6 Closing Remarks

The focus of modern protein science research has moved from the study of individual proteins to protein systems research, targeting the dynamics of the cellular process and interactions on the set of molecules involved using a wide range of techniques from genomics to molecular probe and imaging science. Although the fundamental mechanisms adopted in any biological processes in different species appear to be the same, there have been variations in the regulation mechanisms. For example, proteins sharing high sequence similarities over species which were evolutionarily derived from a common ancestor sometimes show different characteristics with distinguishable mechanisms. Proteins found in different species yet classified within the same nomenclature can exhibit differences in mechanism, since they adapt to changing physiological states (homeostasis) on its evolutionary path to diversity. During the process, proteins must evolve and cope with the host cell to build compensation mechanisms and networks entangled intricately by both intracellular and inter-cell interactions. Therefore, even if one protein derived from

a certain species is characterized well with clear-cut results, its characteristics do not always agree with its counterpart proteins of the same family but from a different species.

By X-ray crystal structure analysis which began in 1960, the three-dimensional protein structures, such as myoglobin, hemoglobin, and lysozyme were determined one after the other. That of the huge protein complex of GroEL was on the cover of the journal *Nature* in 1994. The era has arrived when one can understand the function of a certain protein on the basis of structure at the atomic resolution. At the same time, a concern about whether a protein (or protein complex) coming from a particular species can constitute a representative model for all species has emerged in the author's mind as follows; lysozyme for example, has been investigated thoroughly and is thus frequently featured as a typical model protein in textbooks of protein science. Lysozyme crystals are obtained using NaCl as a precipitant. However, treating lysozyme as the archetypal protein for crystallization trial procedures to be mimicked with other proteins can lead to failure.

It is inevitable that a specific target protein from a particular organism will gain more exposure and significance with time, especially if it was discovered at the early stages of research. Therefore, finding counterexamples to the prevailing theory can at times be misinterpreted as being exceptions to the prevailing model. The Group I chaperonin research has been expanding around the GroEL-GroES derived from *E. coli*, and it is certain that a lot of valuable knowledge has been obtained. It is about time to that we have to recognize that the *E. coli* and the GroEL-GroES complex derived from *E. coli* should not be treated exclusively as the archetype model for the chaperonin complex. As Okamoto et al. have recently reported, the mammalian Group I chaperonin homolog (Hsp60-Hsp10) is poorly understood while GroEL-GroES system of bacterial origin has been analyzed in detail. In mammalian cells, many physiological functions such as protein folding, assembly, transports across membranes are assumed to be mediated by Hsp60, and there is a report that mammalian Hsp60 exists in heptameric single ring structure (Okamoto et al. 2015) and that the Hsp60-Hsp10 reaction cycle is different from the GroEL-GroES reaction cycle (Parnas et al. 2012; Vilasi et al. 2014). Biochemical features of the chaperonins derived from mammals and culture cells have been accumulating recently, and those are not always the same as those confirmed with *E. coli* GroEL-GroES.

Recently, due to the more and more progress in information technology, and the availability of easy crystallization kits, the difficulty threshold of protein structural analysis has been lowering. If one wants to determine a structure of a certain protein by electron microscopic and/or X-ray crystal structure analyses, the structure obtained at the initial stage is sometimes slightly crude with inadequate resolution even after the refinement routine. Because the field of chaperonin continues to be replete with mysteries such as the robustness of the inter-subunit interactions in the GroEL-GroES complexes and the chaperonin function and structure relationship, deeper understanding may require bold feats of imagination. Anyway, it should be important to realize what is measured in the structure-determining method employed. In the protein structure, usually the electrostatic potential at any point varies and can

be represented by a function $\Psi(x, y, z)$ or in polar coordinates $\Psi(r, \theta, \varphi)$. While the values of potential are in the form of a scalar field, for an arbitrary potential distribution it is possible to imagine equipotential surfaces. Considering one equipotential surface to a nearby one along the normal to the equipotential surface, the gradient of the potential can be defined as the interaction forces between such subunits, those form a vector field (Ishii and Sato 2013). Therefore, thermodynamic evaluation of interaction between the constituent subunits in the asymmetric bullet-shaped, symmetric football-shaped complexes and so on should be the next challenging issues. Needless to say, unfounded speculations must be avoided and it is instructive not overstate any conclusions regarding details finer than the real resolution where the structural model is built.

Although the progress in this subcellular biochemistry area for macromolecular chaperonin complexes has been very rapid in recent years, much remains to be clarified. In this article, the author would have tried to summarize the recent progresses on the quaternary structures reported for the GroEL-GroES complexes while taking the chaperonin function into consideration. It would be very satisfying for the chapter author, if this article has successfully conveyed the growing progress in elucidating Group I chaperonin GroEL-GroES complex through its nearly 30-year history. Looking back at its consequences today, Anfinsen's dogma can be read as a suggestion that spontaneous folding occurs if it meets the following conditions; "uniqueness", which means the sequence does not have any other configuration with a comparable free energy; "stability", which means small changes in the surrounding environment cannot give rise to the minimum configuration changes; "kinetical accessibility", which means the final shape can be formed without going through any highly complex changes in the shape. At the same time we have to be aware how far the hypothesis and/or the structure model can be applied, namely whether it is applicable for events in cells, *in vivo* or just for those in a test tube, *in vitro*. Certain protein structure determined by X-ray crystallography only reflects the structure under the crystallization buffer condition therefore it does not guarantee the same in different solution conditions.

This article serves as an introductory exploration into the study of the Group I chaperonin GroEL-GroES complex. It is hoped that this article can serve as a background reference that will motivate the reading of more specialized or advanced accounts in literature, inspiring future generation of researchers in this field.

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