GroEL undergoes numerous conformational alterations in the course of facilitating the folding of various proteins, and the specific movements of the GroEL apical domain are of particular importance in the molecular mechanism. In order to monitor in detail the numerous movements of the GroEL apical domain, we have constructed a mutant chaperonin (GroEL R231W) with wild type-like function and a fluorescent probe introduced into the apical domain. By monitoring the tryptophan fluorescence changes of GroEL R231W upon ATP addition in the presence and absence of the co-chaperonin GroES, we detected a total of four distinct kinetic phases that corresponded to conformational changes of the apical domain and GroES binding. By introducing this mutation into a single ring variant of GroEL (GroEL SR-1), we determined the extent of inter-ring cooperation that was involved in apical domain movements. Surprisingly, we found that the apical domain movements of GroEL were affected only slightly by the change in quaternary structure. Our experiments provide a number of novel insights regarding the dynamic movements of this protein.

Research regarding the specific mechanism by which the Escherichia coli GroE chaperonins bind and assist the folding of various proteins has resulted in the collection of a vast amount of knowledge regarding the specific effects of these proteins on protein folding, the specific factors necessary to achieve these effects, and a partial understanding of the specific processes by which these proteins act (1–4). The general consensus regarding the effects of the chaperonins on protein folding is that these proteins act to minimize nonproductive aggregation of protein folding intermediates by encapsulation of the protein molecule in a dome-like chamber formed by the chaperonins themselves. Our present understanding of the cyclic mechanism of the GroE system is represented by the elegant study performed by Rye et al. (5), which involves the encapsulation of refolding protein in a central cavity formed by the two chaperonin subunits, GroEL and GroES (the cis complex), and a subsequent ejection of the refolding protein into the bulk medium which is initiated by the binding of ATP to the ring situated in trans to the refolding protein. This mechanism presented by Rye et al. provides a very comprehensive view of the process by which GroEL binds, segregates, and releases refolding protein molecules to minimize nonproductive aggregation and promote efficient protein refolding.

The GroEL subunit is composed of three structural domains (equatorial, intermediate, and apical), each possessing a distinct role in the overall mechanism. The apical domain, located at the entrance to the central cavity, is responsible for the recognition, binding, and possibly the release of refolding protein molecules and the co-chaperonin GroES to the GroEL tetradecamer. The x-ray crystal structure of GroEL14-ADP7-GroES, (6) shows that this domain undergoes radical movements that are controlled by the binding of the nucleotide ATP to the equatorial domain and subsequent hydrolysis to ADP and P\textsubscript{i}. This radical movement of the GroEL apical domain allows a modulation of the binding affinity of GroEL toward various protein molecules, by alternatively presenting and hiding various structural motifs located at the domain surface.

The x-ray crystal structures reported to date presumably reveal only two specific conformations that are accessible to the GroEL subunit, and the actual number of conformations utilized, as well as the specific sequence of events which describe the cyclic mechanism, are the subjects of immediate and extensive study (7–10). A study by Ranson et al. (11) has recently elucidated a number of structural characteristics regarding the functional intermediate states of GroEL that suggest that GroEL may undergo a more intricate series of events than a simple closed-open transition in its functional mechanism. Using cryo-electron microscopy, they have shown that in the ATP-bound state of GroEL D398A, the apical domain of GroEL initially rotates ~25° in a counterclockwise rotation, whereas the final open conformation seen in the x-ray crystal structure places the apical domain at a position that is rotated clockwise 90° relative to the initial closed conformation. This result, together with numerous other facts revealed in this study, hint at the extreme complexity that underlies the chaperonin mechanism.

Various biophysical studies regarding the kinetic aspects of GroEL subunit movements have also been performed. Yifrach and Horovitz (12) have performed a detailed kinetic analysis of the various movements of GroEL using a mutant form of GroEL (F44W). In their studies they found an interesting kinetic phase whose rate displayed a bisigmoidal dependence on
the concentration of the nucleotide ATP. Also, in a recent study, Cliff et al. (13) have performed experiments regarding the molecular movements of GroEL that expand on the results of Yifrach and Horovitz. They demonstrated, using a mutant GroEL subunit with a tryptophan residue located in the equatorial domain, that the GroEL subunit undertook no less than four distinct conformational changes upon ATP binding. These conformational changes were respectively attributed to various important stages of the chaperonin functional mechanism, based on their kinetic characteristics and behavior under various experimental conditions.

As part of our studies on the elucidation of the molecular mechanism of GroEL-facilitated protein folding, we were very interested in describing in detail the specific movements of the GroEL apical domain that are initiated as a result of ATP binding. However, the wild-type GroEL subunit does not possess a suitable structural probe, which may be utilized in such studies. To address this, we attempted to construct a mutant GroEL molecule with functional characteristics that were virtually identical to wild-type GroEL, but with a fluorescent tryptophan residue introduced into the apical domain. Our experiments have resulted in the construction and characterization of GroEL R231W. GroEL R231W possesses a single tryptophan residue in the apical domain of GroEL (Fig. 1a), situated in between the two α-helices (helix H and helix I), which are implicated in interactions between GroEL and refolding protein, as well as GroES and the co-chaperonin GroES. In addition, Arg231 may be seen in the x-ray crystal structure of GroEL to move from a relatively hydrophilic position at the top of the GroEL ring to a relatively hydrophobic position at the interface of two adjacent GroEL subunits (Fig. 1b). A fluorescent probe introduced into this site should report on a number of important events during the cyclic mechanism of GroEL, including binding of GroES, as well as various intricate movements of the apical domain.

In this report we summarize our results regarding stopped-flow fluorescence experiments on GroEL R231W, with special emphasis on the sequence of events immediately following the binding of ATP to the equatorial domain of GroEL. We initially observed three major kinetic phases from tryptophan fluorescence above 320 nm. Experiments were performed in 50 mM triethanolamine, pH 7.5, containing 20 mM MgCl2 and 50 mM KCl. Stopped-flow spectroscopy of various GroEL proteins was performed on an Applied Biophysics SX-17MV stopped-flow fluorescence spectrophotometer. The excitation wavelength was 285 nm, and a filter was used to quantify all emission fluorescence above 320 nm. Experiments were performed in 50 mM triethanolamine, pH 7.5, containing 20 mM MgCl2 and 50 mM KCl. Protein samples were dialyzed overnight against this buffer containing 0.5 g/liter of powdered acid-washed charcoal to remove any fluorescent

![Figure 1. Location of arginine 231 in the structure of GroEL.](http://www.jbc.org/)

Purification of GroEL R231W and SR-1/R231W was accomplished according to previously published protocols (14). In the case of GroEL SR-1/R231W, an additional purification step consisting of a 30-min incubation with 1 mM ATP followed by fractionation on a Q-Sepharose column as described by Inobe et al. (15) was required to attain the necessary purity. Details regarding the purification of GroEL R231W are given under “Results.”

Bovine rhodanese and nucleotides were obtained from Sigma. Pig heart malate dehydrogenase was obtained from Roche Applied Science. All other reagents were obtained commercially from Wako Fine Chemicals (Osaka) and Nacalai Tesque (Kyoto).

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild-type GroEL protein was purified from E. coli cells harboring plasmid pUCESL, as described elsewhere (14). GroEL mutants R231W and SR-1/R231W were constructed by using the QuickChange site-directed mutagenesis kit (Stratagene), using either pUCESL (wild-type GroEL) or pEL-SR1 (15) (GroEL, SR-1) as the template. The constructed plasmids were used to transform E. coli JM109. pEL-SR1, the template used in the construction of GroEL SR-1/R231W, was a generous gift from Professor Kunihiro Kuwajima, Tokyo University.

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1 The abbreviation used is: SR-1, single ring mutant.
contaminants. The final concentration of GroEL during measurements was set at 0.5 mg/ml (corresponding to an oligomeric molar concentration of 0.62 μM for GroEL R231W and 1.2 μM for GroEL SR-1/R231W). All experiments were performed at 25 °C. Ten fluorescence traces per sample point were averaged to obtain data suitable for kinetic analyses. For the analysis of individual kinetic phases, regions of each trace where the phase of interest was the major component were selected and then analyzed using the software provided with the stopped-flow system according to a single- or sequential double exponential equation using non-linear least squares fitting. All of the experimental traces described below were obtained at an identical photomultiplier voltage (700 volts) to allow comparisons of the relative amplitudes of each kinetic phase.

**RESULTS**

*Initial Characterization of GroEL R231W—* GroEL R231W was constructed using conventional mutagenic procedures and overexpressed in *E. coli*. In preliminary experiments, we confirmed that this mutant was capable of replacing wild-type GroEL in *E. coli* strain KY1156 cells (19) and was therefore active in *vivo*. Expression of this mutant protein was very high, and purification from crude extracts was relatively straightforward (14), with a single exception. In Q-Sepharose anion exchange column purifications, a fraction of GroEL R231W was seen to elute in inactive form at ionic concentrations lower than the active mutant protein. Although various attempts were made to reconstitute this soluble, inactive form of GroEL R231W, our attempts have so far been unsatisfactory. Care was taken, therefore, to purify only the fractions with ATPase activity, which were eluted at NaCl concentrations of 0.4–0.5 M from a Q-Sepharose gel.

Initial characterization of the GroEL R231W mutant indicated that its behavior toward rhodanese (16, 17) and mitochondrial malate dehydrogenase (20) was indistinguishable from the wild-type protein (data not shown). However, we observed in refolding assays using other, larger proteins that GroEL R231W showed a decreased tendency to bind to larger polypeptides (not shown). This is in agreement with a very recent study (21), which implicated this residue in the binding of substrate proteins and GroES. An ATPase activity that was slightly higher than the wild-type chaperonin was also detected. In the present study, we regarded the effects of these deviations from wild-type-like behavior on the overall mechanism to be negligible.

When GroEL R231W was mixed with various concentrations of ATP and immediately monitored using stopped-flow fluorescence, we observed an intricate series of fluorescence intensity changes, suggesting various movements of the apical domain (Fig. 2). The changes in fluorescence intensity involved an initial sharp increase (Phase A, apparent rate constant \( k_{app} > 300 \text{ s}^{-1} \) at 300 μM ATP, 25 °C, Fig. 2a), followed by a large decrease in fluorescence (Phase B, \( k_{app} = 68 \text{ s}^{-1} \) at 300 μM ATP, 25 °C, Fig. 2a), which was in turn followed by a slow increase in fluorescence (Phase C, \( k_{app} = 4.3 \text{ s}^{-1} \) at 1 mM ATP, 25 °C, Fig. 2b). The rate constants of Phase A and Phase B at these two ATP concentrations were clearly different; the fastest phase A was lost in the dead time of the instrument at 1 mM ATP. The ATP concentrations were clearly different; the fastest phase A (Fig. 2a at 300 μM ATP, 25 °C, Fig. 2a), followed by a large decrease in fluorescence (Phase B, \( k_{app} = 68 \text{ s}^{-1} \) at 300 μM ATP, 25 °C, Fig. 2a), which was in turn followed by a slow increase in fluorescence (Phase C, \( k_{app} = 4.3 \text{ s}^{-1} \) at 1 mM ATP, 25 °C, Fig. 2b). The rate constants of Phase A and Phase B at these two ATP concentrations were clearly different; the fastest phase A was lost in the dead time of the instrument at 1 mM ATP. The residuals derived from a typical analysis of Phases B and C are shown in the lower panels of Fig. 2, a and b. In each case, the residuals consisted of small, random deviations, which suggested that the fitting procedure had been completed properly.

A more detailed examination of the apparent rate constants of each phase with respect to the ATP concentration revealed a number of common characteristics with the kinetic phases observed by Cliff *et al.* (13) in their studies. With regard to Phase A, the apparent rate constants observed under our experimental conditions was too rapid to allow a detailed analysis of its characteristics. However, the value of the apparent rate constant of Phase A in the presence of 300 μM suggested that this kinetic phase was similar to phase a of GroEL Y485W. This extremely rapid kinetic phase most likely reflects movements of the apical domain, which immediately follow ATP binding.

Fig. 3 shows the dependence of the apparent rate constants and relative amplitudes of Phase B with respect to the ATP concentration. As shown in Fig. 3, the dependence of the rate constants of Phase B on the ATP concentration were characterized by an initial increase in apparent rate that leveled off transiently at ATP concentrations from 200 to 300 μM. The rate constants were again increased at ATP concentrations higher than 300 μM, to saturate at ATP concentrations greater than 1000 μM. The overall saturation profile was sigmoidal, a distinct characteristic of a kinetic phase that was previously observed by Yifrach and Horovitz (12) in their experiments using GroEL F44W, and also by Cliff *et al.* for phase b in GroEL Y485W (13). In order to compare the present values with the values for GroEL Y485W, we analyzed the curves in accordance with the phenomenological model utilized by these research groups, more specifically according to Reaction Scheme 1 put forth by Cliff *et al.* (13),

\[
\begin{align*}
K_1 & \quad \text{EL} \quad \text{ATP} \\
\text{EL} + (n+m)\text{ATP} & \quad \text{EL} \text{ATP}_n + m\text{ATP} \\
\text{EL} \text{ATP}_n + m\text{ATP} & \quad \text{EL} \text{ATP}_{(n+m)} \\
\text{EL}^x & \quad \text{ATP}_n + m\text{ATP} \\
\text{EL}^x \text{ATP}_n + m\text{ATP} & \quad \text{EL}^x \text{ATP}_{(n+m)}
\end{align*}
\]

**REACTION SCHEME I**

where GroEL binds to *n* molecules of ATP with an equilibrium constant of \( K_1 \), followed by *m* molecules of ATP binding with an equilibrium constant of \( K_2 \). These two forms of GroEL each undergo conformational transitions with a forward rate con-
amplitude were observed at ATP concentrations greater than 300 μM ATP (Fig. 3b). Analysis of the data according to a simple Hill equation revealed a weak cooperativity (n = 2.0 ± 0.2), which was similar to the value of n obtained using Equation 1 in Fig. 3. From the amplitude data in Fig. 3b it is possible that the structural state attained after the completion of Phase B may be the same in both moderate (−250 μM) and high (−1 mM) concentrations of ATP. Alternatively, the states may be different, but indistinguishable by changes in fluorescence of Trp231. Interestingly, this characteristic was also a feature, which was observed for phase b in GroEL Y485W (13), reinforcing the notion that the two phenomena reflect a common molecular event. Since we are unable to distinguish between the states attained after Phase B at different ATP concentrations by fluorescent probes located at two diverse locations within the GroEL subunit, these states may indeed be structurally equivalent.

With regard to Phase C, the maximum apparent rate was similar to that of phase c as reported by Cliff et al. (13) (Fig. 4). In the present study, the fact that Phase C was detected as a fluorescent increase allowed us to analyze the characteristics of this phase in more detail. The rate of this phase varied with the ATP concentration and saturated at 4.5 s⁻¹ with a K₅₀ of 105 ± 47 μM. Interestingly, the amplitude of this phase saturated at a much lower concentration of ATP (−50 μM). It seems that in the presence of ATP at concentrations from 50 to −300 μM, the rate of the apical domain movements attributed to Phase C was dependent on the ATP concentration while the amplitude remained constant.

A prominent difference between the results of the present study and that of Cliff et al. (13) involved the characteristics of a kinetic phase corresponding to phase d in GroEL Y485W. We found that in GroEL R231W, a slow decrease in fluorescence intensity may indeed be observed after the completion of Phase C, with an apparent rate constant of 0.39 s⁻¹ at 1 mM ATP, 25 °C (data not shown). However, the appearance of this kinetic phase was rather erratic. At present, we would like to refrain from characterizing this kinetic phase in more detail until we are more confident of the specific conditions under which this phase is detected.

The number of similarities between the numerous kinetic phases determined in the present study and the results of Cliff et al. (13), in particular with regard to Phase B, suggested attractively that we were observing functionally significant movements of the GroEL apical domain, and that a number of these movements were part of a global alteration of the GroEL subunit.

Phase B Is Unaffected by the Binding of GroES—When GroEL R231W was mixed simultaneously with 1 mM ATP and
an equimolar concentration of GroES, an additional kinetic phase was observed (Fig. 5, dotted traces). Analyses of the kinetic curves indicated that the new phase consisted of a fluorescence increase with an apparent rate constant of 29 s$^{-1}$. A rough estimate of the rate of association of GroEL and GroES under the experimental conditions used yielded a bimolecular rate constant of $4.6 \times 10^7$ M$^{-1}$ s$^{-1}$, which was in good agreement with the binding constants obtained in other studies (5, 22). The apparent rate of this newly detected phase was dependent on the molar ratio of GroEL and GroES (Fig. 5b). Therefore, we concluded that GroEL R231W was capable of reflecting the binding of GroES to the GroEL ring, in addition to the numerous movements of the apical domain. Since we were able to observe GroES binding and apical domain movements simultaneously using Trp231, and also, since we observed that the apparent rates of the GroES binding phase and Phase B were very similar to each other, we were curious to determine whether there were any dependences between these two phenomena. Specifically, we probed the relationship between GroES binding and Phase B by lowering the concentration of ATP so that the apparent rate of Phase B would be retarded, and monitoring the effects of this retardation on the phase reflecting GroES binding. Fig. 6 shows the kinetic traces obtained at ATP concentrations ranging from 300 to 1000 μM ATP.

As shown in Fig. 6, we found that the kinetic phase reflecting GroES binding remained relatively unaffected by the changes in apparent rate of Phase B. In order to confirm this, we subtracted from the data shown in dotted traces (taken in the presence of GroES) in each panel of Fig. 6 the corresponding traces shown in solid traces (taken in the absence of co-chaperonin), to isolate the GroES binding phase. All three of the derivative traces closely overlapped (data not shown), suggesting that the binding of GroES proceeded with little regard to the completion of the apical domain movements corresponding to Phase B.

Apical Domain Movements in a Single Ring GroEL Mutant—In order to obtain more data to clarify the relationship between apical domain movements and GroES binding, as well as to determine the relationship between the three conformational movements and the quaternary structure of GroEL, we constructed an additional mutant in which GroEL R231W was combined with the single ring mutations of GroEL SR-1 (8, 9). GroEL SR-1/R231W was expressed and purified readily from E. coli cells, and the behavior of this mutant was similar to the original SR-1 mutant, with regard to quaternary structure as determined by gel filtration, permissive rhodanese refolding (8) and ATPase activities (9) (data not shown). When we performed stopped-flow fluorescence experiments on this new mutant, we found that the changes in fluorescence of the heptameric mutant chaperonin retained most, but not all, of the characteristics of tetradecameric GroEL R231W. As shown in Fig. 7, the fluorescence changes of GroEL SR-1/R231W consisted of an initial rapid increase, followed by a large decrease, and finally a slow increase, essentially the same as the fluorescence changes detected in GroEL R231W. The kinetic constants obtained for each Phase B and Phase C in SR-1/R231W were as follows (at 300 μM ATP): Phase B; $k_{app} = 56.9 \pm 1.57$ s$^{-1}$, relative amplitude = 0.105 ± 0.00216, Phase C; $k_{app} = 1.07 \pm 0.0178$ s$^{-1}$, relative amplitude = 0.027 ± 0.000321. Of note was the roughly 4-fold reduction in apparent rate and a large reduction in the relative amplitude of Phase C in the heptamer mutant, indicating a moderate dependence of this kinetic phase to the quaternary structure of GroEL.

![Figure 5](http://www.jbc.org/)

**Fig. 5.** a, changes in the ATP-triggered tryptophan fluorescence changes of GroEL R231W in the presence and absence of an equimolar concentration of GroES. Solid lines represent changes in the fluorescence of GroEL R231W in the absence of GroES, dotted lines indicate fluorescence changes of GroEL R231W in the presence of an equimolar oligomeric concentration of GroES (0.6 μM GroES$_7$). The experimental temperature was 25°C, and the final concentration of ATP was 1 mM. b, the dependence of the apparent rate constants of the newly detected phase on the GroES/GroEL ratio. Ratios are derived from oligomeric concentrations; i.e., [GroES$_7$]/[GroEL$_{14}$].

![Figure 6](http://www.jbc.org/)

**Fig. 6.** A comparison of the changes in fluorescence of GroEL R231W in the presence and absence of GroES at varying ATP concentrations. A comparison similar to that shown in Fig. 5 was performed in the presence of the following ATP concentrations: 300 μM (a), 400 μM (b), 1 mM (c). The experimental temperature was 25°C. Solid lines indicate fluorescence changes in the absence of GroES, dotted lines indicate fluorescence changes in the presence of an equimolar oligomeric concentration (0.6 μM GroES$_7$) of GroES.
Chaperonin Domain Movement and Quaternary Structure

DISCUSSION

The GroEL Apical Domain Undergoes Numerous Conformational Changes upon ATP Binding—A comparison of the x-ray crystal structures of GroEL (23) and the GroEL-ADP\textsubscript{7}-GroES complex (6) show that GroEL is able to assume two very distinct conformations during its functional cycle, as it segregates refolding protein molecules within its unique cylinder-like structure. A detailed understanding of the specific steps by which GroEL transitions from one form (the closed form) to the other (the open, ATP-bound form) is a necessary prerequisite in understanding the functional mechanism of this intriguing protein complex. In this report we provide details regarding the specific movements of the apical domain of GroEL triggered by ATP binding, using the fluorescent GroEL mutant R231W.

GroEL R231W proved to be an extremely useful tool in elucidating the myriad movements of the apical domain after ATP binding. Because the activity of this mutant closely mimicked that of the wild-type chaperonin, various comparisons with previous studies was greatly facilitated. Using this mutant, we observed a total of three distinct movements of the apical domain that were seemingly of great relevance to the chaperonin mechanism. The characteristics of the three phases, Phases A, B, and C, closely resembled the characteristics of the kinetic phases observed in a previous study by Cliff et al. (13) using the equatorial domain mutant GroEL Y485W. In particular, the saturation curves of the rate constants and amplitudes of Phase B with regard to the ATP concentration were extremely similar to the behavior shown by phase b in Cliff et al. A bisigmoidal saturation curve was also observed in fluorescence experiments performed by Yifrach and Horovitz on the GroEL mutant F44W (12), and the kinetic constants obtained in these studies also closely resembled the values obtained in the present study for Phase B. The other two kinetic phases also shared common characteristics such as the apparent rate at certain ATP concentrations. In addition, with regard to Phase A, the apparent rate of this phase was very similar to the rate of a kinetic phase detected by us in a previous study using an equatorial domain mutant of GroEL, GroEL T89W (24) (−350 s\textsuperscript{-1} at 300 μM ATP), suggesting that these three kinetic phases reflect a common event. The extremely rapid conformational change detected in GroEL T89W was postulated to lead to the release of certain refolding protein molecules bound to GroEL (24), and the fact that we were now able to detect an analogous conformational change in the apical domain lends support to this idea. It is reasonable to assume that the fluorescence changes observed in GroEL R231W upon

Fig. 7. Analysis of the tryptophan fluorescence changes of GroEL SR-1/R231W upon binding of ATP. a, changes in the tryptophan fluorescence intensity of GroEL SR-1/R231W at 25 °C upon addition of 300 μM ATP. b, the changes in tryptophan fluorescence of GroEL SR-1/R231W monitored for an extended interval (5 s). The ATP concentration was 300 μM.

When we observed the binding of GroES to this newly constructed fluorescent chaperonin in the presence of various concentrations of ATP, we found that GroES binding was still observable in the heptamer mutant (Fig. 8). This result suggested strongly that movements of the apical domain and GroES binding could occur in parallel on the same GroEL ring. Again, we could observe no relationships between the GroES binding phase and Phase B, suggesting that even in the heptamer mutant, these two events were occurring relatively independent of each other.

When we analyzed the dependence of the rate and amplitude of Phase B on the ATP concentration in GroEL SR-1/R231W, we discovered an additional characteristic of this kinetic phase which was in apparent conflict with previous hypotheses regarding the domain movements of GroEL. As shown in Fig. 9a, the dependence of the apparent rate constants of Phase B on ATP concentration were relatively unchanged in the heptamer mutant, and only a slight decrease in the maximum apparent rate observed at high ATP concentrations was seen. The specific kinetic constants obtained were as follows: $k_1 = 107 \pm 35 \mu M$, $k_2 = 43.6 \pm 17 \text{ s}^{-1}$, $n = 2.2 \pm 1.1$ for the first sigmoidal transition, and $k_3 = 549 \pm 34 \mu M$, $k_4 = 156 \pm 4 \text{ s}^{-1}$, $m = 3.2 \pm 0.4$ for the second sigmoidal transition. The most striking aspect of the kinetic characteristics shown in Fig. 9, however, was the fact that a bisigmoidal dependence of the apparent rates was still observable at all in SR-1/R231W, a heptamer variant of GroEL (Fig. 9a, inset). The presence of bisigmoidal behavior contained in a single heptamer ring of GroEL suggested a previously unsuspected level of complexity regarding the allosteric mechanism of GroEL.

Fig. 8. Detection of apical domain movements and GroES binding in GroEL SR-1/R231W. Experiments were performed at the following ATP concentrations: 300 μM (a), 500 μM (b), 1 mM (c). The solid line indicates the changes in fluorescence detected in the absence of GroES, and the dotted line indicates the changes in fluorescence detected in the presence of an equimolar oligomeric concentration (1.2 μM GroES) of GroES.
Chaperonin Domain Movement and Quaternary Structure

**Fig. 9. A comparison of the dependence on ATP concentration of the apparent rate constants and relative amplitudes of Phase B in GroEL R231W and GroEL SR-1/R231W.**

- Open circles represent GroEL R231W; closed circles represent GroEL SR-1/R231W. Panel a shows a comparison of the apparent rate constants; panel b shows a comparison of the relative amplitudes. Theoretical curves were derived as described in Fig. 3 and "Results." In the inset to panel a, a slightly expanded view of the results for GroEL SR-1/R231W is shown which more clearly displays the bisigmoidal nature of this curve.

The addition of the nucleotide ATP reflect dynamic changes in the domain orientations of the GroEL subunit that are important in the overall molecular mechanism. A complete understanding of the roles of each conformational change would therefore be vital in understanding the detailed molecular mechanism of GroE-facilitated protein encapsulation and folding assistance.

As shown in Fig. 6, we were unable to observe any relationships between GroES binding and the movements of the apical domain. Interestingly, at low concentrations of nucleotide, the increase in fluorescence attributed to GroES binding was initiated before the completion of Phase B (Fig. 6a), demonstrating quite clearly that GroES binding was independent of the completion of this apical domain movement. Furthermore, when we isolated the fluorescence changes attributed to GroES binding by subtracting the kinetic trace obtained in the absence of co-chaperonin from the trace obtained in the presence of it, we found that the derivative traces from each ATP concentration closely overlapped, suggesting that GroES binding was not affected at all by the changes in apical domain movement (data not shown). This was a somewhat unexpected result, as we had initially assumed that GroES binding would be deeply affected by the relative conformational state of the apical domain. Therefore, further experiments were performed to address this result.

A simple explanation for the supposed independence between GroES binding and Phase B would be that the two events are occurring on different GroEL rings of GroEL R231W. To clarify this point, and to also elucidate further relationships between this signature tetradecameric structure and the intricate kinetic movements of the GroEL apical domain, a heptameric derivative of GroEL R231W, GroEL SR-1/R231W was constructed and characterized. When we performed stopped-flow fluorescence experiments on this single ring derivative of GroEL R231W, we found that almost all of the domain movements, with the exception of Phase C, were relatively conserved in a single heptameric ring (Fig. 7), and second, that the binding of GroES was also observable (Fig. 8). In particular, the results shown in Fig. 8 obtained at varied ATP concentrations revealed that again, GroES binding and apical domain movements occurred independently of each other. The results in Fig. 8 ruled out the possibility that GroES binding and Phase B were segregated on separate rings of GroEL. At present, our results point toward the notion that binding of GroES is triggered upon completion of the extremely rapid conformational change that is reflected by Phase A, and completion of all subsequent movements of the GroEL apical domain are unnecessary for its initiation.

It is interesting to speculate on the specific method by which GroEL binds to the co-chaperonin GroES after ATP binding. A single ring of GroEL is composed of seven identical subunits, and the apical domain of each subunit is capable of interaction with both refolding proteins and the co-chaperonin GroES. It is conceivable that during the chaperonin cycle, only a fraction of the seven subunits that comprise the GroEL ring is involved in co-chaperonin binding, while the remaining subunits undergo various domain movements, which alter their interactions with refolding protein molecules. Experimental evidence of chaperonin ternary complexes, where GroES and refolding protein molecules are simultaneously bound to the same ring of GroEL, have been reported. In our own studies, we have succeeded in detecting a productive ternary complex of GroEL, GroES, and refolding protein by utilizing GroEL C138W, a mutant chaperonin capable of temperature-dependent arrest of the chaperonin cycle (19, 25). The ability to simultaneously form interactions between GroES and refolding protein in a single ring of GroEL would conceivably ensure the successful encapsulation of the refolding protein within the GroEL central cavity, preventing non-constructive diffusion of the protein molecule into the surrounding medium and thereby increasing folding efficiency.

Alternatively, it may be that all seven subunits of a given GroEL ring are simultaneously capable of undergoing domain movements and binding interactions with GroES. In the course of our experiments to prove or disprove the above hypothesis regarding specialized GroES-binding and non-binding subunits, we reasoned that if the GroEL subunits of a given ring were indeed partitioning into GroES-binding and non-binding subunits, there should have been a reduction in the relative amplitudes of Phases B and C in experiments performed in the presence of GroES, since a fraction of the subunits in the GroEL ring are supposedly diverted from their respective apical
clonal domain movements when GroES is present. However, we were unable to detect conclusively that the addition of GroES resulted in a measurable decrease in the amplitude of these apical domain movements. The specific binding process of GroES to GroEL, therefore, must be clarified by other methods for us to interpret these experimental results definitively.

Experiments Using a Single Ring Variant of GroEL R231W Reveal an Interesting Relationship between Apical Domain Movement and Quaternary Structure—From characterization of the changes in fluorescence of GroEL SR-1/R231W, an additional, very important relationship between the apical domain movements and the unique quaternary structure of GroEL was revealed, specifically, one involving Phase B. As shown in Fig. 9, when we probed the dependence of the Phase B rate on ATP concentration in GroEL SR-1/R231W, we found that although the maximum apparent rate of Phase B was slightly decreased in the heptameric mutant, a clear bisigmoidal dependence of this rate on the concentration of ATP was still observed. From our experimental results we believe that Phase B reflects a global movement of the GroEL subunit, which has been previously detected in experiments using the GroEL mutants Y485W and F44W. Cliff et al. (13) performed detailed experiments on the characteristics of phase b in GroEL Y485W, and proposed that this kinetic phase was a concerted movement of GroEL, which occurred in one heptameric ring at low ATP concentrations, and in both heptameric rings at higher ATP concentrations. This conclusion was supported by an experimental result which demonstrated that the second sigmoidal transition observed at high ATP concentrations was abolished in the presence of low concentrations of ADP, resulting in a large decrease in the maximum apparent rate. The addition of ADP was presumed to block ATP binding to one of the heptameric rings.

In our studies, we have not addressed the situation where ATP and ADP were simultaneously present in the experimental mixture because in preliminary experiments we found that addition of ADP to GroEL R231W resulted in fluorescence changes which were radically distinct from that observed in the presence of ATP (data not shown), and we wished to avoid any confusion regarding the interpretation of individual kinetic phases. However, in our experiments using an actual heptameric mutant of GroEL R231W, we found that the bisigmoidal behavior of Phase B was retained, and the maximum apparent rate also attained a similar high value, which would be a clear impossibility if this kinetic phase were indeed occurring on two GroEL rings at high ATP concentration. It is our view from the results shown in Fig. 9 that the fluorescence changes of Phase B reflect a concerted movement of a single ring of GroEL, whose apparent rate is modulated according to a bisigmoidal dependence on the ATP concentration.

Such a conclusion poses an interesting question which must be answered. In order to explain the phenomenon of bisigmoidal dependence in a single GroEL heptamer, the presence of two types of nucleotide binding site within the heptameric ring must be postulated. The first site, controlling the first sigmoidal transition, would saturate at relatively low concentrations of ATP. The second binding site would have a lower affinity for ATP; however, binding of nucleotide to this binding site results in a radical increase in the rate of conformational change. We observed that the fluorescence intensities attained after the completion of Phase B under these two experimental conditions were indistinguishable from each other (Fig. 3b). Either the conformational state attained under these two conditions is in fact identical, or they are different but indistinguishable via changes in tryptophan fluorescence. Regardless of this, it seems necessary to postulate the existence of two different types of nucleotide binding site for ATP in the GroEL heptameric ring. In this context, it is extremely interesting that Inobe et al. (15), using pyrenyl-labeled wild-type GroEL and SR-1 GroEL molecules, have detected changes in pyrenyl fluorescence upon the addition of various concentrations of nucleotide which were best explained by the existence of two different types of ATP binding site in the GroEL tetradecamer, with different binding affinities. At present, our data do not allow us to determine whether the different binding sites are due to differences that arise between the binding sites of different GroEL subunits in a given GroEL ring, or if there are two binding sites within the same GroEL subunit, with different affinities. Evidence against the possibility that there may be more than one nucleotide binding site per GroEL subunit is given in studies performed by Terada and Kuwajima (26), as well as by a very recent study performed by Chaudhry (27). Isothermal titration calorimetry of nucleotide binding to GroEL molecules support a stoichiometry of one nucleotide bound per subunit in both studies, and so is unlikely that there may be more than one binding site for adenine nucleotides per GroEL subunit. The notion that there may be inherent differences in the binding affinity between different subunits of a GroEL ring is in apparent conflict to the evidence that these transitions display characteristics of a concerted transition, and also goes against previous results regarding the nested cooperative behavior of the ATPase activity of GroEL (28, 29). It is interesting in this context to note, however, that cryo-electron microscopy studies by Ranson et al. (11) have revealed that in the unliganded form of GroEL, it is possible to distinguish differences in the conformations of the subunits composing the two heptameric rings of GroEL, even in the absence of any apparent factors which would affect GroEL conformation. Perhaps in the initial apo state of GroEL, there is a partitioning of subunits, which may explain this extremely intriguing experimental result. Future experiments should address this question.

The specific role of Phase B is still uncertain. The characteristics of this kinetic phase which have been elucidated in the present study reflect the complexity of this domain movement: First of all, Phase B is independent of GroES binding, even though this event closely involves the apical domain and is clearly reflected by the common structural probe, Trp231. Second, Phase B displays a bisigmoidal dependence of its rate relative to the ATP concentration, and this bisigmoidicity is retained in a heptameric variant, which suggests that this conformational change occurs more or less on a single heptameric ring. A mechanistic interpretation that explains these divergent characteristics simultaneously is extremely difficult to formulate. However, it has been shown in various studies (6, 10, 11, 30) that the conformational changes, which are triggered by the binding of ATP to GroEL involve a net expansion of the heptameric ring structure and an extensive rearrangement of the intra-ring and inter-ring contacts of each subunit. Such a ring expansion would involve change in the relative distance between the individual subunits of GroEL, perhaps resulting in an increased polar environment of the subunit/subunit interface. If the fluorescence changes attributed to Phase B were reflecting this oligomeric expansion, a number of discrepancies discovered in the present study would be explainable. In partial support for this hypothesis, Inobe et al. (31) performed stopped-flow solution x-ray scattering studies on GroEL, focusing on the molecular structures formed immediately after ATP binding. They found that GroEL underwent a rearrangement of structures that was detectable by small angle x-ray scattering at a rate that corresponded closely to the rate of phase b in Cliff et al. (13), allowing for the lower tempera-
tures that were necessary in their experiments. We are presently performing experiments, using the mutant chaperonins constructed in the present and accompanying study, to confirm if the completion of Phase B coincides with ring expansion of the GroEL heptamer.

The results described in the present study reflect the extreme complexity of the GroEL functional mechanism, which must be defined for a complete understanding of this intriguing phenomenon, and also highlight a number of questions which must be answered to achieve this goal. Additionally, this study demonstrates the usefulness of the mutant GroEL R231W in clarifying the relationships between GroEL structure and function. We are presently performing experiments, using the mutant chaperonins that were necessary in their experiments. We are presently performing experiments, using the mutant chaperonins constructed in the present and accompanying study, to confirm if the completion of Phase B coincides with ring expansion of the GroEL heptamer.

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Stopped-flow Fluorescence Analysis of the Conformational Changes in the GroEL Apical Domain: RELATIONSHIPS BETWEEN MOVEMENTS IN THE APICAL DOMAIN AND THE QUATERNARY STRUCTURE OF GroEL

Masaaki Taniguchi, Tatsunari Yoshimi, Kunihiro Hongo, Tomohiro Mizobata and Yasushi Kawata

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