GroEL-Substrate-GroES Ternary Complexes Are an Important Transient Intermediate of the Chaperonin Cycle

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GroEL C138W is a mutant form of Escherichia coli GroEL, which forms an arrested ternary complex composed of GroEL, the co-chaperonin GroES and the refolding protein molecule rhodanase at 25 °C. This state of arrest could be reversed with a simple increase in temperature. In this study, we found that GroEL C138W formed both stable trans- and cis-ternary complexes with a number of refolding proteins in addition to bovine rhodanese. These complexes could be reactivated by a temperature shift to obtain active refolded protein. The simultaneous binding of GroES and substrate to the cis ring suggested that an efficient transfer of substrate protein into the GroEL central cavity was assured by the binding of GroES prior to complete substrate release from the apical domain. Stopped-flow fluorescence spectroscopy of the mutant chaperonin revealed a temperature-dependent conformational change in GroEL C138W that acts as a trigger for complete protein release. The behavior of GroEL C138W was reflected closely in its in vivo characteristics, demonstrating the importance of this conformational change to the overall activity of GroEL.

The chaperonin GroEL from Escherichia coli binds numerous proteins in vivo and in vitro and facilitates their folding by providing a space within its unique quaternary structure where protein molecules can safely complete their folding processes (1, 2). The specific steps involved in binding, isolating, and then releasing the refolding protein molecules involve a series of intricately associated conformational changes that are performed by the GroEL protein. The conformational changes that are controlled by the binding and hydrolysis of ATP and the participation of an accessory chaperonin, GroES. The presently accepted cycle of chaperonin-facilitated protein folding (3, 4) consists of an initial binding of the refolding protein to GroEL and subsequent binding of ATP and GroES, which drops the refolding protein molecule into a cylindrical cavity formed by seven subunits of GroEL and seven subunits of GroES. The bound ATP molecules are hydrolyzed slowly by the GroEL subunit, and this has the effect of priming the cage formed by GroEL and GroES for release. Then, an additional seven molecules of ATP are bound to another heptameric ring bound back-to-back to the initial GroEL heptamer (the trans ring), and this binding triggers the release of the refolding protein molecule, ADP, and bound GroES.

The numerous conformational changes of GroEL may be localized to three distinct domains found in the GroEL subunit (5). The apical domain is responsible for the recognition and binding of refolding protein molecules and the co-chaperonin GroES. The equatorial domain possesses the binding site for the nucleotide ATP, which acts as the modulating factor of these conformational changes. The intermediate domain transfers various signals between the two former domains to produce a concerted movement of the GroEL subunit. Mutations in each of these domains result in alterations in the facet of the chaperonin mechanism that is most reflective of the specific role of each domain (4, 6–10). Many subtle changes may be seen when mutations and other alterations are introduced into the intermediate domain, as this has the effect of altering the cooperation of the other two domains to complete a functional chaperonin cycle (7, 11).

In a previous study, our laboratory evaluated the effects of three mutant GroEL proteins whose mutations were each located in a different domain of the GroEL subunit (7). Two of these mutants, GroEL T89W and GroEL Y203C, produced predictable effects in their respective domains: GroEL T89W (12), in the ATP hydrolytic abilities localized in the equatorial domain, and GroEL Y203C, in the substrate-binding characteristics of the apical domain. However, the most interesting mutant was localized in the intermediate domain. GroEL C138W was a mutant that was apparently indistinguishable from the wild-type chaperonin under normal experimental conditions. However, when we introduced into the experimental field a refolding protein molecule, bovine rhodanese, at 25 °C, we observed a complete arrest of both rhodanese refolding and ATP hydrolysis by GroEL. This arrest was seemingly unrelated to the presence or absence of GroES. Further experiments showed that at 25 °C, GroEL C138W formed a stable ternary complex in which rhodanese and GroES were simultaneously bound to the apical domain. More interestingly, this ternary complex could be induced to complete the chaperonin cycle by a simple increase in temperature to 37 °C. This result was interpreted to reflect the transfer of conformational signals between the equatorial domain and the apical domain, which were necessary for a coordinated movement of the GroEL subunit.

In the present article, we show some further results obtained using the novel characteristics of this mutant chaperonin. We found that the arrested ternary complex formed by GroEL C138W was a general phenomenon, and similar arrested com-
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plexes could be formed with a number of refolding proteins in addition to bovine rhodanese, with molecular masses ranging from 33 to 85 kD. Furthermore, both cis-tetramers and trans-tetramers could be formed in this manner could be induced to resume folding upon a simple increase in the experimental temperature. Stopped-flow fluorescence analysis of the introduced tryptophan residue showed that a conformational change with an apparent rate constant of 82 s⁻¹ in the presence of 1 mM ATP at 37 °C was strongly affected by this mutation. This conformational change became undetectable when the temperature was decreased from 37 to 25 °C, indicating that the mutation specifically rendered this conformational change temperature-dependent. We confirm in this report a previous assertion that this conformational change governs the release of substrate protein from the GroEL apical domain (13), and from the nature of the arrested complex, propose a sequence of events which occur at the apical domain of GroEL immediately after ATP binding, that seemingly assures the efficient transfer of bound protein molecules into the GroEL central cavity.

**EXPERIMENTAL PROCEDURES**

*Cell lines, Proteins, and other Materials—*GroEL C138W was expressed in *E. coli* JM109/pUC19 cells and purified at 4 °C according to published protocols (14). The substrates proteins phosphofructokinase, luciferase, pyruvate kinase, triosephosphate isomerase (TIM), aldolase, rhodanese, and adenylate kinase were obtained from Sigma. Gp23 protein was isolated from cells possessing a plasmid (pET23a+ Vgb23), which contained the structural gene for phage T4 gp23 protein. The gene was inserted from bacteriophage T4 using PCR (the phage samples were a generous gift from Dr. Fumi Ariasaka of the Tokyo Institute of Technology) and ligated into the EcoRI BamHI restriction sites of pET23a (+). In *vivo* activities of GroEL C138W were evaluated using *E. coli* strain KY1156, which possesses a groESL operon placed under the control of the lacUV5 promoter. *E. coli* KY1156 was constructed as follows. lacUV5-groESgroEL, a derivative of Charon 25, was first constructed by in vivo recombination (15). The resultant phage carries a groESL operon placed under the lacUV5 promoter and lacUV5 genes, which were originally contained in plasmid pKV1561. This phage specimen was used to infect *E. coli* strain MC4100 [F⁻ araD ΔargF- lacUV5 p-groES groEL tet]. Then, the ΔgroES::tet mutation of KY1880 (15) was transduced into this A-lysogenic strain in the presence of IPTG by phage P1 to produce strain KY1156, whose genetic copy of groEL had been disrupted by tet, and which possesses an IPTG-inducible groESL operon within the lysogenic insert of lacUV5-groESgroEL.

Taka-amylase A was obtained by purification and recrystallization from a commercial product, Takadiastase Sanka (16). Guanidine hydrochloride (GdnHCl) was obtained from Wako Pure Chemicals. Other reagents were obtained commercially.

**Formation and Analysis of Arrested Chaperonin Complexes at 25 °C**—Each substrate protein was extensively unfolded in the presence of 4 M GdnHCl and subsequently diluted 100-fold into Refolding Buffer A (50 mM Tris-HCl, pH 7.4, containing 100 mM KCl, 10 mM MgCl₂, and 2 mM 2-mercaptoethanol) containing an equimolar concentration of GroEL C138W 14-mer. Immediately afterward, 2 mM ATP was added, and each mixture was incubated for 10 min at 25 °C. Where indicated, an equimolar concentration of GroES heptamer was also added at this point. The mixture was loaded onto a Sephacryl S-300 size-exclusion column (2.5 cm × 56 cm) and equilibrated with Refolding Buffer A (50 mM Tris-HCl, pH 7.4, containing 100 mM KCl, 10 mM MgCl₂, and 2 mM 2-mercaptoethanol) containing an equimolar concentration of GroEL C138W 14-mer. The end result was to produce a series of unfolded rhodanese samples with different rhodanese concentrations but a fixed denaturant concentration of 6 M GdnHCl. Five μl of each unfolded rhodanese sample were then rapidly mixed with 500 μl of reaction mixture containing 60 mM GroEL C138W tetradecamer and 100 mM wild-type GroES heptamer, which had been preincubated for 10 min at 25 °C. The end result was to produce a series of samples containing differing concentrations of arrested rhodanese-GroEL C138W complex. The mixtures were further incubated for 10 min at 25 °C, whereupon the ATPase activities of each sample were assayed by addition of 70 μM ATP (1.4 μM of [γ-³²P]ATP) and incubation at 25 °C. After hydrolysis was allowed to proceed for 30 min, 60-μl aliquots from each reaction mixture were taken and mixed with 40 μl of stop solution (1 M perchloric acid and 1 mM sodium phosphate) and placed on ice for 20 min. Then, 200 μl of 20 mM ammonium molybdate was added to each sample, vortexed vigorously, and incubated for addition of 300 μl of water-saturated isopropl acetate and a second vortexing. The concentration of phosphomolybdate complex that partitioned into the organic phase was measured by taking 150-μl aliquots of the organic phase and counting the radioactivity with a Wallac 1409 liquid scintillation coun ter. Raw values were corrected for spontaneous hydrolysis of ATP. The percent recovery of phosphomolybdate complex into the organic phase was assumed to be 100% in the subsequent conversion of radioactivity to phosphate concentration.

**Stop-flow Fluorescence Spectroscopy**—Stop-flow fluorescence analysis of the changes in tryptophan-derived fluorescent GroEL C138W were measured on a conventional stopped-flow apparatus (SX-17MV, Micro-Needles fluorescence spectrophotometer). The excitation wavelength was 295 nm, and a cutoff filter was used to monitor fluorescence intensities above 320 nm. All samples were prepared in stopped-flow buffer (50 mM triethanolamine, pH 7.5, containing 20 mM MgCl₂ and 50 mM KCl). The experi-
mental temperature was controlled with a water bath. Samples of GroEL C138W were mixed rapidly with ATP so that the final concentrations of GroEL and ATP were 0.5 mg/ml and 1 mM, respectively. Experiments were performed at 25 and 37 °C, and the results from five runs were averaged and were analyzed using the analysis software of the system according to either a single exponential or a sequential two-component exponential mechanism.

In Vivo Assessments of GroEL C138W Function—Plasmid pACYCESLC138W was constructed by ligating a fragment of pUCESLC138W excised by EcoRI and SalI with plasmid pACYC184 prepared by digestion with EcoRI and ScaI. This plasmid was used to transform E. coli strain KY1156 to produce strain KY1156/pACYCESLC138W. This strain was grown on Luria-Bertani plates containing the antibiotics kanamycin (50 μg/ml) and tetracycline (20 μg/ml) at either 37 or 25 °C, in both the presence and absence of 1 mM IPTG. In the absence of IPTG, E. coli KY1156 requires an alternate source of active GroEL protein to be viable at 37 °C. Preliminary experiments showed that in the presence of IPTG, the size of the colonies on agar plates containing kanamycin (50 μg/ml) at either 37 or 25 °C, in both the presence and absence of 1 mM IPTG. In the absence of IPTG, E. coli KY1156 requires an alternate source of active GroEL protein to be viable at 37 °C. Preliminary experiments showed that in the presence of IPTG, the size of the colonies was the same as those seen after a 12-h incubation at 37 °C (data not shown).

**RESULTS**

The Ubiquity of the Arrested Ternary Complex—The GroEL C138W-rhodanese-GroES ternary complex observed in a previous study was postulated to be an integral part of the overall mechanism of GroEL function (7). However, there was the possibility that this ternary complex might have been a special intermediate, which could be observed only during the refolding reaction of rhodanese. If this were the case, the detection of such an intermediate would not hold any relevance to the overall chaperonin cycle. In this study, therefore, we performed a series of experiments in order to demonstrate the ubiquity of this complex in the refolding reactions of numerous substrate proteins, as well as the consistency in the characteristics that are observed. We used the following proteins in our evaluation: adenylate kinase from chicken (molecular mass = 21 kDa), TIM from rabbit muscle (26.6 kDa × 2), bovine rhodanese (33 kDa × 1) (18), aldolase from rabbit muscle (39.2 kDa × 4), Taka-amylase A from Aspergillus oryzae (54 kDa × 1) (16), Gp23 protein from T4 phage (56 kDa × 1), GroEL protein from E. coli (57 kDa × 14), pyruvate kinase from rabbit muscle (58 kDa × 4), luciferase from firefly (61 kDa × 1) (19), and phosphofructokinase from rabbit muscle (85.1 kDa × 4). The subunit molecular masses of the substrate proteins varied from 21 to 85 kDa, and represented a fair sampling of the various protein sizes that may be found in a typical cell. Fig. 1 shows the results of a typical experiment in which a number of these proteins were renatured in the presence of GroEL C138W, ATP, and GroES at 25 °C, fractionated by size-exclusion chromatography, and analyzed by SDS-PAGE. As shown for the small protein adenylate kinase in Fig. 1A, proteins which do not form stable ternary complexes with GroEL C138W and GroES did not co-elute with GroEL C138W under our experimental conditions. Contrary, in the case of aldolase for example, a ternary complex was detected and isolated, as indicated by the co-elution of the band corresponding to aldolase in high-molecular mass fractions containing GroEL C138W (Fig. 1B). Aldolase was by no means the only protein to display this behavior, and as shown in Fig. 1C, TIM, Taka-amylase A, and phosphofructokinase were also shown to form stable ternary complexes with GroEL C138W at 25 °C. Further experiments showed that of the ten proteins sampled in this study, only the smallest, adenylate kinase, was incapable of forming a stable ternary complex with GroEL C138W and GroES at 25 °C (Table 1).

Taking into consideration various recent studies that showed that the size of the protein molecule which could be secluded within the GroEL central cavity was restricted to proteins smaller than 60 kDa (20, 21), it was especially interesting that phosphofructokinase, with a molecular mass of 85 kDa, was capable of forming a stable ternary complex with GroEL and GroES. Therefore, the nature of the stable ternary complex formed by GroEL C138W, GroES and each substrate protein was probed in more detail, to determine the specific orientation of GroEL, the refolding protein, and GroES. Various substrate proteins were first refolded in the presence of either GroEL C138W only or an equimolar concentration of GroEL and GroES with 2 mM ATP. The mixtures were then fractionated using size-exclusion chromatography to isolate arrested chaperonin complexes. Each purified complex was then subjected to a brief treatment with proteinase K, in order to digest any substrate protein that was accessible to the protease. Fig. 2 exemplifies the results of this experiment. As seen in the figure, we found that the substrate proteins we used could be separated largely into two groups. The first group, composed of proteins such as phosphofructokinase and pyruvate kinase, whose subunit molecular masses exceeded 55 kDa, were bound in a stable ternary complex with GroEL but displayed no differences when GroES was present, indicating that these proteins were exclusively bound in the trans conformation in the arrested ternary complex. In contrast, proteins such as Taka-amylase A and TIM, whose subunit molecular masses fell within the boundaries dictated by the size of the central cavity...
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Table I
Formation of stable binary and ternary chaperonin complexes by GroEL C138W at 25 °C

| Substrate protein                  | Total molecular mass (kDa) | Binary complex | Ternary complex |
|-----------------------------------|-----------------------------|----------------|-----------------|
| Phosphofructokinase (rabbit)      | 85.1 × 4                    | +              | +               |
| Luciferase (firefly)              | 61 × 1                      | +              | +               |
| Pyruvate kinase (rabbit)          | 58 × 4                      | +              | +               |
| GroEL (E. coli)                   | 57 × 14                     | +              | +               |
| Gp23 (T4 phage)                   | 56 × 1                      | +              | +               |
| Taka-amylose A (A. oryzae)        | 54 × 1                      | +              | +               |
| Aldolase (rabbit)                 | 39 × 4                      | +              | +               |
| Rhodanese (bovine)                | 33 × 1                      | +              | +               |
| TIM (rabbit)                      | 26 × 2                      | +              | +               |
| Adenylate kinase (chicken)        | 21 × 1                      | +              | -               |

* Ternary complexes were detected, but dissociated protein is also seen in the lower molecular mass fractions, suggesting a less extensive interaction with GroEL.

Table II
Estimation of the percentage of cis-ternary complex by brief digestion of arrested binary (− GroES) and ternary (+ GroES) chaperonin complexes with proteinase K

| Substrate protein (subunit molecular mass) | Binary complex (− GroES) | Ternary complex (+ GroES) |
|-------------------------------------------|--------------------------|---------------------------|
| TIM (26 kDa)                              | 18                       | 45                        |
| Rhodanese (33 kDa)                        | 2.6                      | 48.8                      |
| Taka-amylose A (54 kDa)                   | 4.2                      | 62.5                      |
| Gp23 (56 kDa)                             | 1.3                      | 3.0                       |
| GroEL (57 kDa)                            | 5.2                      | 14.1                      |
| Luciferase (61 kDa)                       | 8.8                      | 10.9                      |
| Pyruvate kinase (58 kDa)                  | 17.2                     | 12.0                      |

* The amount of trapped protein detected at zero time (minus proteinase K) was set to 100%.

Fig. 2. Proteinase K treatment of the arrested ternary complexes allow an analysis of the relative amounts of cis and trans ternary complexes. Results are shown for two different proteins, phosphofructokinase (PFK; 85 kDa) and Taka-amylose A (TAA; 54 kDa). Each protein was refolded in the presence of GroEL C138W at 25 °C, either in the presence or absence of the co-chaperonin GroES. Part of the sample was then subjected to a 10-min treatment with proteinase K. The results are shown side by side. Arrows indicate the relative positions of each refolding protein. The results for phosphofructokinase were detected using Coomassie staining, while the results for Taka-amylose A were obtained by quantitation of Cy5-labeled Taka-amylose bands on a Fuji FLA-2000 image analyzer.

... of GroEL (22), could be detected in significant amounts when GroES was added to the initial mixture, suggesting that a fraction of the substrate protein was shielded from digestion by the co-chaperonin. Table II summarizes the relative percentages of cis ternary complex which was detected for each refolding protein. From the results in Table II, we postulate that refolding in the presence of GroEL C138W at 25 °C results in the formation of stable ternary complexes, and the specific nature of these complexes were dependent mainly on the subunit molecular mass of the refolding substrate protein.

Next, we assayed the refolding reactions of a number of proteins that formed stable ternary complexes in Figs. 1 and 2. Fig. 3 shows the results of refolding assays performed on Taka-amylose A and TIM at 25 °C, the restrictive temperature, and 37 °C, the temperature at which the behavior of GroEL C138W reverts to that of the wild-type chaperonin. For comparison, refolding assays of rhodanese in the presence of GroEL C138W are also shown in a separate panel. Taka-amylose A (16) and TIM are examples of non-stringent proteins, with relatively high spontaneous refolding yields under our experimental conditions. Rhodanese is an example of a protein whose stringency during refolding is modulated by the refolding conditions (23, 24). In this assay, rhodanese reweighting was performed under non-permissive conditions (24). As shown in the figure, at 25 °C each protein was unable to complete folding, regardless of the presence of the co-chaperonin GroES and ATP. At 37 °C, however, the refolding reaction of all three proteins in the presence of GroEL C138W was measurably improved compared with the reactions at 25 °C, demonstrating again the temperature-de-
state of the bound nucleotides. In other words, is the ternary state arrested in an ATP-bound conformation? Or does ATP hydrolysis proceed to a certain extent even after substrate protein and GroEL form an arrested complex? To answer this question, we determined the amount of ATP hydrolyzed by GroEL C138W which were preincubated with increasing concentrations of unfolded rhodanese protein. We reasoned that if GroEL was arrested as an ATP-bound complex, the amount of ATP hydrolyzed at saturating concentrations of substrate protein would approach zero. On the other hand, if ATP hydrolysis still occurred after the arrested complexes had been formed, the amount of ATP hydrolyzed would extrapolate to a certain non-zero value. The results of this experiment are shown in Fig. 5. As seen in the figure, when GroEL (60 nM 14-mer) and GroES (100 nM heptamer) were incubated with increasing concentrations of unfolded rhodanese protein, the ATPase activity of the chaperonin was reduced. At high ratios of rhodanese to GroEL tetradecamer, however, the concentration of ATP hydrolyzed was seen to approach a non-zero value of $1.4 \text{M ATP}$. This result suggests that the ATPase activity of GroEL is not immediately arrested upon formation of the arrested ternary complex, and that GroEL C138W undergoes at least one round of ATP hydrolysis after formation of the arrested ternary complex.

Stopped-flow Analysis of Temperature-dependent Conformational Changes—Recent studies have attempted to characterize the specific conformational changes of GroEL during its functional mechanism using extremely rapid spectrophotometric methods (13, 25–28). In the present case, the introduced tryptophan provides an ideal conformational probe for such studies, and an interesting comparison could be made at the two different experimental temperatures. Fig. 6 indicates a stopped-flow fluorescence experiment performed on GroEL C138W mixed with 1 mM ATP at 25 and 37°C. The changes in fluorescent intensity observed at these two temperatures were dramatically different. At 25°C, a very weak increase in fluorescent intensity could be observed, which could be analyzed according to a single exponential equation to yield an apparent rate constant of about 12.6 s$^{-1}$. Upon increasing the temperature to 37°C, however, the total amplitude of the fluorescence...
change was dramatically increased, and the trace was analyzed according to a sequential two-phase mechanism to yield apparent rate constants of 82.1 s$^{-1}$ and 19.5 s$^{-1}$, respectively. The majority of the increase in fluorescence was due to the newly detected fast phase. The simplest interpretation of these results was that a structural characteristic of the mutant chaperonin limited the fast movement of the intermediate domain at 25 °C, and upon temperature increase, this block was removed. This temperature-dependent difference in the conformational changes of the GroEL subunit agreed well with the switch in chaperonin function seen upon a similar increase in temperature.

In Vivo Characteristics of the C138W Mutant—Finally, we were interested in determining if the characteristics of GroEL C138W determined in the above experiments were reflected in any way in the in vivo behavior of this mutant. Fig. 7 shows cultivation experiments using E. coli strain KY1156, whose cellular expression of GroES and GroEL are controlled by the lacUV5 promoter (15). Under conditions where the groESL operon is not induced by the addition of IPTG, E. coli KY1156 grows extremely slowly at 25 °C and not at all at 37 °C. Transformation of this strain with a plasmid which overproduces wild-type GroES and GroEL C138W resulted in a recovery of wild-type-like growth at 37 °C (Fig. 7B), indicating that GroEL C138W substituted for wild-type GroEL at this temperature. However, at 25 °C, growth of the colonies were extremely slow (Fig. 7A), suggesting that under these conditions, insufficient amounts of active GroEL protein were present. This result indicated clearly that at 25 °C, GroEL C138W was unable to function as a replacement for wild-type GroEL, but was capable of functioning in a manner identical to the wild-type chaperonin at 37 °C.

DISCUSSION

Studies regarding chaperonin-facilitated protein folding are presently concentrating on linking specific movements of the GroEL subunit, detectable by various spectrophotometric means, to the various steps of the functional cycle revealed in refolding assay studies (3, 4, 28). A powerful general strategy would be to map various loss-of-function mutations of the GroEL molecule to specific stages in the presently postulated chaperonin molecular mechanism. As part of our efforts to elucidate in detail the molecular mechanism of chaperonin function, we report here our findings regarding GroEL C138W, a mutant chaperonin that displays temperature-dependent chaperonin activities.

We first set out to determine if we could detect the complex in other refolding reactions in addition to rhodanese, thereby demonstrating the ubiquity of this intermediate under various in vitro experimental conditions. The results shown in Fig. 1 and Table I clearly demonstrate that the arrested complex of GroEL C138W, GroES, and refolding protein may be observed in numerous proteins with molecular masses greater than 30 kDa.

Supporting evidence to the notion that this arrested complex is an integral part of the chaperonin-facilitated protein folding reaction, rather than an artifact of it, was obtained by analyzing the specific composition of the arrested complex, and also the fate of this complex after resumption of the refolding reaction upon temperature increase. From the results shown in Fig. 2 and Table II, we have first found that a number of proteins, specifically, proteins with molecular masses between 30 and 54 kDa, form both cis- and trans-ternary complexes. This limit in size with regard to the ability to form an arrested cis complex is similar in range and agrees well to the values obtained in other studies regarding the maximum size of the proteins that are able to fold inside the GroEL central cavity (20, 21). In
A very important question which must be addressed with regard to the specific characteristics of the arrested ternary complex is the state of the nucleotides which were bound during the initial formation. The state of the nucleotide would be important in determining the relative position of this intermediate in the overall chaperonin mechanism. Our initial assumption regarding this question was that the arrested complex would not undergo additional hydrolysis cycles once formed, in other words, that the arrested complex would be an ATP-GroEL-rhodanese-GroES complex (7). Surprisingly however, the results shown in Fig. 5 suggest strongly an alternate scenario; that once formed, the chaperonin undergoes at least one cycle of heptameric ATP hydrolysis. Although further experimentation is necessary in order to explain this extremely interesting experimental result, the fact that even under these conditions, the arrested complex is capable of resuming its function after temperature increase, compels us to regard this result to be relevant to the overall mechanism. Although it is a highly speculative possibility at this point, it may be that the molecular mechanism which underlies chaperonin function is much more complex than a simple linear and orderly progression of molecular events.

The next question in our study would then be to determine the specific conformational change which is responsible for this arrest in the chaperonin cycle. Fortunately, stopped-flow fluorescence studies of the introduced tryptophan provided a very important clue. As shown in Fig. 6, when GroEL C138W was mixed with ATP at 37 °C, two kinetic phases, one with an apparent rate constant of 82.1 s⁻¹ and the other with an apparent rate constant of about 19.5 s⁻¹ were observed. Strikingly, the fast phase was undetectable at 25 °C, and the increase in fluorescence intensity was composed from a single kinetic phase with a rate constant of 12.6 s⁻¹. The rate constant of the fast kinetic phase seen at 37 °C has been previously observed in other studies using pyrenyl-labeled GroEL. Jackson et al. (13) determined that upon binding of ATP, pyrenyl GroEL displays an increase in pyrenyl-derived fluorescence whose apparent rate constant is identical to the rate seen in the present study. This kinetic phase displayed a maximum apparent rate constant of about 180 s⁻¹ at saturating ATP concentrations, and represents a form of GroEL that strongly binds ATP with a lower affinity for refolded protein molecules. They postulated that this step would be the key conformational change which is responsible for the release of protein molecules from GroEL, and our results have succeeded in demonstrating this more directly, with regard to both cis and trans ternary species. What is most interesting about this intermediate is, however, that during the formation of the ternary complex the binding of the co-chaperonin GroES proceeded without the release of protein folding intermediates into the bulk solution, and a state arose (in the cis-ternary complex) where GroES and refolding protein were simultaneously bound to the same heptameric ring of GroEL. The fact that this intermediate was formed suggests that the binding of GroES precedes the final release of refolding protein into the GroEL cylinder. The specific process of the binding of GroES to the GroEL apical domain and the subsequent release of refolding protein into the GroEL cylinder is the subject of recent intense experimental efforts (3, 4). Our present results suggest strongly the following scenario regarding the specific process by which GroES binds and dislodges the refolding protein molecule into the central cavity: upon ATP binding, the apical domain of GroEL shifts to allow the binding of GroES, while still bound to the refolding protein molecule. GroEL then undergoes a large conformational change involving the intermediate domain that lowers the affinity of the GroEL apical domain for protein folding intermediates, and the protein molecule is dropped into the central cavity. In this process, a transient intermediate is formed where the apical domain of GroEL simultaneously binds GroES and the refolding protein molecule (see Fig. 8).

Such a sequence of events would be an attractive mechanism, as this would ensure that proteins bound to GroEL and subsequently placed in the cis conformation relative to GroES would be efficiently transferred into the GroEL central cavity.
Diffusion of the partially refolded protein into the surrounding medium would be prevented. Also, supportive evidence for the above mechanism may be found in a number of previous studies. Cliff et al. (28) have used various tryptophan mutants to map the various movements of the GroEL subunit. They have shown that GroEL undergoes a very rapid conformational change in addition to the one documented in this study, with an apparent rate constant greater than 100 s⁻¹ in the presence of 175 µM ATP. We ourselves have documented an extremely rapid movement of the GroEL subunit which occurs immediately after ATP binding using the GroEL T89W mutant (12), and inferred from refolding assays that this conformational change was part of the protein release mechanism of GroEL. It is possible that one of these conformational changes results in a GroES-binding conformation. Also, Farr et al. (29) have shown in experiments involving covalent GroEL heptamers that GroEL rings impaired in substrate protein binding activity are nevertheless capable of binding to GroES. This interesting experimental result suggests indirectly the possibility that the refolding protein molecule and GroES may simultaneously bind to the same ring of GroEL in a biologically significant manner. The elucidation of the specific role of this ternary complex will most likely require many more experiments under a variety of conditions.

Recent studies have highlighted the differences between protein folding in vitro and in vivo, placing emphasis on differences such as the concentration of macromolecules, and the extreme heterogeneity of the environment within a typical cell (30, 31). The characteristics of the chaperonin GroEL, which is active under both cellular conditions and also under laboratory conditions, must be relevant to both of these conditions. In order to evaluate roughly the effects of the C138W mutation on the in vivo characteristics of GroEL, we examined if GroEL C138W could substitute for the wild-type chaperin in vitro. As shown in Fig. 7, GroEL C138W was capable of substituting for the wild-type chaperin at 37 °C, but was unable to do so at 25 °C. This excellent correlation of in vivo phenotype to in vitro function indicated that first, the behavior of GroEL C138W at 37 °C was sufficiently identical to the wild-type protein to allow substitution in vivo, and second, that the changes which were brought about by the mutation (temperature-dependent restriction of conformational change) are of strong relevance to the in vivo activity of GroEL. We believe, therefore, that the characteristics of GroEL C138W revealed in the present study are very relevant to the actual molecular cycle utilized by the wild-type chaperin, and provides us with a number of important clues regarding the sequence of events which take place immediately after binding of ATP to the GroEL ring.

Fig. 8 summarizes our conclusions in this study. The main assertion which we make based upon our results involves the sequence by which GroES is bound and refolding protein released following ATP binding to the GroEL subunit. We propose that the binding of GroES occurs prior to the final release of refolding protein into the GroEL cylinder. The key conformational change that dictates protein release was found to involve a large movement in the intermediate domain, with an apparent rate constant of 82 s⁻¹ in the presence of 1 mM ATP. This conformational change, documented in other studies previously, is rendered temperature sensitive in GroEL C138W, which allowed a detailed characterization of this intermediate state. In further experiments, a more detailed process by which GroEL manages to enclose refolding protein molecules into the relative safety of its central cavity, with special regard to the hydrolysis of ATP, should become clear.

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REFERENCES

1. Hartl, F. U., and Hayer-Hartl, M. (2002) Science 295, 1852–1858
2. Fenton, W. A., and Horwich, A. L. (1997) Prot. Sci. 6, 743–760
3. Rye, H. S., Burston, S. G., Fenton, W. A., Beechem, J. M., Xu, Z., Sigler, P. B., and Horwich, A. L. (1997) Nature 388, 792–798
4. Rye, H. S., Roseman, A. M., Chen, S., Furtak, K., Fenton, W. A., Saihil, H. R., and Horwich, A. L. (1999) Cell 97, 325–338
5. Braig, K., Owinskiowski, Z., Hegde, R., Burston, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) Nature 371, 578–586
6. Fenton, W. A., Kashi, Y., Furtak, K., and Horwich, A. L. (1994) Nature 371, 614–619
7. Kawasaki, Y., Kawagoe, M., Hongo, K., Miyazaki, T., Higurashi, T., Mizobata, T., and Nagai, J. (1999) Biochemistry 38, 15731–15740
8. Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saihi, H. R., Fenton, W. A., and Horwich, A. L. (1995) Cell 83, 577–587
9. Yifrach, O., and Horovitz, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1521–1524
10. Mizobata, T., Makino, Y., and Yoshida, M. (1996) J. Biol. Chem. 271, 28229–28234
11. Martin, J. (2002) Biochemistry 41, 5050–5055
12. Mizobata, T., Kawasaki, M., Hongo, K., Nagai, J., and Kawata, Y. (2000) J. Biol. Chem. 275, 25600–25607
13. Jackson, G. S., Staniforth, R. A., Halsall, D. J., Atkinson, T., Holbrook, J. J., Clarke, A. R., and Burston, S. G. (1993) Biochemistry 32, 2554–2563
14. Mizobata, T., and Kawata, Y. (1994) Biochim. Biophys. Acta 1209, 83–88
15. Kanemori, M., Mori, H., and Yura, T. (1994) J. Bacteriol. 176, 4235–4242
16. Kawata, Y., Hongo, K., Mizobata, T., and Nagai, J. (1996) Protein Eng. 11, 1293–1298
17. Horovitz, A., Rochkareva, E. S., Kovalenko, O., and Girshovich, A. S. (1993) J. Mol. Biol. 231, 58–64
18. Mendoza, J. A., Rogers, E., Lorenz, G. H., and Horovitz, P. M. (1991) J. Biol. Chem. 266, 13044–13049
19. Fridmann, Y., Ulitzur, S., and Horovitz, A. (2000) J. Biol. Chem. 275, 37851–37856
20. Houy, W. A., Frishman, D., Eckerstorh, C., Lottspeich, F., and Hartl, F. U. (1999) Nature 402, 147–154
21. Sakikawa, C., Taguchi, H., Makino, Y., and Yoshida, M. (1999) J. Biol. Chem. 274, 21251–21256
22. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) Nature 388, 741–750
23. Mendoza, J. A., Demeler, B., and Horovitz, P. M. (1994) J. Biol. Chem. 269, 2447–2451
24. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., and Hartl, F. U. (1991) Nature 352, 36–42
25. Yifrach, O., and Horovitz, A. (1996) Biochemistry 37, 7083–7088
26. Horovitz, A., and Yifrach, O. (2000) Bull. Math. Biol. 62, 241–246
27. Fridmann, Y., Kafri, G., Danziger, O., and Horovitz, A. (2002) Biochemistry 41, 5938–5944
28. Cliff, M. J., Kud, N. M., Hay, N., Lund, P. A., Webb, M. B., Burston, S. G., and Clarke, A. R. (1999) J. Mol. Biol. 293, 667–684
29. Farr, G. W., Furtak, K., Rowland, M. B., Ranson, N. A., Saih, H. R., Kirchhausen, T., and Horwich, A. L. (2000) Cell 100, 561–573
30. Martin, J., and Hartl, F.-U. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1107–1112
31. Ellis, R. J. (2001) Curr. Opin. Struct. Biol. 11, 114–119
32. Lasemlli, U. K. (1970) Nature 227, 680–685
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