ATP-Bound States of GroEL Captured by Cryo-Electron Microscopy

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Summary

The chaperonin GroEL drives its protein-folding cycle by cooperatively binding ATP to one of its two rings, priming that ring to become folding-active upon GroES binding, while simultaneously discharging the previous folding chamber from the opposite ring. The GroEL-ATP structure, determined by cryo-EM and atomic structure fitting, shows that the intermediate domains rotate downward, switching their intersubunit salt bridge contacts from substrate binding to ATP binding domains. These observations, together with the effects of ATP binding to a GroEL-GroES-ADP complex, suggest structural models for the ATP-induced reduction in affinity for polypeptide and for cooperativity. The model for cooperativity, based on switching of intersubunit salt bridge interactions around the GroEL ring, may provide general insight into cooperativity in other ring complexes and molecular machines.

Introduction

A variety of ATP and GTP-dependent molecular machines perform many of the essential macromolecular processes in living cells, including those critically involved in information transfer: transcription, translation, and protein folding. Machines mediating the last of these steps have been the most recently recognized, and their nucleotide-directed mechanisms pose fascinating problems (Bukau and Horwich, 1998). Of particular interest are the ring assemblies known as chaperonins, the best characterized of which is the GroEL/GroES system in E. coli (Hartl, 1996; Ranson et al., 1998; Sigler et al., 1998). The essential physiological role of chaperonins, together with the experimental accessibility of the GroE system, has stimulated extensive biochemical and theoretical analysis. The resulting information may provide a conceptual model for ring complexes which harness the energy of nucleotide binding for many cellular functions.

The double-ring GroEL binds nonnative proteins in a central cavity, then mediates productive folding inside a sequestered space formed when ATP and GroES bind to the same ring as polypeptide. Each GroEL subunit comprises an equatorial, ATP binding domain joined by a hinge region to a slender intermediate domain that in turn has a hinge connection to an apical domain mediating polypeptide binding (Braig et al., 1994). The two apposed rings of equatorial domains hold the cylindrical oligomer together through both intra- and inter-ring contacts.

The cooperative binding of ATP to a GroEL ring allows rapid binding of GroES to that ring, converting it from a polypeptide-accepting to a folding-active state. At the same time, ATP binding allosterically discharges the ligands from the opposite ring. These events and the series of states in the chaperonin reaction cycle are depicted in Figure 1. Figures 1a and 1b represent states that have been extensively studied in vitro to characterize the chaperonin system, but are unlikely to form part of the in vivo cycle. A nonnative polypeptide binds to hydrophobic sites on the apical domains of an open GroEL ring (Figure 1a or 1e). Positively cooperative binding of ATP to one of the two GroEL rings promotes a conformational change in the chaperonin (Figure 1b or 1e) and is a prerequisite for binding of the cochaperonin GroES to that ring (Chandrasekhar et al., 1986; Gray and Fersht, 1991; Jackson et al., 1993; Todd et al., 1994). Negative cooperativity with respect to ATP binding results in asymmetric complexes, with the two rings adopting different conformations (Yifrach and Horovitz, 1995; Burston et al., 1995). Binding of GroES to the ATP-bound ring brings about major conformational changes that involve rigid body movements of the intermediate and apical domains, the latter elevating 60° and twisting 90° clockwise. As a result, the hydrophobic polypeptide binding sites move into the interfaces between GroEL subunits, and the walls of the folding chamber become hydrophilic. The binding of ATP and GroES thus acts to release the substrate polypeptide from its apical binding sites into a hydrophilic folding chamber capped by the dome-shaped GroES (Figure 1c; Roseman et al., 1996; Xu et al., 1997). The encapsulated polypeptide is free to undergo productive folding in this protected space (Weissman et al., 1995; 1996; Mayhew et al., 1996; Rye et al., 1997). Subsequently, ATP hydrolysis (∼10 s) weakens the binding of GroES (Figure 1d), and the free GroEL ring can now bind ATP and nonnative polypeptide (Figure 1e), providing the signal for release of GroES and polypeptide and enabling GroES to bind to the ATP-bound ring, forming a new folding-active complex (Fig-
Figure 1. Schematic Diagram of GroEL Functional States
(a) Nonnative polypeptide substrate (wavy black line) binds to an open GroEL ring. (b) ATP binding to GroEL alters its conformation, weakens the binding of substrate, and permits the binding of GroES to the ATP-bound ring. (c) The substrate is released from its binding sites and trapped inside the cavity formed by GroES binding. (d) Following encapsulation, the substrate folds in the cavity and ATP is hydrolysed. (e) After hydrolysis in the upper, GroES-bound ring, ATP and a second nonnative polypeptide bind to the lower ring, discharging ligands from the upper ring and initiating new GroES binding to the lower ring (f) to form a new folding active complex on the lower ring and complete the cycle. (f) and (c) are related by 180° rotation. Thus, the rings of GroEL alternate between folding-active and polypeptide-accepting states. (a) and (b) are shown in brackets to indicate that they represent states that are functional in vitro, even though they are unlikely to be significantly populated in vivo. The in vitro cycle includes cycling between the asymmetric states (c)–(f).

The asymmetric forms shown in Figures 1c–1f are likely to represent in vivo states, with Figure 1d as the predominant acceptor state for nonnative polypeptide.

It is clear that ATP binding plays a pivotal role in chaperonin function, but the structural consequences of ATP binding remain poorly understood. Cryo-EM studies of GroEL-nucleotide complexes at 30 Å resolution all showed extension and twisting of the apical domains and asymmetry between the rings (Roseman et al., 1996, 2001; White et al., 1997). Although the crystal structure of GroEL containing the nonhydrolyzable ATP analog ATPγS has been determined, both rings were fully occupied with nucleotide (Boisvert et al., 1996). Perhaps as a consequence, the structure did not capture the domain rotations or asymmetry between the two GroEL rings that had been observed in solution. Even more problematic, it has been observed that nonhydrolyzable ATP analogs can neither promote productive unfolding of stringent substrates nor drive dissociation of folding-active complexes, suggesting that the analogs cannot mimic the stereochemical action of ATP on GroEL (Rye et al., 1997). Thus, the question remains as to what conformational changes are accomplished by the action of ATP that enable GroES binding to the ATP-bound ring and discharge of ligands from the opposite ring. The available low-resolution cryo-EM images have suggested that the apical domains in a GroEL-ATP complex move partway toward the GroES-bound state (Roseman et al., 1996), but the details and mechanisms of these movements are unclear. Moreover, the nature of the contacts mediating the cooperative action within and between GroEL rings remains obscure.

To further probe the conformational changes promoted by ATP binding to GroEL, we have examined the structure of an ATPase mutant of the chaperonin, GroEL(D398A) complexed with ATP (corresponding to Figure 1b), at a resolution extending to 10 Å. Fitting the subunit domains en bloc into this cryo-EM structure shows that ATP binding changes the pattern of interactions throughout the double ring structure. A 20° downward rotation of the intermediate domains in the ATP-bound ring communicates a conformational change around the ring. The apical domains, released from an initial salt bridge constraint, twist counterclockwise by ~25°, requiring a net ~115° clockwise excursion of the substrate binding sites during the subsequent encapsulation of folding protein by GroES binding. Owing to tilts of the equatorial domains, the inter-ring interface is distorted, suggesting a pathway for propagating negative cooperativity to the opposite ring. A similar counterclockwise twist is also observed when ATP binds to the open ring of GroES-ADP−GroEL to form GroES-ADP−GroEL-ATPγ (corresponding to Figure 1e), suggesting a mechanism by which ATP might trigger dissociation of the GroES-capped folding chamber on the opposite ring.

Results

Solution Structures of Unliganded GroEL and GroEL(D398A)-ATP
To improve the resolution of our EM maps to 10 Å, it was necessary to collect large datasets on a microscope with a coherent source and correct for electron optical distortions (Böttcher et al., 1997; Conway et al., 1997). To validate our microscopy and single particle image processing methods, we determined the structure of unliganded GroEL and compared it to the known crystal structure. The resulting GroEL density map is shown in Figure 2a. The isolated domains were docked separately as rigid bodies into each ring of the EM density map using the program DockEM (Roseman, 2000), which provided an excellent fit. The map resolution was 10.8 Å at a Fourier shell correlation of 0.5 and 7.9 Å at the 3σ cutoff. The resolution curve is available in Supplemental Figure S1a at http://www.cell.com/cgi/content/full/108/1/869/DC1.

Although GroEL has a relatively weak ATPase activity, mixing and freezing a sample for cryo-EM takes several seconds, allowing significant ATP turnover before vitrification. A structure determined from the resulting mixture...
of GroEL-ATP and GroEL-ADP complexes describes the ensemble of structures present in solution, but the mixture of states limits resolution (Roseman et al., 2001). In the present work, we used a mutant GroEL with compromised ATPase activity to prevent ATP turnover before freezing. As shown previously, the D398A mutation in GroEL reduces the ATPase activity to ~2% of wild-type while allowing the mutant GroEL to bind ATP and GroES with normal affinity. GroEL(D398A) is also able to fold stringent substrate proteins such as Rubisco and mitochondrial malate dehydrogenase (Rye et al., 1997, 1999). These functional properties strongly suggest that

the defect in D398A is solely in catalysis of ATP hydrolysis, and that the mutant chaperonin can undergo the full range of allosteric rearrangements induced by ATP binding in the wild-type chaperonin.

The structure of GroEL(D398A) in the presence of 250 μM ATP is shown in Figure 2b. The resolution is 14.9 Å at 0.5 correlation, and 9.7 Å at the 3σ cutoff (Supplemental Figure S1b). The intermediate and equatorial domains of each ring were fitted using DockEM. The apical domains of this map had to be fitted manually, because their increased mobility in ATP results in some missing density. However, the apical domain orientations are

Figure 2. The Solution Structure of GroEL and GroEL-ATP
Surface representation of the side view of (a) GroEL and (b) GroEL(D398A)-ATP, showing that the GroEL-ATP complex is elongated compared to unliganded GroEL. The cryo-EM maps are contoured in blue mesh, with the atomic coordinates for the GroEL equatorial (green), intermediate (yellow), and apical (red) domains fitted in. In the upper ring of (b), the intermediate domain is reoriented so that it contacts the neighboring equatorial domain. In the lower ring of (b) and both rings of (a), the contact is to a neighboring apical domain. (c and d) Schematic diagrams of the deduced intersubunit salt bridges, shown as blue and red circles, in the three front subunits of each structure. (a) and (b), as well as Figures 3 and 5–7, were produced with Bobscript and Raster 3D (Kraulis, 1991; Merritt and Murphy, 1994; Esnouf, 1997).
constrained by their asymmetric shape and the connecting hinge to the intermediate domain. This allowed their orientations to be confidently assigned by manual docking in O (Jones et al., 1991). The accuracy of these fits is demonstrated by a central slice through the EM density and fitted atomic coordinates (Supplemental Figure S2).

Domain Rotations and Asymmetry
In the orthorhombic crystal structure of unliganded GroEL, the two rings are related by 2-fold crystallographic symmetry. However, in our map of unliganded GroEL, a small but distinct asymmetry is visible. Figures 3a and 3b show views of the structure from each end of the GroEL oligomer. The differences in the density envelope are explained by a 10° rotation of the intermediate domains in Figure 3b relative to Figure 3a and the crystal structure, seen in the overlay of a fitted subunit from each ring (Figure 3c). Asymmetry has not been observed previously in unliganded GroEL, and could result from either contaminating peptides in the sample binding to the chaperonin, or from the homo-oligomeric chaperonin being inherently asymmetric. This asymmetry between the GroEL rings, even in the absence of nucleotide, suggests that the intermediate domain is independently mobile, and its orientation may be a crucial factor in the allosteric transitions of GroEL. Tests to verify the asymmetry of the GroEL map are described in the Supplemental Data at http://www.cell.com/cgi/content/full/108/1/869/DC1.

The GroEL(D398A)-ATP map (Figure 2b) is considerably elongated and extremely asymmetric, with all domains in different orientations from those seen in unliganded GroEL. The upper ring in Figure 2b has a large downward rotation of its intermediate domain (Figure 3f), and the intersubunit contact between intermediate and apical domains, involving R197 and E386, is lost (see below). The lower ring in Figure 2b is more similar to a ring of unliganded GroEL, and retains density at the original intersubunit contact. This comparison suggests that the upper, more altered ring with the broken salt bridge has converted to an allosteric R (relaxed) state, whereas the lower ring is in an allosteric T (tense) state. This assignment is consistent with clear kinetic evidence for disruption of this salt bridge in the R state (Yifrach and Horovitz, 1988a). We conclude that the upper, R ring is occupied by ATP. In both rings, the apical domains extend vertically and twist counterclockwise. The twist in the R ring is ~25° (Figure 3d) and in the T ring is ~15° (Figure 3e). The apical domains bear the hydrophobic residues responsible for the binding of non-native proteins, and their counterclockwise twist has two consequences. Firstly, the twist partially buries some of the exposed hydrophobic surface in the intersubunit interface (note the position of the white-gray space-filling circles in Figure 3d versus Figure 3a). Second, it causes a radial expansion of the ring of substrate binding sites, increasing their separation (compare the central “hole” in Figure 3d with that in Figure 3a). Since the intersubunit contacts in the T ring are preserved, the 15° twist may introduce significant strain into this conformation.

ATP Binding Weakens Polypeptide Binding
The apical domain twisting should reduce the accessibility of the substrate binding sites to nonnative polypeptide. In order to test this prediction directly, a binding experiment was performed. Under physiological conditions, polypeptide and ATP bind to GroEL before GroES (Rye et al., 1999). Thus, there is a brief period (~1 s) in an ongoing folding reaction during which polypeptide is exposed to ATP-induced conformational changes which partly remove the hydrophobic binding sites from the central cavity before GroES binds. The question arises as to whether a stringent protein substrate such as Rubisco can be released from GroEL before GroES binding. To address this question, a binary complex was made between fluorescein-labeled Rubisco and SR1, a single ring version of GroEL that is fully competent to refold labeled Rubisco in the presence of ATP and GroES (Rye et al., 1997, 1999). ATP was added to this binary complex and polypeptide release was assessed by monitoring transfer of Rubisco to GroEL(D87K), a mutant chaperonin able to bind but not release substrate protein even in the presence of ATP (Figure 4a; Fenton et al., 1994). Transfer was detected by gel filtration, as the SR1 and D87K complexes are readily separable. We observed that at 4 s, the shortest time of manual mixing, 23% of Rubisco was transferred to the trap molecule (Figure 4b). At longer times of ATP exposure, 30 s and 60 s, the amount of transfer increased to 35% and 45%, respectively. By contrast, ADP produced no significant time-dependent release. Thus, ATP binding specifically weakens the affinity of a GroEL ring for a stringent polypeptide substrate.

Although the affinity of an ATP-bound GroEL ring for even stringent polypeptides like Rubisco is weakened, GroES binding could still occur rapidly enough to prevent escape of the substrate protein. To test this, GroES was added to the mixture of Rubisco-SR1 binary complex and D87K trap prior to addition of ATP, allowing GroES, whose binding to GroEL requires ATP, to compete with departure of polypeptide induced by ATP binding. In this case (Figure 4b, lane 4), no transfer of polypeptide was observed, indicating that GroES binds to an ATP-bound ring with sufficient speed to completely block polypeptide release, even though ATP binding to a GroEL ring produces a conformational change that weakens the affinity for polypeptide.

Switching of Intersubunit Contacts
Figure 5 shows the intermediate domain interactions of the T (Figure 5a) and R rings (Figure 5b) of GroEL(D398A)-ATP. The intermediate domain in the T ring is oriented toward the underside of the apical domain of its neighboring subunit, allowing E386 to retain its original salt bridge contact to R197. In contrast, the intermediate domain in the R ring rotates downward by ~20° (Figure 3f), directing the loop containing E386 to make a new contact. This interaction, seen as a new, discrete bridge of density in Figures 2b and 5b, occurs with the top of helix C (aa 66–84) in the equatorial domain of the neighboring subunit. This new intermediate domain orientation is similar to that seen in the GroES-bound ring of the crystal structure of the wild-type GroES-ADP–GroEL complex (Xu et
The so-called ADP bullet. There, the downward rotation is somewhat larger (≈25°) and residues at the tip of the intermediate domain make contact with M73 and E76 in the neighboring equatorial domain. The smaller rotation seen in our structure locates the new contact further up the helix. The residues involved cannot be precisely determined by fitting the domains as rigid bodies, since we have no basis for reorienting side chains. However, our density is consistent with the contact being made with K80 and D83, with K80 being the most plausible candidate, as this would create a new E386-K80 salt bridge to replace the lost intermediate-apical salt bridge. In order to test this proposal, K80A and K80E mutants of GroEL were made and the cooperativity of ATP binding was measured. Mutation of K80 to A or E reduces positive cooperativity (Hill coefficient is lowered by ≈40%).

Although the intermediate domain orientation in the R ring is similar to that seen in the GroES-bound ring of the ADP bullet crystal structure, here it can be identified as an allosteric rearrangement induced solely by nucleotide. The new contact in GroEL-ATP provides a
Figure 4. Effect of ATP on GroEL-Bound Substrate Protein
(a) Schematic illustration of experiment assessing whether a polypeptide substrate, fluorescein-labeled Rubisco, is released by ATP from the single ring version of GroEL, SR1. If release occurs, the Rubisco molecule can be captured by the GroEL “trap” mutant, D87K.
(b) Transfer of fluorescent Rubisco measured on gel filtration profiles resolving SR1 from D87K. In the absence of nucleotide, a small amount of Rubisco is transferred to the trap in a time-independent manner, and this was substracted from the other measured values. ATP causes transfer that increases with time. In the presence of GroES, there is no transfer above background.

means by which ATP binding in one subunit can be communicated to the equatorial domain of its neighboring subunit in the GroEL ring. Moreover, the site of this contact is extremely close to the phosphate binding loop (aa 86–90), which is involved in coordinating Mg-ATP.

Distortion of the Inter-Ring Interface
The effect of ATP binding on the ring-ring interface was investigated by examining the fits of the equatorial domains of both rings of the GroEL-ATP map. The most striking difference with unliganded GroEL (Figure 6a) is that the centers of mass of the two rings of equatorial domains have moved apart (Figure 6b). This increased separation suggests that ATP binding weakens the inter-ring interface and is consistent with biochemical evidence that ATP allows the formation of “mixed ring” GroEL complexes (Burston et al., 1996). As detailed in Figure 3f, the equatorial domains of the ATP-bound R ring change orientation, tipping radially inward and tilting sideways as viewed from inside the complex. This rotation pushes the end of helix D (aa 88–104, pink in Figure 6) down into the ring-ring interface, shortening the distance from 4 Å to 6 Å at one contact (right side, Figure 6b), and stretching it from 5 Å to 11 Å at the other (left side, Figure 6b). The equatorial domains of the T ring (lower subunit in Figure 6b) undergo a smaller rotation, in a similar direction. These tilts are reflected in opposite changes in the distances between α-carbon atoms of contact residues.

ATP Binding to a Preformed GroEL-GroES-ADP Complex
In order to examine the effect of ATP binding in a more physiological setting, GroES-ADP-,GroEL (ADP bullet) complexes were prepared with wild-type GroEL, and a 2-fold molar excess of SR1 was added. This mixture was briefly exposed to 1 mM ATP and vitrified within a few seconds. The SR1 mutant of GroEL binds GroES in the presence of nucleotide but cannot release it (Weissman et al., 1995). Therefore, the added SR1 traps both free GroES and any GroES released from the original ADP/GroES-bound ring upon ATP binding to the opposite ring. This trapping action effectively prevents subsequent binding of GroES to the newly ATP-bound ring. By vitrifying this reaction for cryo-EM, a snapshot of the mixture of chaperonin complexes is captured (see Experimental Procedures for the distribution of states). Trapping of released GroES ensures that any bullet-shaped complexes observed in this snapshot contain ADP in the GroES-bound ring and ATP in the free ring (Figure 1e). This experimental design allows us to specif-

Figure 5. Intersubunit Contacts
(a) A closeup view of the intermediate domain orientation in the unliganded T ring of GroEL-ATP.
(b) A similar view of the intermediate domain orientation in the ATP-bound R ring. The equatorial (green), intermediate (yellow), and apical (red) domains of two adjacent subunits are shown inside a blue/yellow wire mesh surface representing the EM density. The change in color of the mesh indicates the boundary between the two subunits in each view. The T ring contains density at the E386-R197 salt bridge. In the R ring, E386 makes a new contact near K80 and D83 in the neighboring equatorial domain.
physically probe the effect of a new round of ATP binding to the open ring of a GroES-ADP-GroEL complex. The solution structure of a GroES-ADP-GroEL-ATP$_1$ complex calculated from all bullet-shaped images in the dataset, with resolution extending to $\sim 12.5$ Å ($3\sigma$ cut-off—for resolution curve see Supplemental Figure S1c at http://www.cell.com/cgi/content/full/108/1/869/DC1), is shown in Figure 7. The EM density of the GroES-bound ring fits the GroES-bound ring from the ADP bullet complex crystal structure extremely well, aside from a possible small twist in the GroES mobile loops that contact GroEL (Figure 7a). The apical domains of the open ring, now bound with ATP, have undergone significant rotations, however. In order to fit these domains to the open ring, a $15^\circ$-$25^\circ$ counterclockwise rotation is required (compare the orientation line in Figure 7b with that duplicated from Figure 3a). On the other hand, density corresponding to the intersubunit R197-E386 salt bridge (circled near the bottom of Figure 7a) remains intact in that ring, suggesting that the effect of ATP binding to the GroES-ADP-GroEL complex is very similar to that described for the T ring of the GroEL-ATP complex (cf. Figure 3e).

**Discussion**

The cryo-EM structure of a GroEL-ATP complex, containing data to 10 Å resolution, provides new information about an essential intermediate state in the GroEL-GroES reaction cycle. Under normal cellular conditions in the presence of the cochaperonin GroES, ATP binding to a GroEL ring directs that ring to bind GroES. At the same time, it sends an allosteric signal to the opposite ring that discharges previously bound GroES, releasing polypeptide that had been folding in the encapsulated cavity (Rye et al., 1997). Although it has so far been

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**Figure 6. Inter-Ring Contacts**

Equatorial domain contacts in unliganded GroEL (a) and GroEL(D398A)-ATP (b). The EM density is contoured in blue mesh. Three fitted equatorial domains are in green, with helix D in pink. Residues forming the inter-ring contacts are shown in space-filling representation, colored red for negative, blue for positive, and gray for nonpolar side chains. The distances showing changes in contact length are separations between $\alpha$-carbon atoms of residue A109 in opposing subunits (for the K105/E434 contact on the right of each panel) and of residue V464 in opposing subunits (for the R452/E461 contact on the left). Residues 109 and 464 are neutral residues involved in each contact.

**Figure 7. Domain Twisting Caused by ATP Binding to the Open Ring of GroEL-GroES-ADP**

Cryo-EM structure to 12.5 Å resolution of the GroES-ADP-GroEL-ATP$_1$ complex isolated from a reaction mixture. The cryo-EM map is shown as a blue transparent surface, with the atomic structure docked in. The GroEL domains are colored as in Figure 2, and GroES is cyan. The precision of the fit is demonstrated by the side view (a). The white circle in (a) indicates the position of the R197-E386 contact. The free apical domains (lower ring in [a]) are docked into the end view (b) with a counterclockwise rotation of $20^\circ \pm 5^\circ$. The white dotted lines are orientation markers for comparison of the apical rotation here with that in Figure 3a.
impossible to study ATP-bound states of GroEL by crystallography, vitrification of the slowly hydrolysing D398A mutant before ATP hydrolysis allows a uniform population of molecules to be examined by cryo-EM and single particle image processing.

The observation that ATP binding alone is sufficient to activate major structural changes agrees with findings accumulating for other molecular machines in which the recognizable work is triggered by the energy of nucleotide binding (e.g., Wang and Oster, 1998). Hydrolysis is used simply to drive the machine forward through its reaction cycle. Indeed, previous studies on the GroE system have shown that the folding-active complex is the ATP-GroES bound state (Weissman et al., 1996; Rye et al., 1997).

The movements observed here provide a structural basis for the fundamental features of ATP binding that have been recognized through biochemical study over past years. Critically important to the proper function of the chaperonin machine is the cooperative nature of ATP binding, positive within a GroEL ring, but negative between rings. This intricate allosteric mechanism establishes the inherent asymmetry of the GroEL-GroES reaction cycle, such that only one ring is folding-active at a time (see Figure 1). Here, the allosteric movements that dictate this complex pattern of cooperativity have been observed.

**Mechanism of Positive Cooperativity**

Our results suggest that communication between the ATP binding sites of subunits within the ring is crucial for propagation of positively cooperative binding of ATP by GroEL, and that intermediate domain movement effects this communication. ATP binding to one ring causes the intermediate domains of that ring to rotate downward, consistent with a molecular dynamics simulation of the structural changes accompanying ATP and GroES binding to GroEL (Ma et al., 2000). The experimentally determined structure of the apical domain conformation in GroEL-ATP differs substantially from that in the simulation, however. The rotation of the intermediate domain allows it to form a new contact with the equatorial domain of the neighboring subunit in the ring, in close proximity to the neighboring ATP binding site. We propose that this new intermediate-equatorial contact is a major route by which information on the ATP binding state of a GroEL subunit is passed to the ATP binding site of the neighboring subunit and onward around the GroEL ring. Such communication of occupancy between binding sites, which are initially functionally equivalent, is precisely what might be envisaged in a positively cooperative system where initial ligand binding increases the affinity of successive sites.

The effects of mutations at two sites in GroEL support the idea that cooperativity in ATP binding by GroEL is mediated by the switching of salt bridges. Disruption of the intermediate-apical salt bridge present in unliganded GroEL by the mutation R197A causes a large decrease in positive cooperativity (Yifrach and Horovitz, 1994, 1998b). Here we show that disruption of the alternate intermediate-equatorial contact in the R ring of GroEL-ATP by mutation of K80 to A or E also reduces cooperativity, although to a lesser degree.

The proposed mechanism is also consistent with transient kinetic studies which reveal multiple phases upon ATP binding (Yifrach and Horovitz, 1998b; Clift et al., 1999). A first, very fast phase may correspond to the structural rearrangement of the intermediate domains.

This rapid change in intermediate domain orientation could then act as a trigger for the large, concerted change in apical domain orientation indicated by a subsequent slower phase (Clift et al., 1999). This large, concerted change primes the ATP-bound GroEL ring for GroES binding and drives ligand release from the other ring.

**Mechanism of Negative Cooperativity**

So far, the only structural analysis of the mechanism for negative cooperativity has been based on the crystal structure of the ADP bullet complex (Xu et al., 1997). Although the equatorial domains in the GroES-bound ring of this structure were tilted, those in the other ring were tilted in a complementary direction so that the interface was undistorted. It was proposed that negative cooperativity results from en bloc movements of the subunits.

In contrast, the GroEL-ATP structure here in solution suggests a very different mechanism of negative cooperativity. The equatorial domains of the R ring have the same tilts as those in the GroES-bound ring of the bullet complex: they are tilted radially inward, but also sideways, as seen from inside the oligomer (Figure 3f). In both structures, the sideways component of the tilt is larger, although only the radially inward tilt is mentioned in Xu et al. (1997). The altered interface observed here occurs because the equatorial domains of the T ring do not follow those in the R ring. Unlike the situation in the bullet crystal structure of Xu et al. (1997), the equatorial domains of the T ring have a small tilt opposing that of the R ring. The contact at the end of helix D (pink in Figure 6) leading from the ATP binding site is shortened, and the other contact is lengthened (Figure 6). Overall, the rings are further apart, consistent with observations that the interface is weaker in the presence of nucleotide. We have also observed an expansion of the interface in a lower resolution analysis of wild-type GroEL-ATP (Roseman et al., 2001). This distortion identifies essential elements of the negative cooperativity mechanism, in which the effect of ATP binding is transmitted through changes in the inter-ring interface. A possible pathway for transmission of allosteric changes includes helix D (pink in Figure 6), which connects the nucleotide binding sites of the two rings via an inter-ring contact.

There are two possible sources for the discrepancy between the preserved interface in the ADP bullet structure and the distorted one in GroEL-ATP. It is possible that the interface in an ADP bullet structure is restored following ATP hydrolysis after being distorted initially by ATP binding. However, it is also possible that conditions in the crystal reverse conformational changes in the unliganded ring of the ADP bullet complex that existed in solution. The difference between the nucleotide-bound crystal form (Boisvert et al., 1996) and our observations on GroEL-nucleotide complexes in solution (Roseman et al., 1996; 2001; White et al., 1997; present data) provide a precedent for such an effect.
Movements of the Substrate Binding Sites
Disruption of the E386-R197 salt bridge between the intermediate and apical domains of adjacent subunits of the ATP-bound ring increases the mobility of the apical domains, and allows them to rotate upward and twist. This rearrangement has major implications for a substate polypeptide bound to the apical domain hydrophobic sites lining the central cavity of GroEL. Stringent substrate proteins require multivalent interactions with the hydrophobic binding sites of at least three adjacent GroEL apical domains to bind productively (Farr et al., 2000). The 25° counterclockwise twist of the apical domains upon ATP binding partially buries the substrate binding sites in the subunit interface. In addition, the twisting causes the ring of binding sites to expand radially.

Previous binding measurements have shown that the ATP-bound GroEL state has reduced affinity for polypeptide. In the absence of GroES, certain substrate proteins, such as lactate dehydrogenase, are productively released from GroEL by ATP alone (Staniforth et al., 1994). Here we demonstrate that even the stringent GroEL substrate Rubisco interacts more weakly with the twisted apical domains of GroEL-ATP. Nevertheless, the fast rate of GroES binding relative to that of polypeptide release ensures that stringent substrates become encapsulated in the GroEL-GroES cavity, rather than escaping into bulk solution, when both GroES and ATP are present.

The counterclockwise twist of the apical domains was unexpected, given that the ATP-bound state is an obligate intermediate on the pathway from unliganded GroEL to the GroEL-GroES-ADP complex (see Figure 1). The apical domains bound to GroES are twisted ~90° clockwise relative to the orientation seen in unliganded GroEL. Thus, the counterclockwise twist described here means that binding of the GroEL mobile loops causes the apical domains of GroEL-ATP to undergo ~115° (~25° to +90°) of clockwise twist. Although the pathway followed by the apical domains to achieve the clockwise twist upon GroES binding is unknown, it is unlikely to pass back through the original unliganded conformation, because of the presence of ATP in the binding sites. Such extreme movements of the apical domains could be consistent with a proposed action of forced unfolding by separation of the binding sites during the encapsulation process (Shitilerman et al., 1999), although recent results do not support this proposal (Chen et al., 2001). Counterclockwise apical domain twisting also occurs in the physiological situation when ATP binds to a complex with GroES and ADP already resident on the opposite ring. This twist, in a ring which retains the R197-E386 salt bridge, may introduce strain. This strain may be critical for the biological role of ATP in this context, which is to trigger discharge of substrate protein and GroES from the previous folding-active chamber on the opposite ring.

Conclusions
The switching and distortion of salt bridge contacts between subunits, both within and between rings, provides a structural framework for the allosteric mechanism of GroEL. We propose that the change in orientation of the intermediate domain (and the resulting salt bridge switching) is a controlling element, and a series of conformational changes which explain the biochemical properties of the GroEL-ATP state follow from this initial movement. These conformational changes mediate the communication of ATP binding to neighboring subunits around the GroEL ring in a positively cooperative mechanism. ATP binding also brings about changes in the orientation of the equatorial domains of both rings, mediating negative cooperativity. Furthermore, the loss of the salt bridge to the substrate binding apical domain upon ATP binding permits those domains to twist, reducing their affinity for substrate proteins and priming the ATP-bound ring for GroES binding. In the ATP-bound ring of the GroEL-ATP structure, the apical domains are twisted in a counterclockwise direction, opposite to their orientation in the GroES and ADP-bound state that follows in the GroEL cycle. The salt bridge from the intermediate to apical domain in unliganded GroEL switches to form its new contact with the equatorial domain. In contrast, the empty ring of GroEL-ATP and the ATP-bound ring of the GroES-ADP-GroEL-ATP; complex show similar counterclockwise twisting of their apical domains but retain density at the intermediate to apical domain salt bridge. The proposal that intermediate domain orientation plays a pivotal role is supported by the asymmetric conformation of the intermediate domains in all GroEL complexes observed in solution and by the global effects of mutations in this region.

Switching of salt bridges upon conversion between the allosteric states of cooperative proteins has been described for a textbook example of allostery, the enzyme aspartate transcarbamoylase (ATC). In ATC, conversion between T and R states is also accompanied by the loss of interglobular salt bridges and by the formation of new bridges (Kantorowitz and Lipscomb, 1990). Here, the oligomeric ring structure of GroEL and the presence of negative cooperativity create a more complex interplay of allosteric signals. These signals direct global tertiary and quaternary changes to alternately create and discharge a protein folding chamber. The switching of interglobular salt bridges allows for propagation of positive cooperativity around a GroEL ring, transmission of negative cooperativity across the ring interface, and modulation of substrate binding affinity. Such switching may form the basis of a general mechanism for cooperative ligand binding in a range of ring-shaped molecular machines.

Experimental Procedures

Proteins and Reagents
Wild-type GroEL, GroES, and the GroEL(D398A) mutant were prepared as described previously (Kad et al., 1998; Weissman et al., 1995; Rye et al. 1997). All EM experiments were performed in 12.5 mM HEPES (pH 7.5), 5 mM KCl, and 5 mM MgCl₂.

Rubisco Binding
GroEL-Rubisco binary complex was prepared by diluting 9 µM acid-denatured, fluorescein-labeled Rubisco 10-fold into buffer containing 1 µM SR1 (Rye et al., 1999). GroEL(D87K) was added to a final concentration of 0.5 µM, and 50 µl samples were then incubated in the presence of 1 mM ATP, 5 mM ADP, or 1 mM ATP/3 µM GroES, as indicated. The nucleotide reaction was quenched at the indicated times by the addition of 20 mM CDTA, and the samples were frac-
tionated on a Tosohaas G4000SW_HPLC gel filtration column. Fluorescein-labeled Rubisco was detected by in-line fluorescence monitoring.

**EM Data Collection and Image Preprocessing**

Grids with holey carbon films were prepared and vitrified for cryo-EM by standard methods (Dubochet et al., 1988). Samples were imaged on 200K-FEG transmission EMs equipped with a Gatan cryotransfer stages. Micrographs were recorded on Kodak EM film using low dose protocols (~10 e/Å²), and digitised using a ZI Imaging Phototscan-TD scanning microdensitometer. Magnification calibration parameters were used for each microscope to correct the final specimen sampling for each dataset (see Supplemental Table S1 at http://www.cell.com/cgi/content/full/108/1/869/DC1).

Micrograph defocus and astigmatism were determined using the program CTFFINDD2 provided by Nikolaus Grigorieff and now distributed with the MRC suite of programs (Crowther et al., 1996). Micrographs showing significant drift or astigmatism were discarded. All micrographs were assumed to be of untitled areas. All particles recognizable as chaperonin side views and not in contact with other particles were interactively selected using the program Xmip (Crowther et al., 1998). For details of the number of particles and the defocus range in each dataset, see Supplemental Table S1. Particles were then extracted into 160 × 160 boxes, band-pass filtered between 250 and 4 Å, and normalised to a constant mean and standard deviation using SPIDER (Frank et al., 1996).

**Starting Models for Refinement**

To avoid bias, the crystal structures of GroEL were not used as starting models for either the unliganded GroEL or the GroEL(D398A)-ATP data. To create a starting model, 5000 unliganded GroEL images were interpolated into 64 × 64 boxes and band-pass filtered between 250 and 25 Å. A low-resolution GroEL map was created by angular reconstitution in IMAGIC (van Heel et al., 1996). This map was used as the starting model for refinement of both unliganded GroEL and GroEL(D398A)-ATP data. The crystal structure of the GroEL-GroES-ADP complex (Xu et al., 1997) was used to refine the GroEL-GroES dataset after all high resolution features were removed by Fourier filtration to 40 Å resolution.

**Angular Refinement with CTF Correction**

The refinement procedure used was essentially that described previously (Roseman et al., 1996, 2001), but modified to increase processing speed and incorporate astigmatic contrast transfer function (CTF) correction and merging of 2D image data with different defoci. In contrast to a previous study (Roseman et al., 2001), all procedures for alignment, manipulation of the CTF, and reconstruction were performed within SPIDER, using the 7-fold rotational symmetry of the complexes. A detailed description of the refinement procedure used is included in the Supplemental Data. The merging and correction of the CTF is more usually done on 3D maps. However, performing the correction on 2D images removes the constraint that each micrograph must contain enough molecular views to yield a 3D reconstruction. This is useful for GroEL-GroES complexes, which contain predominantly end views (7-fold axis perpendicular to the image plane), which are less useful for 3D reconstruction.

**Classification of Aligned Images**

After several rounds of refinement, aligned images were interpolated to 80 × 80 pixel boxes, band-pass filtered between 250 and 25 Å, and classified using IMAGIC. Classes containing spurious or persistently misaligned images were interactively selected, and their constituent raw images were excluded from the dataset for subsequent iterations of the refinement procedure.

**Structure of GroES-ADP-GroEL-ATP**

3982 images of complexes in the GroEL-ADP-GroES-SR1-ATP mixture were classified into end views (8.3% of all complexes), free GroEL (13.3%), GroES-bound GroEL (45.2%), free SR1 and SR1-GroES (15.7% and 8.6%), and GroEL with two GroES-bound (9.1%). Images corresponding to the pseudosymmetrical complex of GroEL with two GroES-bound were further classified and were found to contain mostly incorrectly aligned, singly-ligated GroEL-GroES complexes. True, doubly liganded GroES-GroEL-GroES complexes were rare (~1%–2%). The GroEL-GroES complexes (1803) were refined (see Supplemental Data at http://www.cell.com/cgi/content/full/108/1/869/DC1 for details), to give a map extending to 12.5 Å resolution, from a starting model derived from the crystal structure of GroEL-GroES-ADP filtered to 40 Å resolution. A resolution curve for this dataset is included in the Supplemental Data. The crystal structure of the GroEL-ADP-GroES complex (Xu et al., 1997; pdb code 1AON) was manually docked into the EM map using the program O (Jones et al., 1991).

**Fitting of Atomic Coordinates into EM Maps**

Initial fits of the GroEL subunit domains were generated by manually placing the atomic coordinates of the GroEL-ATP·S structure (Boisvert et al., 1996; pdb code 1DER) into the electron density using the program O (Jones et al., 1991). The fitted atomic coordinates were converted into density maps, and all maps were Fourier filtered to 10 Å using a square cutoff filter. The orientations of the manual fits were then refined using the program DockEM (Roseman, 2000), which maximises local correlation between the search object density and the electron density of the GroEL map. The highest ranking fit was selected in all cases.

**Acknowledgments**

We thank Richard Westlake and David Houldershaw for computer support, and Shaoxia Chen for EM support. We also thank Elena Orlova and Pawel Penczek for advice on image processing and Nikolaus Grigorieff for his defocus determination program. We are grateful to Tony Clarke, Michelle Hayes, and Amnon Horovitz for discussion, and to Christine Slingsby for discussion and comments on the manuscript. We thank Stephen Fuller and EMBL, Heidelberg, Germany for use of EM facilities. H.R.S is a member of The Bloomsbury Centre for Structural Biology. This work was funded by a Programme grant from the Wellcome Trust and used BBSRC-funded facilities at Imperial College.

Received January 29, 2001; revised December 4, 2001.

**References**


Accession Numbers

The density maps of unliganded GroEL, GroEL(D396A)-ATP, and GroES-ADP-GroEL-ATP, together with the fitted coordinates for all domains, have been submitted to the Protein Data Bank under accession numbers 1GR5, 1GR6, and 1GRU, respectively.