Probing Open Conformation of GroEL Rings by Cross-linking Reveals Single and Double Open Ring Structures of GroEL in ADP and ATP

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Two heptamer rings of chaperonin GroEL undergo opening-closing conformational transition in the reaction cycle with the aid of GroES and ATP. We introduced Cys into the GroEL subunit at Ala-384 and Ser-509, which are very close between adjacent GroEL subunits in the open heptamer ring but far apart in the closed heptamer ring. The open ring-specific inter-subunit cross-linking between these Cys indicated that the number of rings in open conformation in GroEL was two in ATP (GroELOO), one in ADP (GroELADP), and none in the absence of nucleotide. ADP showed an inhibitory effect on ATP-induced generation of GroELADP. The isolated GroELADP and GroELOO, which lost any bound nucleotide, could bind GroES to form a bullet-shaped 1:1 GroEL-GroES complex and a football-shaped 1:2 GroEL-GroES complex, respectively, even without the addition of any nucleotide. Substrate protein was unable to form a stable complex with GroELOO and did not stimulate ATPase activity of GroEL. These results favor a model of the GroEL reaction cycle that includes a football complex as a critical intermediate.

Chaperonin facilitates the folding of other proteins using the energy of ATP hydrolysis (1–4). GroEL, an Escherichia coli chaperonin, consists of 14 identical 57-kDa subunits arranged in two heptamer rings. Each ring contains a central open cavity, and the two rings are stacked back-to-back (5). Denatured protein binds to the apical end of the central cavity of the heptamer ring of GroEL (6–10). In the presence of ATP, a disk-shaped GroES binds to the same apical end as a lid to seal the cavity and generates a chamber. The denatured protein is discharged into the chamber, making this heptamer ring folding-active, where productive folding proceeds (11, 12). After several seconds, the GroES lid is detached from GroEL, and the substrate protein is free to escape into solution.

Two heptamer rings of GroEL undergo opening-closing conformational transition, coupled with attachment and detachment of GroES, in the functional cycle (13). In the transition from “closed” to “open” conformation, apical domain of each GroEL subunit in the ring is shifted upward and outward, and the cleft between apical and equatorial domains opens. GroES is associated with the open ring, and two kinds of GroEL-GroES complexes are formed. An asymmetric “bullet”-shaped complex is a 1:1 GroEL-GroES complex in which GroES attached to one of two heptamer rings in GroEL (14–16). A symmetric “football”-shaped complex is a 1:2 GroEL-GroES complex in which GroES attached to both heptamer rings of GroEL (17–22). The football complex contains two open rings; the bullet complex contains one closed and one open ring, and free GroEL is made up of two closed rings.

Previously, we generated the GroEL in which two rings in GroEL were locked in a closed conformation by disulfide cross-link between apical and equatorial domains in the same GroEL subunits (23). This GroEL can bind ATP and denatured protein but fails to process further reaction steps such as ATP hydrolysis, GroES binding, and release of substrate protein. We report here the opposite version; open conformation-specific inter-subunit cross-links were introduced into the GroEL ring. Using this cross-linking as a probe of open conformation, we found that one ring was open in ADP (GroELADP), although two rings were open in ATP (GroELOO). The isolated GroELADP and GroELOO, which were nucleotide-free, formed a stable bullet and football complex with GroES even in the absence of any nucleotide. These results support a GroEL mechanism that includes a football complex as a critical intermediate.

EXPERIMENTAL PROCEDURES

Reagents and Proteins—Pig heart mitochondrial malate dehydrogenase (MDH)1 and NADH were purchased from Roche Applied Science; ATP, ADP, pyruvate kinase, lactate dehydrogenase, and hexokinase were from Sigma. The trace amount of contaminating ATP in the ADP solution was eliminated by hexokinase/glucose treatment (24). Mutant proteins were generated by site-directed mutagenesis using the PrimeSTAR mutagenesis basal kit from Takara. GroEL mutants and GroES were purified as described previously (25, 26). GroEL with mutations A384C/S509C/C138A/C458A/C519A (27) (termed GroEL(2Cys)) was stored in HKM buffer (25 mM HEPES-KOH, pH 7.4, 5 mM MgCl2, and 100 mM KCl) containing 5 mM dithiothreitol. Before use, dithiothreitol was removed from the stored GroEL by ammonium sulfate precipitation. The stored GroEL was precipitated by 65% saturated ammonium sulfate and resolved in HKM buffer. The trace amount of contaminating ATP in the ADP solution was eliminated by hexokinase/glucose treatment (24). Mutant proteins were generated by site-directed mutagenesis using the PrimeSTAR mutagenesis basal kit from Takara. GroEL mutants and GroES were purified as described previously (25, 26). GroEL with mutations A384C/S509C/C138A/C458A/C519A (27) (termed GroEL(2Cys)) was stored in HKM buffer (25 mM HEPES-KOH, pH 7.4, 5 mM MgCl2, and 100 mM KCl) containing 5 mM dithiothreitol. Before use, dithiothreitol was removed from the stored GroEL by ammonium sulfate precipitation. The stored GroEL was precipitated by 65% saturated ammonium sulfate, and precipitation was washed by HKM buffer containing 65% saturated ammonium sulfate and resolved in HKM buffer. Cy3-labeled GroES (GroES-Cy3) and BODIPY-labeled MDH (MDH-BODIPY) were prepared as described previously (28, 29). Proteins were stored at –80 °C.

1 Supported by research fellowships from the Japanese Society for the Promotion of Science for Young Scientists.

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3 The abbreviations used are: MDH, mitochondrial malate dehydrogenase; HPLC, high pressure liquid chromatography; WT, wild type; BeF3, fluoroberyllate 3 or 4.
GroEL\textsubscript{O} and GroEL\textsubscript{OO} were isolated as follows. For isolation of GroEL\textsubscript{O}, GroEL(2Cys) (1 \mu m) was incubated with 5 mM ADP in HKM buffer for 50 h at 25 °C. For isolation of GroEL\textsubscript{OO}, GroEL(2Cys) (0.5 \mu m) was incubated with 5 mM ATP and 10 \mu m diamide in HKM buffer for 5 h at 25 °C. The solutions were loaded to a butyl-650 TOYOPEARL column equilibrated with 25 mM HEPES-KOH, pH 7.4, 100 mM KCl, and 1 mM EDTA. The column was washed with the same buffer, and the protein was eluted with 25 mM HEPES-KOH, pH 7.4.

**Cross-linking**—Cross-linking assays were carried out as follows. GroEL(2Cys) (0.5 \mu m) was incubated with 20 \mu m diamide in the presence or absence of 5 mM nucleotide (ATP or ADP) for 2 h at 25 °C unless otherwise stated. The ATP regeneration system (10 mM phosphoenolpyruvate and 5 \mu g/ml pyruvate kinase) was added to the assay mixture in the case of incubation with ATP. When indicated, GroES (2 \mu m), BeCl\textsubscript{2} (1 mM) plus NaF (10 mM), and urea-denatured MDH (1 \mu m) were added. Reaction was quenched by adding 10 mM N-ethylmaleimide. Cross-linking was analyzed by nonreducing SDS-PAGE using 5–20% gradient gel.

**Binding Assays with Gel Filtration**—Gel filtration HPLC was used to detect binding of GroES and substrate protein to GroEL. A column (G3000SW\textsubscript{XL}) was equilibrated with HKM buffer containing 50 mM Na\textsubscript{2}SO\textsubscript{4}. The flow rate was 0.5 ml/min. To test GroES binding, GroES-Cy3 (0.5 \mu m) and GroEL (0.2 \mu m) were mixed, and an aliquot (10 \mu l) was applied to the column. To test substrate protein binding, MDH-BODIPY (12.4 \mu m) denatured in 6.6 M urea was diluted to 0.5 \mu m in HKM buffer containing 0.2 \mu m GroEL. The mixture was incubated for 5 min and centrifuged (15,000 \times g, 5 min), and supernatant (10 \mu l) was applied to the column. To test substrate protein binding, MDH-BODIPY (12.4 \mu m) denatured in 6.6 M urea was diluted to 0.5 \mu m in HKM buffer containing 0.2 \mu m GroEL. The mixture was incubated for 5 min and centrifuged (15,000 \times g, 5 min), and supernatant (10 \mu l) was applied to the column. The elution was monitored by an in-line fluorometer (for GroES-Cy3, excitation at 550 nm, emission at 570 nm, and for MDH-BODIPY, excitation at 490 nm, emission at 520 nm).

**Quantification of Bound GroES**—To know the stoichiometry of the bound GroES versus GroEL\textsubscript{O} and GroEL\textsubscript{OO}, GroEL (1 \mu m) and GroES (5 \mu m) were mixed and incubated with 1 mM ATP, ADP, or none. The mixture was loaded to gel filtration HPLC equilibrated with HKM buffer containing the indicated nucleotide (0.2 mM), and the fraction containing GroEL was analyzed by SDS-PAGE. The intensities of the bands stained by Coomassie Brilliant Blue were quantified by Scion Image. The 1:1 wild-type GroEL-GroES complex made in ATP were used for calibration. SR398, a single-ring version of GroEL with the additional mutation D398A, has very weak ATPase and is unable to dissociate GroES even in ATP.

**Electron Microscopy**—GroEL\textsubscript{O} and GroEL\textsubscript{OO} (each 47 nm) were incubated with 3-fold excess of GroEL (147 nm) in HKM buffer for 10 min. An aliquot of the solution was applied directly on a specimen grid covered with a carbon support film. The specimen was immediately stained with 2.0% uranyl acetate and observed with an electron microscope (JEM-1230, JEOL).

**ATPase Assay**—ADP hydrolyzed by GroEL was measured spectrophotometrically with an ATP-regenerating system. The assay mixture contained 0.2 mM NADH, 5 mM phosphoenolpyruvate, 50 \mu g/ml pyruvate kinase, 50 \mu g/ml lactate dehydrogenase, and 1 mM ATP. When indicated, GroES (1 \mu m) and urea-denatured MDH (1 \mu m) were included. The reaction was initiated by injection of GroEL (final concentration, 0.1 \mu m) into the assay mixture. The decreases in the absorbance at 340 nm were monitored continuously with a spectrophotometer (V550, Jasco, Japan).

**RESULTS**

Specific Cross-linking for the Open Ring Was Designed—In the crystal structure of 1:1 GroEL-GroES bullet complex containing ADP (Protein Data Bank code 1AON (14)), Ala-384 of one GroEL subunit in the open heptamer ring attached by a GroES lid is very close (C\textsuperscript{α–C\textsuperscript{α}} distance, ~6.4 Å) to Ser-509 of the neighboring subunit (Fig. 1). A corresponding pair of residues in the heptamer ring of the opposite side, which is in a closed conformation and does not have a GroES lid, lies distant to each other (C\textsuperscript{α–C\textsuperscript{α}} distance, ~26.8 Å). Starting from a cysteine-less version of GroEL in which three endogenous cysteines of the wild-type GroEL were replaced with alanine (C138A, C458A, and C519A (27)), we further replaced Ala-384 and Ser-509 with cysteines. The resultant mutant GroEL(2Cys) showed nearly equal activities to WT-GroEL in facilitating MDH folding (data not shown) and in ATP hydrolysis (see below). Under oxidizing conditions, introduced cysteines in GroEL(2Cys) were expected to form a disulfide cross-link between neighboring subunits only when the heptamer ring was in the open conformation. If oxidation is complete, seven GroEL subunits in the open ring would make a circular (or linear) cross-linked heptamer. Therefore, cross-linking is a good probe to know whether the ring is in open conformation.

Two Rings Are Open in ATP, and One Ring Is Open in ADP—In the presence of diamide as an oxidizing reagent, GroEL(2Cys) was incubated with none, ADP, or ATP and was analyzed with nonreducing SDS-PAGE (Fig. 2A). Without nucleotide, only a protein band of monomer GroEL subunit was observed (Fig. 2A, lane 1). In ADP, the density of the monomer subunit band decreased to about half, and two high molecular weight bands appeared (Fig. 2A, lane 2). In ATP, almost all the monomer GroEL subunit was shifted to two high molecular weight bands (Fig. 2A, lane 3). When oxidation was interrupted
GroEL Has Two Open Rings in ATP

**A.**

| nucleotide | none | ADP | ATP | none | ADP | ATP | ladder | reduced |
|------------|------|-----|-----|------|-----|-----|--------|---------|
| GroES      | -    | -   | +   | -    | +   | +   |        |         |

Cross-linked products

GroEL monomer

monomer loss (%)

0 42 96 47 97

**B. +MDH**

| nucleotide | none | ADP | ATP | none | ADP | ATP |
|------------|------|-----|-----|------|-----|-----|
| GroES      | -    | -   | +   | -    | +   | +   |

monomer loss (%)

13 62 96 13 66 96

**C. +BeFx**

| nucleotide | none | ADP | ATP |
|------------|------|-----|-----|
| GroES      | -    | +   | +   |

monomer loss (%)

9 52 98 15 50 96

*FIGURE 2. Cross-link formation in GroEL(2Cys) under various conditions.*

A, GroEL(2Cys) was incubated with 20 μM diamide in the absence or presence of nucleotide (5 mM ATP or ADP) and GroES for 2 h. Mixtures were analyzed by nonreducing SDS-PAGE. In the presence of nucleotide, high molecular weight cross-link products were observed. “Ladder” was the sample incubated in ATP with 1 mM diamide, in which disulfide bond formation was stopped prematurely by modification of free sulfhydryl groups because of the high concentration of diamide. Ladder bands correspond to (from top) cross-linked heptamer (circular), heptamer (linear), hexamer, pentamer, and so on. Cross-linked products disappeared by reducing treatment, and monomer band intensity was recovered (Fig. 2A, lane 8). For each sample, loss of the monomer band intensity, compared with the corresponding sample with prior reducing treatment, was measured (Fig. 2A, monomer loss). The number of open rings in a GroEL molecule in ADP and in ATP, estimated from (2 × monomer loss), was 0.84 and 1.9, respectively. Thus, GroEL with single open ring (GroEL<sub>OO</sub>) was generated in ADP and GroEL with double open rings (GroEL<sub>O0</sub>) in ATP. Similar results were obtained by oxidation with other oxidants, such as CuCl₂, H₂O₂, and air oxygen (data not shown). Interestingly, the results were not influenced by the presence of GroES (Fig. 2A, lanes 4–6) and/or a substrate protein (denatured MDH) (Fig. 2B). Multiple turnover in ATP hydrolysis is not necessary to produce GroEL<sub>OO</sub> because the inclusion of BeF<sub>x</sub>, an inhibitor of ATP hydrolysis (22), did not change the nucleotide dependence of cross-linking (Fig. 2C). These results imply that open and closed conformations of the two rings of GroEL are governed by nucleotide: two open rings in ATP, one in ADP, and none without nucleotide.

Generation of GroEL<sub>OO</sub> and GroEL<sub>O0</sub> Is Saturated at 50 μM ATP and 50 μM ADP, Respectively—Generation of GroEL<sub>OO</sub> and GroEL<sub>O0</sub> was examined at various ATP and ADP concentrations. Even though cross-link formation was irreversible under the conditions and therefore tended to give overestimation of the open ring state, the results could provide approximate estimation of ATP and ADP concentrations required for the conformational transition. Incubation of 0.5 μM GroEL(2Cys) in 5 μM ATP resulted in a 45% loss of the monomer band (Fig. 3A), indicating that occupation of seven nucleotide-binding sites of one GroEL ring by ATP is sufficient to induce the closed-to-open transition of one ring. Further increase in ATP concentration up to 50 μM induced the transition of another ring, producing GroEL<sub>O0O</sub>. Contrary to the two-step generation of GroEL<sub>O0O</sub>, GroEL<sub>O0</sub> generation by ADP showed simple dependence on ADP concentration and was saturated at 50 μM ADP (Fig. 3B). Next, GroEL(2Cys) was incubated in the presence of 1 mM ATP plus varying concentrations of ADP. The extent of monomer loss caused by 1 mM ATP was not affected by ADP below 0.6 mM, but it started to decrease as ADP concentration increased to 0.8 and 1 mM ADP (Fig. 3C). Because GroEL<sub>O0</sub> was generated in ADP, the observed decrease in the extent of monomer loss should be due to the inhibition of GroEL<sub>O0O</sub> generation from GroEL<sub>O0</sub>. Thus, ADP inhibits moderately ATP-induced GroEL<sub>O0O</sub> generation, most likely by competing for the same nucleotide-binding sites on GroEL.

GroEL<sub>O0O</sub> Generates a Football Complex and GroEL<sub>O0</sub> a Bullet Complex by GroES Binding—GroEL<sub>O0O</sub> was purified from the incubated mixture of GroEL(2Cys), ATP, and diamide. GroEL<sub>O0O</sub> was formed by air oxidation in ADP and was purified. The purified GroEL<sub>O0O</sub> as well as GroEL<sub>O0</sub> did not contain any endogenously bound nucleotide. Transition from the purified GroEL<sub>O0</sub> to GroEL<sub>O0O</sub> depended on ATP, and ADP counteracted

by sulfhydryl modification that was caused by a high concentration of diamide, a ladder of eight bands, including two high molecular weight bands, was seen (Fig. 2A, lane 7). The top band did not contain the free sulphydryl group because when stained by the SH-reactive fluorescent reagent, only the top band was not stained. Therefore, these bands corresponded to (from the top) the circularly cross-linked heptamer, the linearly cross-linked heptamer, hexamer, pentamer, and so on. By reducing treatment prior to electrophoresis, these high molecular bands disappeared, and the monomer band intensity was recovered (Fig. 2A, lane 8). For each sample, loss of the monomer band intensity, compared with the corresponding sample with prior reducing treatment, was measured (Fig. 2A, monomer loss). The number of open rings in a GroEL molecule in ADP and in ATP, estimated from (2 × monomer loss), was 0.84 and 1.9, respectively. Thus, GroEL with single open ring (GroEL<sub>OO</sub>) was generated in ADP and GroEL with double open rings (GroEL<sub>O0</sub>) in ATP. Similar results were obtained by oxidation with other oxidants, such as CuCl₂, H₂O₂, and air oxygen (data not shown). Interestingly, the results were not influenced by the presence of GroES (Fig. 2A, lanes 4–6) and/or a substrate protein (denatured MDH) (Fig. 2B). Multiple turnover in ATP hydrolysis is not necessary to produce GroEL<sub>OO</sub> because the inclusion of BeF<sub>x</sub>, an inhibitor of ATP hydrolysis (22), did not change the nucleotide dependence of cross-linking (Fig. 2C). These results imply that open and closed conformations of the two rings of GroEL are governed by nucleotide: two open rings in ATP, one in ADP, and none without nucleotide.
GroES complex (bullet complex), respectively. Electron microscopic images of these complexes stained by uranyl acetate were very similar to those of the typical bullet complex and football complex (Fig. 4C). The stability of the GroEL\textsubscript{OO}-GroES football complex was assessed by the loss of GroES-Cy3 from the GroEL\textsubscript{OO}-GroES-Cy3 football complex in the presence of a large excess of nonlabeled GroES. In 1 h, half of GroES-Cy3 was lost in the absence of nucleotide but only 5% was lost in ATP and ADP (data not shown).

GroEL\textsubscript{OO} Cannot Form a Stable Complex with Substrate Protein—The binding of denatured protein to GroEL\textsubscript{OO} and GroEL\textsubscript{OOO} was examined by using fluorescently labeled MDH-BODIPY (Fig. 5). Urea-denatured MDH-BODIPY was diluted into the solution of GroEL. The aggregation was removed by centrifugation, and the supernatant was applied to gel filtration HPLC monitored with fluorescence. In the case of WT-GroEL and reduced GroEL(2Cys), MDH-BODIPY was eluted at the position of GroEL, indicating stable binding of these substrate proteins to GroEL. GroEL\textsubscript{OO} also bound MDH-BODIPY, although the bound amount was less than WT-GroEL. In the case of GroEL\textsubscript{OOO}, very little fluorescence was eluted at the position of GroEL, indicating that GroEL\textsubscript{OOO} cannot form a stable complex with denatured substrate protein.

GroEL\textsubscript{OO} Can Hydrolyze ATP but There Is No Stimulation by Substrate Protein—As is well known, ATPase activity of GroEL is suppressed by GroES and stimulated by substrate protein (denatured MDH) (16, 22, 30) (Fig. 6). ATPase activity of the reduced GroEL(2Cys) also showed the same response to GroES and substrate protein. ATPase activity of GroEL\textsubscript{OO} was somewhat small (~20% of the reduced control). It was suppressed by GroES and was stimulated 4-fold by denatured MDH. GroEL\textsubscript{OOO} had larger ATPase activity than GroEL\textsubscript{OO}, and this activity was suppressed by GroES. However, in agreement with loss of ability to bind substrate protein as described, it was not stimulated by denatured MDH. It is worth noting that the GroEL\textsubscript{OOO}-GroES complex is very stable in ATP, as stated before, and therefore catalytic turnover of ATP hydrolysis does not induce dissociation of GroES from the complex.

DISCUSSION

Even though the conformation-locked GroEL cannot carry out the whole functional cycle, it exhibits the partial functions exclusively attributable to the GroEL in the defined conformational states. We previously showed (23) that the GroEL locked in the double-closed ring conformation can bind substrate protein and ATP, but it cannot hydrolyze ATP nor bind GroES. Here we showed that the GroEL locked in the double open ring conformation can bind GroES and hydrolyze ATP but cannot bind substrate protein in a stable manner. These features have been inferred before and here we show them unambiguously.

We adopted open conformation-specific cross-link formation as a probe of conformational transition of GroEL, and we found that ATP generates GroEL\textsubscript{OO} whereas ADP generates GroEL\textsubscript{OOO}. Without nucleotide, the open ring is not generated in GroEL. Isolated GroEL\textsubscript{OO} and GroEL\textsubscript{OO} lack endogenously bound nucleotide, and they can bind GroES in the absence of nucleotide, generating football complex and bullet complex, respectively. It is now clear that ATP and ADP induce opening

![FIGURE 3. Effect of ATP and ADP concentrations on cross-link formation.](image-url)
motion of one and two rings, respectively, but they are not essential for subsequent GroES binding. Another interesting observation is the inhibitory effect of ADP on ATP-induced GroELOO formation. This observation implies that the population of GroEL molecules in the double open ring conformation is dependent on ATP/ADP balance as indicated previously (31). Inhibition by ADP is moderate, and therefore, it is reasonable to assume that in normal physiological conditions, where ATP is severalfold more abundant than ADP, most GroEL molecules can take the double open ring conformation.

It has been widely accepted that two rings of GroEL alternate the GroES-sealed, folding active state during the functional cycle. The critical reaction intermediate in this model is a bullet-shaped GroELO-GroES complex. This complex is thought to be the state in which substrate protein binding occurs. However, the presence of such a structure has not been directly observed.

Recent advances in FRET and electron microscopy have allowed for the visualization of GroEL-GroES complexes. Figure 4 shows the binding of GroES to GroELO and GroELOO. GroES-Cy3, 2.5-fold excess over GroEL, was mixed with WT-GroEL, reduced GroEL(2Cys), GroELO, or GroELOO in the absence of nucleotide. The mixtures were analyzed by gel filtration HPLC. Elution of GroES-Cy3 fluorescence (excitation at 550 nm and emission at 570 nm) was monitored in-line. GroEL and free GroES-Cy3 were eluted at 12 and 18 min, respectively. GroELOO was mixed with 5-fold excess GroES with or without nucleotide (ATP or ADP), and the GroEL-GroES complex was isolated by gel filtration HPLC. The complex was analyzed by SDS-PAGE, and bound GroES was quantified. The 1:1 WT-GroEL-GroES complex made in ADP and the 1:1 SR398-GroES complex made in ATP were used for calibration standards as 1:1 and 1:2 GroEL-GroES ratios, respectively. SR398 is a single ring version of GroEL with a slow ATP-hydrolyzing mutation (D398A) that retains GroES tightly even in ATP.

Figure 5 shows the binding of substrate protein to GroELOO. MDH-BODIPY was denatured in 6.6 M urea and diluted 24.8-fold into the buffer containing WT-GroEL, reduced GroEL(2Cys), GroELO, or GroELOO. After a 5-min incubation, the solution was centrifuged, and supernatant was applied to gel filtration HPLC. Fluorescence of MDH-BODIPY (excitation at 490 nm and emission at 520 nm) was monitored in-line. GroEL-bound MDH-BODIPY was eluted at 12 min.

Figure 6 illustrates the ATPase activity of GroELOO. ATPase activities of WT-GroEL, reduced GroEL(2Cys), GroELO, and GroELOO were measured. GroES and denatured MDH (dMDH) were added as indicated. Details of experimental conditions are described under “Experimental Procedures.”

The observations and experiments described in this report provide insights into the molecular mechanisms of GroEL function and may have implications for understanding the folding of substrates and the regulation of GroEL activity.
complex (16). However, this model was recently challenged by the findings that a football complex can be an active reaction intermediate. Sameshima et al. (31) monitored the fluorescence resonance energy transfer between GroEL and GroES and found that nearly equivalent amounts of football and bullet complexes coexist in the mixture during the functional reaction cycle. Koike-Takeshita et al. (32) reported that a slow ATP-hydrolyzing GroEL mutant can bind substrate protein and GroES at both rings of the GroEL to generate a football complex. These two studies also suggested that the football complex is generated in ATP, and the bullet complex is in ADP. The results reported here are in good agreement with the above observations and add more support for the mechanism that includes a football complex as an intermediate.

Acknowledgment—We thank Dr. K. Mitsuoka for the help with observing electron microscopic images.

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