One of the most interesting facets of GroEL-facilitated protein folding lies in the fact that the requirement for a successful folding reaction of a given protein target depends upon the refolding conditions used. In this report, we utilize a mutant of GroEL (GroEL T89W) whose domain movements have been drastically restricted, producing a chaperonin that is incapable of utilizing the conventional cyclic mechanism of chaperonin action. This mutant was, however, still capable of improving the refolding yield of lactate dehydrogenase in the absence of both GroES and ATP hydrolysis. A very rapid interconversion of conformations was detected in the mutant immediately after ATP binding, and this interconversion was inferred to form part of the target release mechanism in this mutant. The possibility exists that some target proteins, although dependent on GroEL for improved refolding yields, are capable of refolding successfully by utilizing only portions of the entire mechanism provided by the chaperonins.

The process by which prokaryotic chaperonins such as Escherichia coli GroEL and GroES facilitate the folding of proteins has been elucidated in great detail (1–3). The mechanism that is presently accepted is highly ordered and involves the binding of the target protein in the GroEL central channel (4–6), encapsulation within a chamber composed of GroEL and GroES (4–6), release of the target protein into this chamber to allow folding to proceed unhampered by nonspecific hydrophobic interactions that may lead to aggregation, and finally the release of protein molecules into the bulk solution, triggered by the dissociation of GroES. ATP binding and hydrolysis regulate this process by modulation of domain movements in GroEL, which result in changes in binding affinities for target protein and GroES (7, 8).

Most of the aspects of the above mechanism have been elucidated using target proteins such as rhodanese (9) and Rubisco (10), which, in initial studies, showed little or no spontaneous refolding, and absolutely required both the GroEL and GroES proteins and the hydrolysis of ATP in order to attain maximum refolding yields. Such proteins have conventionally been termed “stringent” target proteins (7, 8, 11), to denote this requirement for all three components of the chaperonin mechanism. In contrast, “non-stringent” targets are released from GroEL in the absence of one or more of the accessorial factors such as the participation of GroES or ATP hydrolysis (12–16). However, further studies regarding the concept of stringency revealed that for each stringent protein target, refolding conditions could be altered so that the requirement of various components could be relaxed (17). The concept of “permissiveness” was then coined to explain this phenomenon; under “non-permissive” conditions, target proteins were capable of refolding successfully only in the presence of all three components of the chaperonin mechanism, whereas under “permissive” conditions, ATP hydrolysis and/or the presence of GroES could be omitted. Although this term explains the behavior of a broader fraction of target proteins reported to date compared with the term “stringency,” the behavior of a small subset of the non-stringent proteins are still not readily explained. Specifically, although a number of non-stringent proteins are capable of folding spontaneously with high yields, and only interact transiently with GroEL during folding without displaying any differences in the overall refolding yield (14), a small number, e.g. tryptophanase (15) and glutamine synthetase (18), display a very low spontaneous yield which improves dramatically in the presence of GroEL and various nucleotides. In the case of tryptophanase, the participation of GroES and ATP hydrolysis are both unnecessary for an improved yield (15). In this context, a unified mechanism that describes the behavior of GroEL toward all target proteins reported to date is far from completion.

An important problem arises from the concept of permissive and non-permissive protein folding reactions vis à vis the specific mechanistic steps of the chaperonin cycle that accommodate these reactions. Specifically, for a given target protein, is the conventional cyclic mechanism important under permissive conditions as well as for non-permissive conditions? If so, what is the specific sequence of events that lead to an orderly release of protein molecules from GroEL, especially in the absence of GroES? These questions must be answered for a complete understanding of the mechanism of chaperonin-facilitated protein folding, encompassing both permissive and non-permissive refolding conditions.

We, among many other research groups, have been attempting to clarify the mechanism of chaperonin-facilitated protein folding by characterizing the numerous conformations that GroEL is presumed to form during this process. A popular experimental method in these studies is to insert a specific mutation into GroEL that is targeted to affect a specific aspect of the mechanism and to characterize the consequences of this mutation (1, 4, 5, 7, 8, 19, 20). In a previous study, we have addressed the phenomenon of inter-domain communication
within the GroEL subunit by using three GroEL mutants (T89W, C138W, and Y203C) (21). Each of these mutants is located in a different domain of the GroEL subunit as follows: Thr-89, equatorial domain; Cys-138, intermediate domain; Tyr-203, apical domain. By using these three mutants, we were able to demonstrate the presence of interdomain communications that were important for the functional mechanism of GroEL (21).

GroEL T89W was engineered to disrupt specifically the ATPase activity of GroEL by replacing Thr-89, which is located in the vicinity of the γ-phosphate-binding region of the ATP-binding site of GroEL, with a bulky tryptophan residue. As expected, this mutant was characterized by a total lack of ATPase activity and also by an inability to bind the co-chaperonin GroES. However, this mutant was able to bind ATP, as detected by changes in the fluorescence intensity of the inserted tryptophan residue. The binding abilities of the mutant GroEL with regard to the target protein enolase were altered, so that GroEL T89W was able to bind only a fraction of the refolding intermediates recognized by the wild-type protein. Interestingly, however, GroEL T89W could also improve the refolding yield of Staphylococcus sp. LDH2 relative to spontaneous yields in an ATP-dependent manner, indicating that chaperonin function was partially retained in this mutant. In this report we describe in greater detail the structural and functional characteristics of GroEL T89W. When we analyzed the conformational changes that occurred during ATP binding to GroEL T89W by labeling the mutant chaperonin with a fluorescent label N-(1-pyrenyl)maleimide and monitoring the changes in pyrenyl-derived fluorescence, we found that, unlike the wild-type chaperonin, GroEL T89W underwent almost no conformational changes upon ATP binding which were detectable by the fluorophore. By utilizing the presence of the tryptophan residue in the nucleotide-binding site, we characterized an extremely rapid conformational change by stopped-flow fluorescence. We propose that this extremely rapid interconversion of conformations observed for GroEL T89W upon nucleotide binding may act as part of a mechanism to release proteins that refold under permissive conditions from GroEL, in the process producing a passive chaperoning effect by reduction of aggregates. The results obtained with the present mutant demonstrate that in the case of certain target proteins, a net improvement in refolding yield may be achieved via a "minimal" mechanism that requires neither GroES binding nor ATP hydrolysis.

EXPERIMENTAL PROCEDURES

Materials—Gdn-HCl (specially purified grade) was obtained from Nacalai Tesque (Kyoto, Japan). ATP and ADP were purchased from Sigma and were used after checking the purity (14). All other reagents were of the highest grade commercially available.

Wild-type GroEL protein was expressed in E. coli DH-1/pKY206 and purified according to published protocols (14, 15). GroEL T89W was prepared as reported in a previous study (21). Purification of the mutant protein was accomplished by using the method for the wild-type protein (22). The concentration of GroEL T89W was determined with a protein dye reagent (Protein Assay Kit; Bio-Rad) using bovine serum albumin as a standard (23).

Refolding Assays of Target Protein—Refolding assays of yeast enolase and Staphylococcus LDH were performed as previously reported (14, 24). The concentrations of enzyme and Gdn-HCl during the refolding reaction were 18 μM and 80 mM for enolase and 2 μM and 50 mM for LDH, respectively. Refolding assays of bovine rhodanese were performed under two differing conditions (25) as follows: permissive refolding conditions outlined by Mendoza et al. (26) to monitor recovery of activities and non-permissive refolding conditions according to Mar-
Fig. 1. Location of threonine 89 in the overall subunit structure of GroEL. Two separate conformations of the GroEL subunit are superimposed; the gray subunit is the conformation of GroEL in the absence of nucleotide (closed conformation), and the gold subunit is the conformation of GroEL when ADP and GroES are bound (open conformation). Both conformations were produced from coordinates derived from the Protein Data Bank code 1AON (33). Superposition of the two conformations was accomplished by fitting the backbone carbons from residues 409 to 500 in the equatorial domain so that overlapping was maximized. The ADP molecule bound to the gold conformation is shown in red. Thr-89, shown in cyan (backbone atoms) and orange (side chain atoms), are shown as space-filling forms. The M-helix in each conformation is highlighted by coloring the interior of the helix in either magenta (gray closed conformation) or green (gold open conformation). Note the movement of the M-helix that is postulated to occur as a result of nucleotide and GroES binding. A shows the overall view of the subunits, and B shows a zoomed view of the nucleotide-binding site from the perspective indicated by the arrow in A. Superposition of the two conformations and construction of the graphic images were performed using MOLMOL (40).

Fig. 1, gray/magenta) is reoriented to cover the nucleotide-binding site (Fig. 1, gold/green). Thr-89 is located in close proximity to this dynamic conformational change. Additionally, Thr-89 is located at the N-terminal of the “D-helix” of GroEL. This helix, which extends through the equatorial domain to interact with GroEL subunits located on the opposite ring, is implicated in the transmission of conformational changes between the two GroEL rings (32, 33). Therefore, Thr-89 is in a unique position to affect both the various conformational perturbations of individual GroEL subunits and also to influence inter-ring communications transmitted via the D-helix.

As described in a previous study (21), expression and purification of GroEL T89W were straightforward, and the static structural aspects of the mutant were, with the exceptions of the fluorescence spectra and near-UV CD spectra, indistinguishable from the wild-type protein. Regarding the functional aspects of GroEL T89W, we determined that GroEL T89W completely lacks ATPase activity (21). However, ATP binding to the mutant could be observed by an increase in tryptophan-derived fluorescence which was dependent on the ATP concentration.

Differences in Chaperoning Activity—Fig. 2 shows the refolding profiles of yeast enolase and Staphylococcus sp. LDH in the presence of GroEL T89W. As reported in a previous study (21), GroEL T89W was capable of binding to and arresting the folding of a small fraction of enolase refolding molecules and subsequently releasing them upon addition of ATP. The fraction of protein molecules that are bound is about 30%, under the conditions used in Fig. 2a. The fraction of molecules bound to GroEL T89W behaved subsequently in a manner indistinguishable from that in the presence of wild-type chaperonin. In contrast, the refolding of LDH was arrested completely in the presence of GroEL T89W. Addition of ATP resulted in the partial recovery of LDH activity (Fig. 2b), which was significantly higher than the yield recovered spontaneously (Fig. 2b, solid line). Delaying the addition of the nucleotide for up to 6 h after the initial formation of the LDH-T89W complex resulted in almost no degradation of the final refolding yield of LDH, indicating that the LDH-T89W complex was stable for this length of time. The results in Fig. 2 indicate that, although reduced in potency, GroEL T89W retains partial chaperonin activity toward a subset of refolding target proteins; specifically, proteins such as LDH, which do not require the presence of the co-chaperonin GroES for significant refolding. The reduction in potency does not seem to be due to a reduction in stability of the GroEL-LDH complex, as this complex was demonstrated to be stable for at least 6 h (Fig. 2b).

A further detailed look at the refolding profiles of these two target proteins revealed another interesting characteristic. In Fig. 3, we assayed the ability of GroEL T89W to bind the refolding intermediates of enolase and LDH, by refolding these two proteins in the presence of various concentrations of GroEL and no nucleotide. The activity recovered under these conditions reflects the fraction of protein molecules that were able to escape interaction with GroEL. As seen in Fig. 3, the introduction of the mutation resulted in a decrease in the affinities for both of the protein targets. However, an important difference between the two protein targets was apparent. In the case of LDH, the mutation caused a decrease in the initial binding affinity between GroEL and target protein, such that an in-
increased concentration of chaperonin was required to arrest completely the refolding of LDH. However, the refolding of LDH was completely suppressed under saturating concentrations of GroEL. In the case of enolase, although increasing concentrations of GroEL resulted in a decrease in the refolding yield, about 50% of the target was still capable of spontaneous refolding in the presence of a 20-fold excess of GroEL T89W oligomer, suggesting that more than half of the refolding enolase molecules could not be bound by GroEL T89W, even at saturating concentrations of chaperonin.

The refolding reaction of rhodanese contrasted sharply with the results obtained for enolase and LDH. Fig. 4 shows the recovery of unfolded bovine rhodanese in the presence of GroEL T89W, monitored by refolding yields and the extent of aggregation. Refolding yields were monitored under permissive conditions, whereas aggregation was monitored under non-permissive conditions, in order to detect the release of any rhodanese molecules with the highest sensitivity. As seen in the figure, in the presence of GroEL T89W and ATP, neither increases in refolding yield nor increases in aggregation were observed, suggesting that rhodanese remained stably bound throughout the assay. Release of rhodanese and recovery of activity was observed only in the presence of wild-type GroEL and the co-chaperonin GroES.

Detection of Conformational Changes upon ATP Binding Using Pyrenyl-labeled GroEL—In an attempt to monitor the differences between the conformational changes that occur in both types of GroEL subunit, wild-type GroEL and GroEL T89W were labeled with N-(1-pyrenyl)maleimide (28, 29, 34), and the changes in pyrenyl fluorescence were monitored upon addition of ATP. Fig. 5 shows the pyrenyl fluorescence spectra of GroEL wild-type and GroEL T89W in the presence and absence of 1 mM ATP. Prior to this experiment, we confirmed that both wild-type GroEL and GroEL T89W were labeled to a similar extent and in a virtually identical manner, as determined by UV spectra analysis (30) and reversed phase-high pressure liquid chromatography analysis of samples digested with lysyl endopeptidase (data not shown). Also, the labeling of GroEL T89W by N-(1-pyrenyl)maleimide resulted in negligible differences in both ATP binding ability, as monitored by tryptophan fluorescence, and the ability to bind and release enolase folding.

**FIG. 2.** GroEL-facilitated refolding reaction of enolase (a) and LDH (b). Circles indicate wild-type GroEL, and triangles indicate GroEL T89W mutant. In both panels, open symbols represent refolding in the presence of a 5-fold molar excess of GroEL 14-mer without nucleotide. Closed symbols indicate the changes in the activity when 2 mM ATP was added at the time indicated by the arrow to the refolding mixture. The solid line without symbols represents spontaneous refolding. In b, the results of refolding assays in which the addition of ATP was delayed for 3 h and 6 h after initiation of folding are overlaid onto the main figure, in order to demonstrate that the GroEL-LDH complex, once formed, is stable during this period.

**FIG. 3.** Binding of wild-type GroEL and GroEL T89W in the absence of nucleotide to the refolding intermediates of enolase and LDH. Enolase and LDH were denatured in Gdn-HCl and diluted into buffer containing the indicated concentration of either wild-type (open symbols) or T89W (closed symbols) GroEL. Triangles represent the residual refolding yields of enolase for each condition; circles represent the same values for LDH. Residual refolding yields were measured 15 min (enolase) or 6 h (LDH) after initiation of folding.

**FIG. 4.** Refolding of bovine rhodanese in the presence of wild-type and mutant GroEL. Experiments were performed at 25 °C. a, time-dependent refolding yields of rhodanese under permissive refolding conditions in the presence of reducing agent and substrate (26). Closed squares indicate spontaneous folding; closed circles indicate refolding in the presence of wild-type GroEL, GroES, and 2 mM ATP; closed triangles indicate refolding in the presence of GroEL T89W, GroES, and 2 mM ATP. b, the extent of irreversible aggregation observed during refolding under non-permissive conditions, monitored by light scattering at 320 nm. Closed squares indicate spontaneous folding; closed circles indicate refolding in the presence of wild-type GroEL and 2 mM ATP; closed triangles indicate refolding in the presence of GroEL T89W and 2 mM ATP. In samples containing GroES, the molar ratio of GroEL to GroES was 1:1 (oligomer).
intermediates (data not shown). As shown in the figure, the addition of 1 mM ATP to pyrenyl-labeled wild-type GroEL resulted in a large increase in the fluorescence intensity of the pyrenyl group (Fig. 5a). On the contrary, the addition of an identical concentration of ATP to pyrenyl-labeled GroEL T89W resulted in almost no changes in the fluorescence intensity (Fig. 5b). This tendency was also confirmed in kinetic experiments. Fig. 6 shows the changes in fluorescence intensity that are observed when ATP is added to pyrenyl-labeled GroEL (29). When an equimolar concentration of ATP relative to GroEL subunit is added to pyrenyl-labeled GroEL wild-type (Fig. 6), the fluorescence intensity of the pyrenyl group displays an initial increase in fluorescence intensity due to ATP binding, followed by a gradual decrease in intensity as the bound ATP is hydrolyzed and the GroEL oligomer relaxes into its initial conformation (29). In sharp contrast, the addition of ATP in either stoichiometric (not shown) or larger concentrations (100 mM final concentration) relative to the subunit concentration of GroEL T89W failed to produce any changes in fluorescence intensity, except for a gradual decrease that was observed for a duration greater than 500 s (Fig. 6, inset). From this it may be concluded that the addition of ATP does not elicit the conformational changes observed upon ATP binding in the wild-type subunit.

Conformational Changes Induced by ATP Binding to GroEL T89W—The fact that we were unable to detect any changes in conformation upon binding of ATP to pyrenyl GroEL T89W prompted us to reexamine the characteristics of ATP binding to this mutant using the introduced tryptophan. Fig. 7 shows the tryptophan fluorescence intensity of GroEL T89W in various concentrations of the nucleotide ATP. As shown in the figure, the binding curve of GroEL T89W with respect to ATP differs radically from that of the wild-type protein, in that no observable positive cooperativity is apparent. Analysis of the binding curve according to the Hill equation obtained a Hill constant of 0.65, which indicated a slight negative cooperativity. The apparent dissociation constant was calculated to be 0.3 mM, which was 1 order of magnitude greater than the wild-type chaperonin. Addition of GroES to the experiment resulted in a negligible effect on the binding curve, confirming previous results that indicated that GroEL T89W is incapable of binding to GroES (21).

Kinetic experiments performed on this fluorescence increase were also performed using stopped-flow spectroscopy. Fig. 8 shows the results of stopped-flow fluorescence experiments performed by mixing GroEL T89W (concentration during measurement, 0.5 mg/ml (8.73 μM subunit)) with various concentrations of ATP and monitoring the increase in tryptophan-derived fluorescence. As shown in the figure, upon addition of ATP the tryptophan-derived fluorescence intensity was seen to increase with time. The apparent rate constants of this fluorescence increase, as shown in Fig. 8b, were seen to increase with ATP concentration. The rates, however, seemed to increase in a saturable manner, indicating that the increase in fluorescence reflected a conformational change that was triggered by the binding of ATP, rather than the direct binding of ATP to the nucleotide-binding site, which would be expected to increase linearly with ATP concentration.

DISCUSSION

In this report we have described some characteristics of a mutant GroEL protein containing a tryptophan residue in the vicinity of the ATP-binding site. GroEL T89W was characterized by a reduced binding affinity for ATP and a total lack of...
Apparent first order rate constants of the raw fluorescence traces measured at concentrations higher than 500 μM were omitted from this analysis. The apparent first order rate equation. Note that the values corresponding to zero time at 750 μM do not converge to the value of the other traces. This divergence is due to errors brought about by the extreme rapidity of the signal change at high ATP concentrations (>500 μM ATP). Apparent first order rate constants of the raw fluorescence traces measured as shown in (a). Due to the divergence in zero τ values at high ATP concentrations (see (a)), observed rate constants obtained at ATP concentrations higher than 500 μM were omitted from this analysis. The solid line is a theoretical curve derived by analyzing the rate constants according to the mechanism outlined in the text.

ATP hydrolysis ability. Nevertheless, this mutant retained partial chaperonin activities and could produce a net increase in refolding yield of the target protein LDH. The mechanism by which GroEL T89W achieved this feat sheds some light on the variety of interactions that are possible between numerous target proteins and the chaperonin GroEL.

Interactions of GroEL T89W with Target Proteins—The yields of spontaneous recovery of enolase in the presence of excess amounts of GroEL T89W were considerably higher than in the presence of wild-type GroEL (Fig. 2a, compare open triangles to open circles). Enolase is an example of a strictly permissive folding reaction; that is, it is capable of near-quantitative refolding in buffer alone but is bound by GroEL during refolding and released in an ATP-dependent manner (14). Therefore, this increase in spontaneous refolding yield in the presence of GroEL T89W indicates that a good portion of the refolding molecules escape binding to this mutant and are allowed to refold. The simplest explanation for this reduced binding would be that the static affinity for enolase was decreased as a result of the mutation, and as a consequence, only a fraction of the protein molecules are bound. However, it should then be theoretically possible to increase the concentration of mutant GroEL protein so that all of the enolase molecules can be bound and refolding arrested completely. The results shown in Fig. 3 argue against this explanation since spontaneous refolding is observed even in the presence of high concentrations of GroEL. Therefore, an alternative explanation is required to explain the behavior of GroEL T89W toward enolase.

In order to obtain additional data that might explain this complex behavior of GroEL T89W toward various target proteins, we monitored the changes in domain conformation that are brought about as a result of ATP binding by introducing a pyrenyl group into the subunits of the wild-type and mutant GroEL proteins. The characteristics of pyrenyl-labeled wild-type GroEL have been documented extensively (28, 29, 34) and facilitate the interpretation of experimental results. From the results shown in Figs. 5 and 6, we concluded that GroEL T89W was incapable of undergoing the rapid domain movement (detectable by the pyrenyl label) observed for the wild-type protein (34). The kinetic experiments, in particular, were performed at a variety of ATP concentrations in light of the fact that the affinity of GroEL T89W for ATP was diminished as a result of the mutation (Fig. 7). Nevertheless, we could not detect any changes in the fluorescence spectra upon ATP addition. Taken together, the results indicate that changes in the domain conformations of GroEL T89W are, if not nonexistent, sufficiently small that they are incapable of being detected by changes in pyrenyl fluorescence.

This experimental result provides a simple explanation for a variety of characteristics of this mutant. For example, the fact that GroEL T89W is unable to bind GroES would be due to the fact that GroEL T89W is incapable of forming the specific conformation required for binding. The relative rigidity apparent in this mutant would most likely affect the initial phase of target peptide binding as well, as demonstrated by the altered binding of enolase unfolding intermediates to this mutant. To summarize, we conclude that GroEL T89W is a mutant chaperonin in which large scale domain movements are drastically reduced, forming a "rigid" chaperonin incapable of implementing the orthodox cycle proposed for chaperonin-facilitated protein folding (7, 8).

Binding of ATP to GroEL T89W—Considering the position of Thr-89 in the overall structure of the GroEL subunit (Fig. 1 (31)), it was to be expected that GroEL T89W would fail to display any measurable ATPase activity. Interestingly, however, the binding of ATP to this mutant chaperonin could be confirmed by the changes in fluorescence of the introduced tryptophan residue (Figs. 7a and 8a). Upon analyzing the ATP binding curves of GroEL T89W (Fig. 7), we found that the positive cooperativity seen in the wild-type chaperonin was absent in this mutant, and a slight negative cooperativity was detected instead. This negative cooperativity was represented by a Hill coefficient of 0.65, as derived from direct fitting of the saturation curves (Fig. 7). It seems that as a result of the mutation, all intra-ring communication of the GroEL subunit is suppressed. The lack of cooperativity shown in Fig. 7 is understandable when we consider that large scale domain movements, which would be important for structural feedback, would be precluded in this mutant.

Of greater interest were the rapid kinetic experiments of ATP binding to GroEL T89W. As shown in Fig. 8a, the kinetics of ATP binding to GroEL could also be monitored using stopped-flow fluorescence spectroscopy. Analysis of these traces could be performed according to a single exponential process. The apparent first order rate constants were seen to increase with increased nucleotide concentrations and seemed to saturate to a certain maximum value at high ATP concentrations (Fig. 8b). The fact that this rate constant saturated at high nucleotide concentrations may be taken as evidence that this increase in fluorescence intensity reflects an indirect change in the GroEL subunit triggered by the binding of ATP and not the direct binding of ATP to the nucleotide-binding...
site. Previously, Jackson and co-workers (28) analyzed an analogous set of results on pyrenyl-labeled wild-type GroEL in a similar manner. From the results shown in Fig. 7, we may conclude that the allosteric behavior detected in experiments using the wild-type protein is suppressed in this mutant. The rate constants in Fig. 8b may then be analyzed according to the simple mechanism (Reaction 1) shown below involving the binding of MgATP to the GroEL subunit,

\[
K_1 \text{GroEL} + \text{MgATP} \rightleftharpoons K_2 \text{GroEL} \cdot \text{MgATP} \rightleftharpoons \text{GroEL} \cdot \text{MgATP}
\]

**REACTION 1**

where GroEL ... MgATP is the initial collision complex of GroEL subunit and MgATP; GroEL*-MgATP is the fluorescence active species formed immediately after initial binding; \(K_1\) is the binding constant of GroEL to ATP; and \(k_2\) and \(k_{-2}\) are the forward and reverse rate constants of the following conformational change, respectively.

The apparent rate constant of this two-step reaction may be described as follows in Equation 1,

\[
k_{obs} = \frac{k_2 [\text{MgATP}]}{K_1 + [\text{MgATP}]} + k_{-2}
\]

(Eq. 1)

where [MgATP] denotes the concentration of added MgATP complex.

When the data shown in Fig. 8b were fit directly to the above equation, the values obtained were as follows: \(K_1 = 0.77 \text{ mM}, k_2 = 640 \text{ s}^{-1}\), and \(k_{-2} = 190 \text{ s}^{-1}\). The value of \(k_2\), which represents the maximum rate of the conformational change detected in the present experiment, is nearly 1 order of magnitude faster than the conformational change detected by Jackson et al. (28) in their experiments on pyrenyl-labeled wild-type GroEL and is most likely a newly detected conformational change of GroEL T89W that is triggered by nucleotide binding. Interestingly, this conformational change is characterized by a significantly high rate constant in the opposite direction, indicating that although ATP binding favors the fluorescent-active form, this conformational change is reversible. We postulate that the increase in tryptophan-derived fluorescence intensity detected in the present study reflects the rearrangement of active site amino acids immediately after the binding of ATP. The reversibility of this conformational change indicates that this conformational change represents an extremely rapid oscillation between two conformations of GroEL T89W.

**Significance of the Rapid Conformational Changes Induced by Nucleotide Binding—**GroEL T89W may be characterized to be a chaperonin with a rigid structure relative to wild type, unable to form the open conformation (Fig. 1, gold) formed by binding of ATP and GroES, which is apparently required for successful refolding under non-permissive conditions. Of extreme interest, however, is the fact that despite this deficiency, GroEL T89W is capable of assisting the refolding of LDH and to improving the refolding yields of this enzyme relative to the spontaneous yield in an ATP-dependent manner. This suggests that the assisted folding of this target protein is accomplished by a mechanism distinct from the conventional mechanism of chaperonin-facilitated folding.

In the mutant, a newly detected, very rapid interconversion of conformations was observed upon addition of ATP. The fact that ATP binding triggers both the release of LDH and this reversible conformational change implies that these two events are related. It is reasonable to conclude that the conformational changes triggered by ATP binding are, at the very least, part of a series of events that result in the release of LDH from the GroEL cylinder.

A possible mechanism through which LDH refolds with improved refolding yield from GroEL T89W may be via the so-called passive chaperoning effect. Passive chaperonin-facilitated folding is achieved when a significant portion of the refolding protein molecules is bound to GroEL, thereby decreasing concentration-dependent aggregation of refolding protein species. The subsequent release of protein molecules from GroEL must be gradual, not too slow as to preclude recovery of activity in a reasonable time frame, but also not too fast as to result in a resumption of aggregation. If we postulate that the fluorescence-active conformation detected in the present study culminates in a conformation with reduced affinity for the target protein LDH, this would result in the release of LDH molecules in a gradual manner. The conditions for an efficient release at this point would be dependent upon the affinity of the target protein for this newly formed GroEL conformation and also upon the relative lifetime of this species. Proteins such as rhodanase, which seemingly bind very tightly to GroEL, would be expected to be unaffected by this conformational change. This was readily confirmed by the results in Fig. 4 which showed that the refolding of rhodanase and recovery of activity was observed only in the presence of wild-type GroEL and the co-chaperonin GroES. To summarize, various reversible conformational transitions in GroEL T89W (one of which was characterized in the present study) may act, toward certain target proteins, as a minimal mechanism which provides a passive chaperonin effect consisting of the suppression of aggregation and gradual release into the bulk medium.

The GroEL T89W mutant used in the present study displayed a range of characteristics that were radically different from that of the wild-type protein. The altered characteristics of this mutant include drastic reduction in domain movement, abolishment of almost all cooperative binding behavior toward ATP, and an inability to hydrolyze this nucleotide. Consequently, the results obtained using such a radically altered chaperonin are not easily extrapolated to various events occurring in the wild-type protein. However, there is evidence provided from other studies that very rapid conformational transitions such as those detected in our experiments may indeed exist in the wild-type chaperonin. A very recent study by Cliff et al. (35) used the introduction of single tryptophan mutants and fluorescence spectroscopy to elucidate a large number of conformational changes in GroEL which were relevant to the overall chaperonin mechanism. Their results plot a course through the chaperonin mechanism from initial binding of ATP to hydrolysis and subsequent target release. Among the conformational changes documented was an extremely rapid transition that was observed upon adding ATP to GroEL Y485W (165 s⁻¹ at 175 μM ATP) which was attributed to a transition of the GroEL heptamer from a T to an R state. This rate is similar to our observed rate constants in Fig. 8b. At present we are unable to state definitively whether the rapid conformational change detected in our experiments corresponds to any conformational transitions detected by Cliff et al. (35). However, we are presently involved in mapping the characteristics of GroEL T89W to various domain movements in the apical domain which, if successful, will allow us to address this interesting matter in more detail.

The numerous proteins that have been reported to interact with GroEL to date display a range of characteristics that are not easily classified in a clear-cut manner; among them are proteins such as lysozyme (36), RNase T1 (37), α-lactalbumin (38), and barnase (39), whose folding pathways are affected by a transient interaction with GroEL; proteins such as tryptophanase (15) and LDH (24), which display an initial low spontaneous yield which increases in the presence of GroEL and...
ATP and the absence of GroES; and proteins such as rhodanese (9) and Rubisco (10), which are tightly bound to GroEL during folding and whose requirements for a successful folding reaction depend on the conditions of refolding (17). Each group of proteins reflects a potential mechanism by which GroEL may interact with various protein species in the actual cellular environment and, as such, must be studied in order to achieve a unified mechanism that explains the myriad interactions between GroEL and folding protein molecules. The results shown in the present study suggest that an additional level of complexity may be found in the mechanism of GroEL-facilitated protein folding, and this idea definitely warrants further study.

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