Functional Communications between the Apical and Equatorial Domains of GroEL through the Intermediate Domain†

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ABSTRACT: The *Escherichia coli* GroEL subunit consists of three domains with distinct functional roles. To understand the role of each of the three domains, the effects of mutating a single residue in each domain (Y203C at the apical, T89W at the equatorial, and C138W at the intermediate domain) were studied in detail, using three different enzymes (enolase, lactate dehydrogenase, and rhodanese) as refolding substrates. By analyzing the effects of each mutation, a transfer of signals was detected between the apical domain and the equatorial domain. A signal initiated by the equatorial domain triggers the release of polypeptide from the apical domain. This trigger was independent of nucleotide hydrolysis, as demonstrated using an ATPase-deficient mutant, and, also, the conditions for successful release of polypeptide could be modified by a mutation in the apical domain, suggesting that the polypeptide release mechanism of GroEL is governed by chaperonin-target affinities. Interestingly, a reciprocal signal from the apical domain was suggested to occur, which triggered nucleotide hydrolysis in the equatorial domain. This signal was disrupted by a mutation in the intermediate domain to create a novel ternary complex in which GroES and refolding protein are simultaneously bound in a stable ternary complex devoid of ATPase activity. These results point to a multitude of signals which govern the overall chaperonin mechanism.

It is well-known that the double-ring-structured chaperonin GroEL (14-mer) from *Escherichia coli* mediates the folding of various proteins in vivo and in vitro with the co-chaperonin GroES (7-mer) (*1*−*3*). The subunit structure of GroEL is composed of three domains: apical, intermediate, and equatorial (*4*−*6*). Each domain has a specific role during folding; i.e., the apical domain interacts with folding intermediates and the flexible loop interface of GroES, the equatorial domain hydrolyzes ATP and controls the overall chaperonin mechanism by this activity, and the intermediate domain connects these apical and equatorial domains. According to various detailed studies (*2*, *3*, *7*, *8*), GroEL first binds protein folding intermediates at its apical domain, and, in the presence of ATP, proceeds also to bind the cofactor GroES in such a way that GroEL and GroES form a capsule around the refolding protein. Since GroES is unable to bind to GroEL in the absence of ATP, the nucleotide is presumed to prime the GroEL protein for binding to GroES. Binding of GroES in the presence of nucleotide, in turn, results in a massive conformational change in the apical domain of GroEL which results in a 2-fold enlargement of the GroEL central cavity (*6*, *9*). The refolding protein is released inside this cavity by the conformational change, where, protected from other similar protein particles, it is able to complete its refolding reaction without interruption. After hydrolysis of ATP in the GroES-bound cis-ring, GroES is released by the further binding of ATP at the trans-ring, at the same time releasing the target protein into solution. Thus, in this functional mechanism, the conformational changes of the apical domain induced by nucleotide binding/hydrolysis at the equatorial domain, as well as the nucleotide-dependent GroES binding at the apical domain, are very important for efficient fulfillment of the function. Although detailed aspects regarding the specific sequence of events in the chaperonin cycle that facilitates protein folding have been extensively studied (*2*, *3*, *7*, *8*), further details on the functional relationships between the domains, especially the functional role of the intermediate domain, remain unclear.

In the present study, to elucidate these aspects of nucleotide- and GroES-dependent release of protein folding intermediates and also to probe for any functional roles of the intermediate domain, we constructed three mutants (Y203C,[1] T89W, and C138W), each contained in a separate domain of GroEL, and performed experiments to evaluate

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[1] Abbreviations: CD, circular dichroism; C138W, GroEL mutant in which the cysteine residue at position 138 was replaced by a tryptophan residue; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; MOPS, 3-morpholinopropanesulfonic acid; NEM, *N*-ethylmaleimide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; T89W, GroEL mutant in which the threonine residue at position 89 was replaced by a tryptophan residue; Y203C, GroEL mutant in which the tyrosine residue at position 203 was replaced by a cysteine residue.

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the effects of each mutation on the functional mechanism of GroEL. Experiments using these mutants revealed that various signals were being exchanged between the equatorial and apical domains during the crucial stage of polypeptide release and subsequent ATP hydrolysis. The intermediate domain was essential to these signal exchanges, as demonstrated by a mutation in the intermediate domain which could arrest the chaperonin cycle in a previously undetected conformation.

MATERIALS AND METHODS

Materials. Gdn-HCl (specially purified grade) was obtained from Nacalai Tesque (Kyoto). *Taq* DNA polymerase and restriction enzymes were purchased from Takara (Kyoto). Primers for mutations and sequencing were synthesized with a Pharmacia DNA synthesizer (Gene Assembler Special). ATP and ADP were purchased from Sigma and used after checking the purity (*10*). All other reagents were of the highest grade commercially available.

Preparation of Mutant Proteins. Genes encoding the GroEL mutants were prepared by the overlap extension method (*11*) as follows. Plasmid pUCESL was first constructed by subcloning the DNA fragment containing the *groE* operon from pKY206 (*Eco*RI−*Sma*I fragment) into the multicloning site of pUC118. Several steps of polymerase chain reaction (Perkin-Elmer DNA Thermal Cycler) of this *groE* operon region using primers (+, − strands) containing the mutation [ACC (Thr) to TGG (Trp) for T89W, TGC (Cys) to TGG (Trp) for C138W, and TAC (Tyr) to TGC (Cys) for Y203C] and the M13 primers P7 and P8 resulted in a DNA fragment containing the desired mutation. These fragments were again cut with *Eco*RI and *Sma*I and ligated into the multicloning site of pUC118 to produce pUCESL mutant forms, which were then used to transform *E. coli* strain JM109. The incorporation of each mutation at the desired positions was confirmed by DNA sequence analysis of the entire GroEL coding region using an Applied Biosystems 373A DNA sequencer. The mutants were expressed in the presence of 1 mM isopropyl-1-thio-β-galactopyranoside at amounts comparable to JM109/pUCESL, i.e., approximately 10−30% of the total soluble protein at 30 °C. Under these conditions, the amount of endogenous GroEL protein from host cell was found to be less than 3% of the expressed mutant proteins by quantitation of the SDS−PAGE band with a Densito-Pattern Analyzer EPA-3000 (Maruzen Petrochemical). Therefore, we concluded that the effect of contaminant wild-type GroEL protein in the purified preparations of mutant forms was negligible in further experiments. The concentrations of GroEL mutants were determined colorimetrically with a protein dye reagent (Protein Assay Kit; Bio-Rad Laboratories) using bovine serum albumin as a standard.

Purification of GroEL Proteins. Wild-type GroEL and GroES proteins were expressed in *E. coli* DH-1/pKY206 and purified at 4 °C as follows. Briefly, after lysis and removal of nucleic acids by addition of streptomycin, the 55% ammonium sulfate precipitate was subjected to a Sephacryl S-300 gel-filtration column (ϕ 2.8 × 130 cm) which had been equilibrated with 50 mM Tris-HCl buffer, pH 7.8, containing 2 mM EDTA and 1 mM 2-mercaptoethanol (buffer A), at a flow rate of 0.5 mL/min. GroEL and GroES fractions were pooled and loaded separately onto a Q-Sepharose anion-exchange column (ϕ 3.4 × 45 cm) which had been equilibrated with buffer A and eluted with a linear concentration gradient of 1 L of buffer A to 1 L of buffer A containing 1 M KCl at a flow rate of 40 mL/h. The purified GroEL and GroES proteins were pooled separately and desalted by dialysis thoroughly with buffer A, and then concentrated.

Mutant GroEL proteins were also purified according to the protocol for wild-type GroEL protein at 4 °C. For fluorescence spectroscopy and capillary electrophoresis experiments, wild-type and mutant proteins were further purified according to the published protocols (*12*). The purity of the highly purified GroEL proteins was such that no tryptophan fluorescence due to contaminated polypeptides in the preparations was detected under the conditions we used in the present study.

Identification of Amino Acid Substitution of GroEL Mutants. Confirmation of the desired mutation at the amino acid level was also accomplished by peptide mapping and sequencing. Samples of purified GroEL mutants were extensively digested by a lysyl endopeptidase (*Achromobacter lyticus* protease I) according to the published protocols (*13*), and separated on a C_{18} reversed-phase HPLC column with detection by both absorbance at 215 nm and fluorescence at 350 nm (excitation: 295 nm). Peaks of interest were collected and sequenced on a Shimadzu PPSQ-10 amino acid sequencer.

Measurements of CD and Fluorescence Spectra, and Relative Molecular Mass. CD spectra were measured on a JASCO J-720 spectropolarimeter equipped with a constant-temperature cell holder. The buffer used was 10 mM HEPES−KOH buffer, pH 7.0, containing 10 mM KCl, 5 mM $Mg(CH_3COO)_2$, and 0.1 mM DTT. Fluorescence spectroscopy was performed on a Hitachi F-4010 fluorescence spectrophotometer equipped with a constant-temperature cell holder. The spectra were measured in 50 mM HEPES−KOH buffer, pH 7.0, containing 10 mM KCl, 10 mM $Mg(CH_3COO)_2$, and 1 mM 2-mercaptoethanol. The relative molecular mass of each GroEL mutant protein was determined by HPLC gel-filtration chromatography performed on a Bio-Gel SEC 40XL size-exclusion column (ϕ 7.8 × 300 mm; Bio-Rad Laboratories), using a Gilson HPLC system at a detection wavelength of 280 nm. The running buffer was 50 mM Tris-HCl buffer, pH 7.8, containing 0.2 M NaCl, and a flow rate of 0.5 mL/min was used for analysis.

Capillary Electrophoresis Analysis. Free-solution capillary electrophoresis analysis was performed on a Quanta 4000E system (Waters) with a fused silica capillary tube (ϕ 75 μm × 45 cm) at 25 °C, as reported previously (*14*). The buffer used was 50 mM MOPS−KOH, pH 7.0, containing 10 mM $Mg(CH_3COO)_2$, 10 mM KCl, 0.5 M trimethylammonium propanesulfonate, and 2 mM ATP.

ATPase Activity Measurements. ATPase activities of GroEL mutants were measured at 25 or 37 °C, using the method of Lanzetta et al. (*15*), as modified by Martin et al. (*16*). Briefly, after incubation for 10 min, 2 mM ATP was added into 600 μL of ATPase assay buffer [50 mM Tris-HCl, pH 7.4, containing 20 mM $Mg(CH_3COO)_2$, 100 mM KCl, and 2 mM 2-mercaptoethanol] containing 0.1−1 μM GroEL or GroEL and GroES oligomer. Routinely, at 10 min

intervals (usually the reaction was traced for 60 min), 50 μL aliquots of the assay mixture were mixed with 200 μL of 1 M perchloric acid, vortexed briefly, and incubated on ice for 30 min. This solution was centrifuged, and 50 μL of the supernatant was mixed with 800 μL of a solution containing 0.034% Malachite Green oxalate, 3.2% ammonium molybdate, 3 N HCl, and 0.1% Triton X-100. After mixing, 100 μL of 34% (w/v) sodium citrate dihydrate was added, and the mixture was allowed to stand for about 30 min at room temperature for the color to develop. The absorbance of this mixture was quantitated at 660 nm. Concentrations of inorganic phosphate were determined by using a standard solution of phosphate in the same buffer.

Nucleotide Binding Measurements. Binding of nucleotides to T89W was measured by monitoring the changes in fluorescence at 340 nm. The excitation wavelength was 295 nm. Various concentrations of ATP were mixed with 62 nM (50 μg/mL) T89W protein (14-mer), and the spectra were subsequently measured. The raw data were corrected for buffer effects by subtraction of a reference spectrum containing an identical concentration of nucleotide.

Refolding Assays. Refolding assays of yeast enolase (*10*) and *Staphylococcus* LDH (*17*) were carried out as previously reported. Refolding of bovine rhodanese was performed under two different conditions: 'permissive' (*18*) and 'nonpermissive' (*16*), with some modifications in protocol. Briefly, rhodanese (50 μM) was unfolded in 40 mM Tris-HCl buffer, pH 7.4, containing 6 M Gdn-HCl and 1.5 mM DTT at 25 °C for 1 h. For permissive refolding, the refolding reaction was started by a 100-fold dilution into 'permissive buffer' (50 mM Tris-HCl, pH 7.8, containing 50 mM $Na_2S_2O_3$, 10 mM $MgCl_2$, 10 mM KCl, and 400 mM 2-mercaptoethanol). For nonpermissive refolding, the dilution was into 'nonpermissive buffer' (30 mM Tris-HCl buffer, pH 7.2, containing 50 mM KCl). The temperature and protein concentration of rhodanese during the refolding reaction were 25 °C and 0.5 μM, respectively; 2 mM ATP, 4 mM Mg-$(CH_3COO)_2$, and a 1.5-fold molar excess of GroEL and GroES oligomer relative to rhodanese were present in the refolding reaction. At appropriate times during the refolding reaction, 200 μL of the mixture was withdrawn and assayed for rhodanese activity. The assay was performed at 25 °C as described previously (*19*). Refolding yield was determined as the percentage ratio of the activity of the refolding enzyme relative to that of native enzyme.

Analysis of Ternary Complex Formation of GroEL−Folding Intermediate−GroES. The nature of the C138W−folding intermediate−GroES ternary complex was examined as follows: rhodanese (50 μM) and LDH (50 μM monomer) were unfolded in Gdn-HCl and diluted 100-fold into 'nonpermissive' buffer containing equimolar concentrations of C138W GroEL 14-mer and GroES 7-mer. Immediately after dilution, 2 mM ATP was added, and this mixture was incubated for 10 min at 25 °C. Three hundred microliters of the refolding mixture thus prepared was then loaded onto a Sephacryl S-300 gel-filtration column (ϕ 12 × 250 mm) which had been equilibrated with the same buffer containing 0.4 mM ATP. Fractions (800 μL) collected from the column were precipitated by addition of trichloroacetic acid (final concentration of 10%) and placed on ice for 15 min. The precipitates were centrifuged and washed with 1 mL of ice-cold water and subjected to SDS−PAGE (15% polyacrylamide gel).

The proteinase K digestion experiments were carried out as follows. C138W−rhodanese complex (0.7 μM) prepared as described above was incubated at 25 °C in the presence of GroES (0.7 μM) and ATP (2 mM). The digestion reaction was started by addition of proteinase K (7 nM; 200 ng/mL) to this mixture. At appropriate times, phenylmethanesulfonyl fluoride dissolved in 8% 2-propanol was added to stop the reaction (1 mM), and then the proteins were precipitated with trichloroacetic acid and analyzed by SDS−PAGE (15% polyacrylamide gel). The quantitation of the bands was performed using the public domain software *NIH Image* (developed at the U. S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov).

RESULTS

Structural Features of GroEL Mutant Proteins. The locations of the three mutation sites, Y203C, T89W, and C138W, are shown in Figure 1a,b. Tyr203 is located in the apical domain, and the side chain projects out toward the interior of the central cavity; Thr89 is located at the ATP binding site in the equatorial domain and interacts with the γ-phosphate group of ATP (*20*) when ATP is bound to GroEL; and Cys138 is located at a hinge region (*9*) connecting the intermediate and the equatorial domains. Each site was selected in the expectation that incorporation of a mutation would result in the following effects on the chaperonin mechanism: a change in the affinity for folding intermediates and GroES (Tyr203), a change in ATP binding/hydrolysis (Thr89), and a change in the general chaperonin mechanism (Cys138). As a result, we hoped to study the functional role of each domain of the GroEL subunit. The tryptophan mutations, if successful, would also allow fluorescence experiments to be performed.

Each mutant was overproduced in *E. coli* in amounts comparable to the wild-type protein. Although small differences in the near-UV CD spectra were observed for T89W and C138W due to incorporation of a tryptophan residue, we concluded that the difference in overall structure compared to wild-type was minimal from the analysis of far-UV CD spectra and gel-filtration chromatography profiles (data not shown).

Functional Characteristics of GroEL Mutant Proteins. (1) ATPase Activity and Binding of GroES. We first examined the ATPase activity of each GroEL mutant. As indicated in Figure 2a, each mutant protein showed different activities. Whereas T89W exhibited no ATPase activity regardless of the presence or absence of GroES, C138W and Y203C showed almost the same ATPase activity profiles as the wild-type GroEL. When the ATPase activities were measured for 60 min, a gentle-slope curve was produced (data not shown). From the curves, almost the same initial rates of ATP hydrolysis (0.08 s^{-1}) as the wild-type GroEL in the absence of GroES (0.08−0.1 s^{-1}) (*21, 22*) were estimated for C138W and Y203C. In the presence of an equimolar concentration of GroES, C138W behaved like the wild-type protein, with a 50% decrease in the ATP hydrolysis activity (*21*), while that of Y203C was decreased by only about 30%. The slight decrease in ATP hydrolysis of Y203C

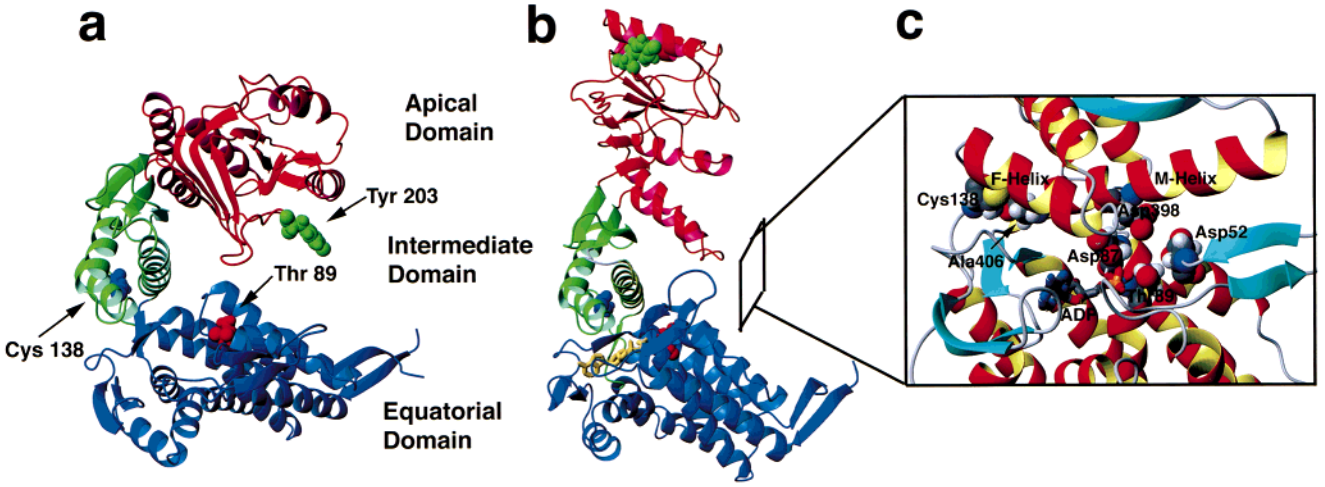

FIGURE 1: Structure of GroEL subunit. Graphical images were created using the program MOLMOL (*45*), using downloaded PDB files. The apical domain is indicated in red, equatorial in blue, and intermediate in green. Mutated residues are represented by a space-filling model [Tyr203 (green), Thr89 (red), and Cys138 (blue)] in the structure of GroEL 14-mer (from 1DER in PDB) (a) and of GroEL−GroES 21-mer (from 1AON in PDB) (b). A magnified structure of the hinge region between the equatorial and the intermediate domains viewed from the left side of (b) is shown in (c).

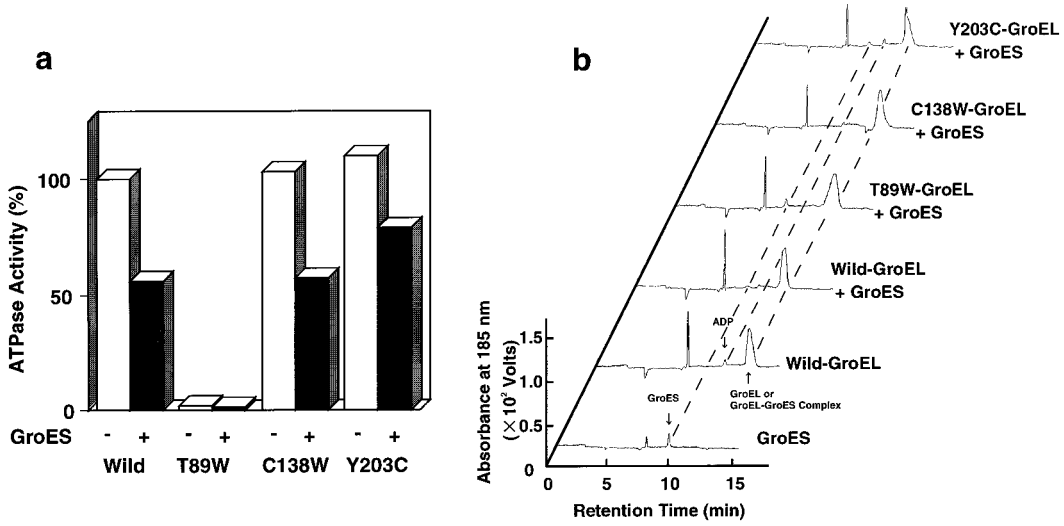

FIGURE 2: ATPase activity (a) and capillary electrophoresis analysis (b) of wild-type and GroEL mutant proteins. Aliquots of 1 μM each of GroEL and GroES oligomer and 2 mM ATP were used for the ATPase activity assay at 25 °C (see Materials and Methods for detailed conditions). The quantity of released inorganic phosphate ion after 60 min reaction was compared to that of wild-type GroEL. For the capillary electrophoresis analysis, a 50 ng protein sample was applied, and the changes in absorbance at 185 nm were monitored (*14*). A sharp peak at 7.5 min was due to neutral net-charge solutes. GroEL and GroEL−GroES complex were not separated under these conditions.

in the presence of GroES suggested that the affinity to bind GroES in the presence of ATP was weakened by the mutation. To detect a direct interaction of each GroEL mutant with GroES, we studied the complex formation of these mutants using capillary electrophoresis (*14*). Binding of GroES to GroEL can be monitored by the disappearance of a peak at 9 min, which represents the GroES 7-mer (Figure 2b). As shown in Figure 2b, C138W was able to form a GroEL−GroES complex under equimolar conditions, similar to the wild-type GroEL protein, while for Y203C under the same conditions, 30% of the GroES remained free in solution. This indicates that Y203C has a decreased GroES binding ability, which is consistent with the weak suppression in ATPase activity seen in Figure 2a. T89W was not able to bind GroES in the presence of ATP.

(2) Chaperonin Activity. We next examined the ability of these mutants to trap refolding intermediates in the absence of ATP and to facilitate protein folding (refoldase activity) in the presence of ATP and GroES at 25 °C. As substrate proteins, we used three different proteins, each of which displays distinct characteristics during refolding in the presence of GroE. The first target protein, yeast enolase (dimer, M_w 47 500 × 2), is a protein which displays a high spontaneous folding yield but whose refolding intermediates are bound efficiently by GroEL. Addition of either ATP singly or ADP and GroES triggers the release of enolase refolding intermediates (*10*). The second target protein, *Staphylococcus* LDH (dimer, M_w 32 000 × 2), refolds very poorly in buffer but is greatly assisted by GroEL in combination with either ATP singly or ADP and GroES (*17*). The third target protein, bovine rhodanese (monomer, M_w 33 000), displays different characteristics according to different buffer conditions. Under the so-called 'nonpermissive conditions', rhodanese is absolutely dependent on GroEL, GroES, and ATP for efficient refolding to occur (*16*).

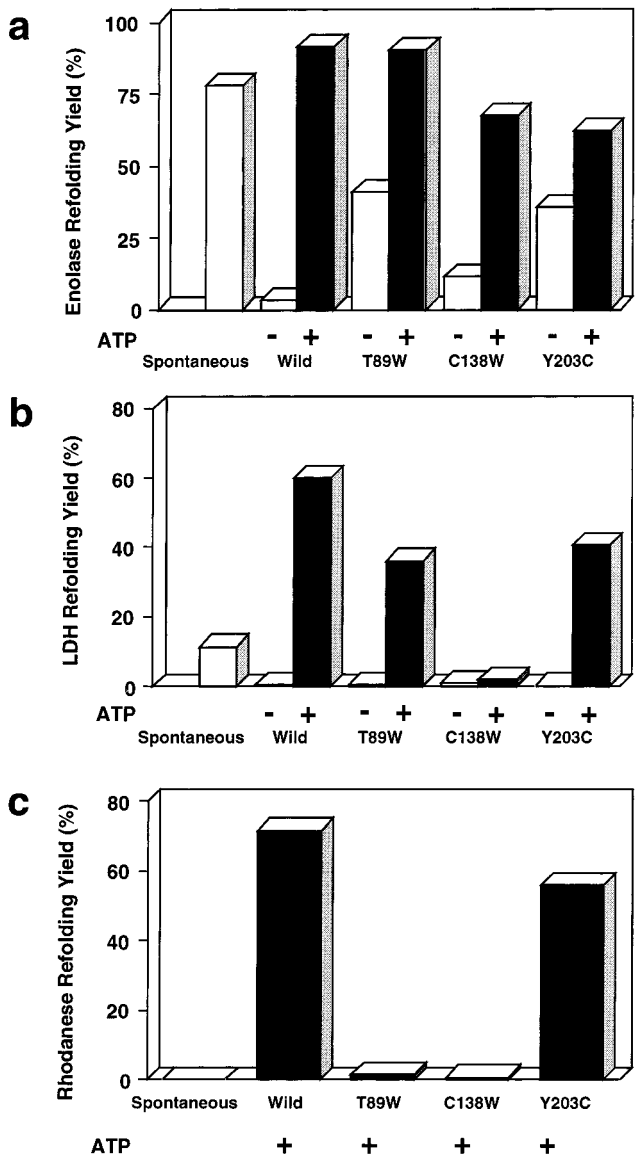

FIGURE 3: Chaperonin-facilitated refolding reaction of enolase (a), LDH (b), and rhodanese (c). The refolding reaction was carried out at 25 °C, and the final refolding activity after reaching plateau (about 20 min for enolase, 6 h for LDH, and 50 min for rhodanese) is indicated by a bar. The final protomer concentrations of enolase, LDH, and rhodanese during the reaction were 380, 50, and 460 nM, respectively. A 5-fold molar excess of chaperonin proteins (GroEL 14-mer and GroES 7-mer) was used for the refolding of enolase and LDH, and a 1.5-fold molar excess was used for the refolding of rhodanese. The concentrations of Gdn-HCl and ATP during refolding were 40−70 and 2 mM, respectively.

As shown in Figure 3a, the efficiency of binding enolase refolding intermediates in the absence of ATP was decreased in the order wild-type > C138W > Y203C = T89W, and the trapped intermediates were all released upon addition of ATP. For LDH, on the other hand, whereas all of the mutants were able to bind the refolding intermediates efficiently in the absence of ATP, the refolding yield upon addition of ATP was varied in the order wild-type > Y203C > T89W (Figure 3b). C138W was unable to facilitate refolding. With regard to rhodanese refolding under 'nonpermissive' (*23*) folding conditions (see Materials and Methods), as shown in Figure 3c, only Y203C could facilitate the refolding reaction at the same efficiency as wild-type, and T89W and C138W could not facilitate it at all. To summarize, Y203C

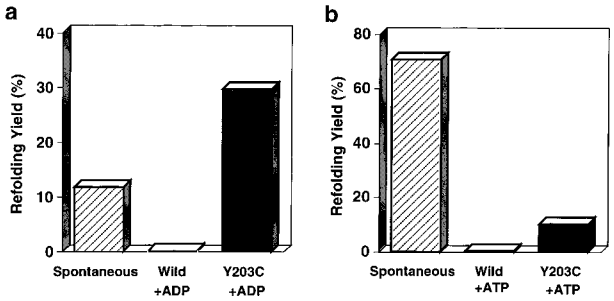

FIGURE 4: Refolding characteristics of Y203C for LDH (a) and rhodanese (b). (a) The refolding reaction was performed in the presence of a 5-fold molar excess of GroEL 14-mer and 2 mM ADP at 25 °C. (b) The refolding reaction was carried out in the presence of a 1.5-fold molar excess of GroEL 14-mer and 2 mM ATP at 25 °C under the permissive conditions as described under Materials and Methods. All other conditions were the same as in the legend for Figure 3.

was capable of refolding all of the substrate proteins in the presence of GroES and ATP, whereas T89W was able to refold enolase and LDH but not rhodanese, and C138W was able to refold only enolase. To study the different characteristics for the different substrate proteins further and also to understand the functional mechanism in terms of GroEL domains, we studied the behavior of each mutant during the folding reaction in greater detail.

Mutation Effects of Tyr203 at the Apical Domain. As shown in Figure 3, Y203C showed a weakening in affinity for refolding intermediates of enolase during the initial formation of the chaperonin complex. Y203C also displayed a difference during the release of LDH and rhodanese upon addition of nucleotides (Figure 4). For wild-type GroEL, in the absence of GroES, the refolding intermediates of LDH are released by the addition of ATP but not ADP (*17*). As shown in Figure 4a, however, Y203C released the refolding intermediates of LDH upon addition of ADP even in the absence of GroES. A similar result was observed for rhodanese (Figure 4b). As shown in the figure, under conditions where rhodanese refolded spontaneously ('permissive conditions') (Figure 4b, left), wild-type GroEL could not release the refolding intermediates of rhodanese by the addition of ATP singly (Figure 4b, center), and GroES was also required for efficient release. However, Y203C released the intermediates to a small but significant extent upon the addition of ATP only (Figure 4b, right). This result, that Y203C displayed a decreased affinity for both GroES and refolding intermediates, is probably attributed to a decrease in hydrophobicity (*16*, *24*−*27*) of the apical domain for folding protein and GroES. A similar conclusion was reached in a previous study which converted this same residue to glutamic acid (*28*). In this study, this decrease in affinity also resulted in a change in the conditions required for productive release of various folding proteins, indicating that the nucleotide specificities of various refolding proteins during chaperonin-facilitated protein folding are affected strongly by the relative affinities between the apical domain of GroEL and various refolding proteins.

Mutation Effects of Thr89 at the ATP Binding Site of the Equatorial Domain. As shown in Figure 2, T89W was found to have no ATPase activity and no GroES binding ability. We next examined whether T89W was able to bind a nucleotide but not hydrolyze it, or was unable to bind

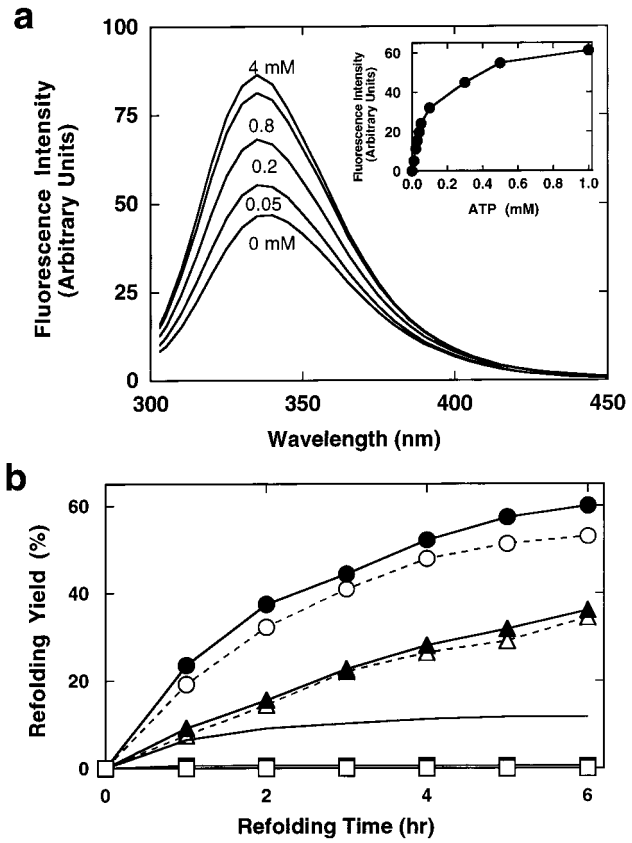

FIGURE 5: Characteristics of T89W GroEL mutant. (a) ATP binding characteristics of T89W monitored by tryptophan fluorescence changes at various concentrations of ATP. The tryptophan fluorescence spectra were measured upon excitation at 295 nm at 25 °C. The inset indicates the fluorescence changes at 340 nm as a function of the concentration of ATP. (b) Refolding reaction of LDH in the presence of a 5-fold molar excess of T89W at 25 °C. Symbols indicate as follows: wild-type GroEL only (open squares); T89W only (closed squares); wild-type GroEL, GroES, and 2 mM ATP (closed circles); T89W, GroES, and 2 mM ATP (closed triangles); wild-type GroEL and 2 mM ATP (open circles); and T89W and 2 mM ATP (open triangles). Spontaneous refolding was represented by the solid line. All other conditions were the same as in the legend for Figure 3.

nucleotides at all. As Thr89 is located in the vicinity of the γ-phosphate of ATP in the ATP binding form (*20*), we could be expected to detect a fluorescence change of the introduced tryptophan upon nucleotide binding in T89W. As shown in Figure 5a, the fluorescence intensity was increased gradually by increasing concentrations of ATP in a saturating manner. Interestingly, the ATP binding curve of T89W did not show positive cooperativity. This finding suggested that the mutation in T89W at the equatorial domain also affected various intersubunit interactions of GroEL. This interesting characteristic of T89W is presently being studied (manuscript in preparation). Although the apparent half-saturation concentration (145 μM) was slightly higher than the wild-type (*10*, *29*), this result clearly shows that this mutant has the ability to bind ATP. The apparent half-saturation concentration was not affected by the addition of an equimolar concentration of GroES 7-mer to the solution (data not shown), which also indicated the inability of this mutant to bind GroES. Therefore, T89W was a mutant where the nucleotide binding ability was apparently decoupled from further steps in the chaperonin mechanism.

Interestingly, although T89W was able to proceed only partially through the postulated chaperonin mechanism, nevertheless it was still capable of enhancing the refolding yield of certain proteins in a nucleotide-dependent manner. As shown in Figure 5b, in the absence of nucleotide, the refolding of LDH was completely arrested by this mutant. Upon addition of ATP, an efficient refolding reaction with a refolding yield greater than spontaneous refolding was observed, although the final refolding yield was lower than that mediated by wild-type GroEL. This result clearly shows that a signal triggered by ATP binding in the equatorial domain is transmitted to the apical domain to release the refolding intermediates of LDH.

Mutation Effects of Cys138 at the Intermediate Domain. Although C138W showed the same characteristics as wild-type GroEL in ATPase activity and GroES binding ability (Figure 2), C138W had no refoldase activity in the refolding reactions of LDH and rhodanese, as shown in Figure 3b and Figure 3c, respectively. To clarify this discrepancy, further detailed experiments were performed as shown below. A time-dependent refolding reaction of rhodanese in the presence of GroEL protein under 'nonpermissive' conditions is shown in Figure 6a. C138W was not able to mediate the refolding of rhodanese even in the presence of GroES and ATP, in contrast to wild-type GroEL. Surprisingly, when we measured the ATPase activity during the reaction, we found that the ATPase activity of C138W was completely arrested under these conditions (Figure 6b). The arrest of ATPase activity could also be achieved by the addition of rhodanese alone, indicating that the binding of rhodanese to C138W resulted in the simultaneous arrest of ATPase activity on both rings. Such evidence of interring communications has been obtained in other studies by monitoring the cooperativity in ATPase activity (*30*) and various conformational changes (*31*). To confirm that the refolding intermediates were trapped by the C138W mutant protein, gel-filtration of the refolding reaction mixture followed by SDS−PAGE analysis was performed. Notably, as shown in Figure 6c, it was found that under these conditions C138W formed a complex not only with rhodanese refolding intermediates but also simultaneously with GroES. The same result was obtained in experiments with LDH (data not shown).

Next, to determine the nature of the ternary complex, we utilized the fact that the cis chaperonin complex protects the refolding protein intermediates from proteinase K digestion (*32*, *33*). In the present experiment, an equivalent molar concentration of rhodanese relative to GroEL and GroES oligomers was used. After 12 min, the trapped intermediates were completely digested when GroES was absent [Figure 6d(iii)], and partially protected when GroES was present [Figure 6d(ii)]. In the presence of GroES, the quantity of rhodanese remaining after a 12 min digestion [Figure 6d-(ii)] was about half of that at 0 min [Figure 6d(i)] as determined by densitometric analysis, suggesting that the stable ternary complex of C138W−rhodanese−GroES is a 1:1 mixture of cis and trans complexes. The absence of any ATPase activity shows that both of these conformations are locked. Interestingly, when the refolding reaction was carried out at 37 °C or when the temperature was shifted from 25 °C to either 30 or 37 °C (Figure 6e), the productive refolding of rhodanese was resumed, concomitant with the restoration of GroEL ATPase activity. This indicates that the charac-

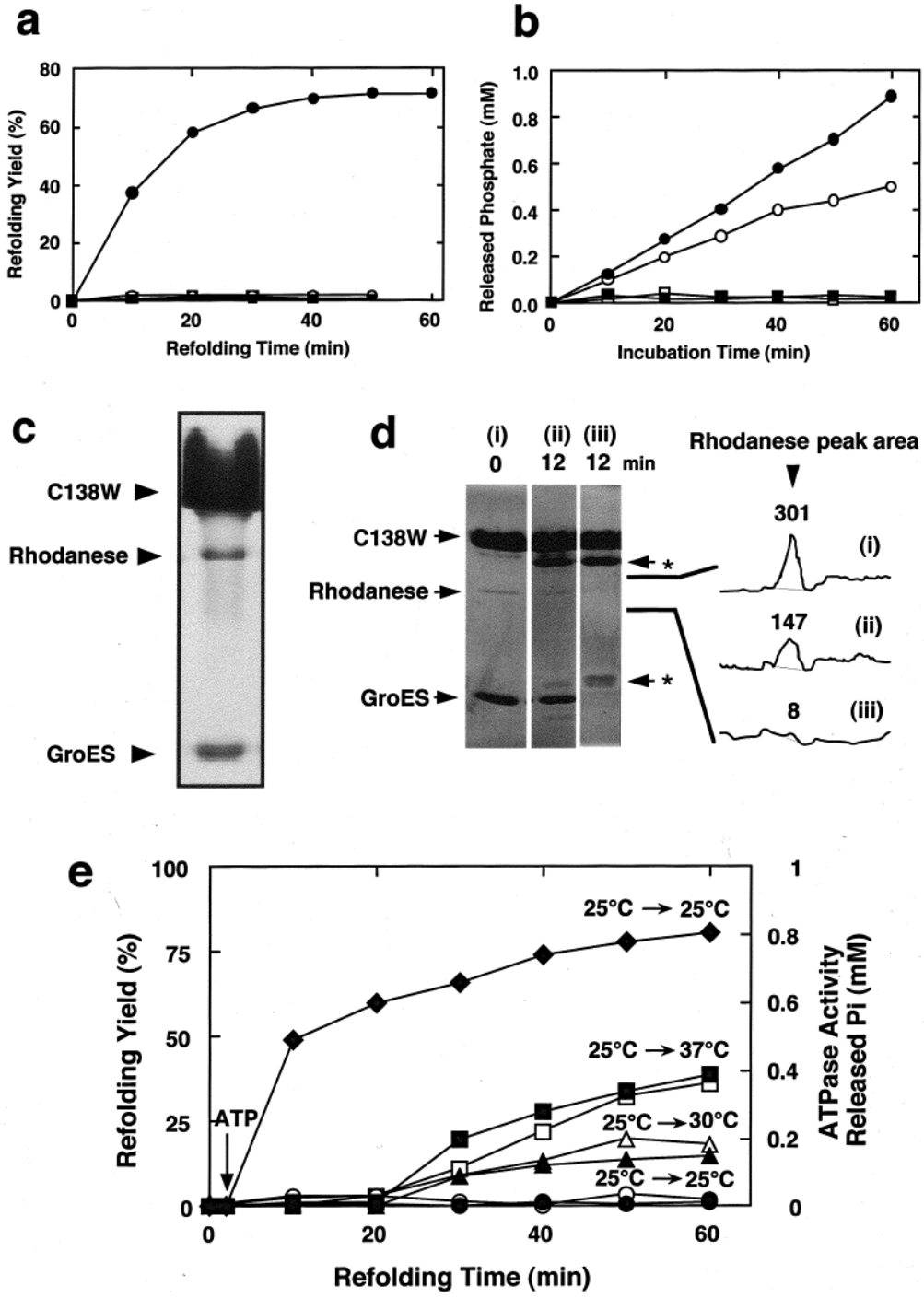

FIGURE 6: Characteristics of C138W GroEL mutant. (a) Refolding reaction of rhodanese (0.5 μM) in the presence of GroEL (14-mer) proteins (0.5 μM), GroES (7-mer) (0.5 μM), and 2 mM ATP at 25 °C. Symbols represent as follows: wild-type GroEL, GroES, and 2 mM ATP (closed circles); wild-type GroEL and 2 mM ATP (open circles); C138W, GroES, and 2 mM ATP (closed squares); and C138W and 2 mM ATP (open squares). (b) ATPase activity of C138W GroEL mutant in the presence or absence of refolding intermediates of rhodanese at 25 °C. GroEL (and GroES) oligomer (0.5 μM) and rhodanese (0.5 μM) and 2 mM ATP were used: C138W only (closed circles); C138W−GroES (open circles); C138W−rhodanese intermediates (closed squares); C138W−rhodanese intermediates−GroES (open squares). (c) SDS−PAGE of the ternary complex of C138W−rhodanese−GroES. See Materials and Methods for the detailed experimental conditions. (d) Protection experiments of C138W−rhodanese−GroES complex from proteinase K digestion. SDS−PAGE (left) and densitometric analysis around the rhodanese band (right) of the samples of the C138W−rhodanese−GroES complex at 0 time (i) and at 12 min (ii), and of C138W−rhodanese complex at 12 min (iii). The results in lanes (i) and (ii) represent aliquots obtained from a common sample at the indicated times. All three lanes are from the same gel. The bands indicated by an asterisk indicate a polypeptide derived from digestion of the C138W mutant GroEL protein (*34*). (e) Refolding of rhodanese in the presence of wild-type GroEL or C138W mutant, GroES, and ATP, and the ATPase activity during the refolding reaction when the reaction temperature was shifted from 25 °C to 30 and 37 °C. Closed symbols represent the refolding yield of rhodanese, and open symbols represent the ATPase activity of GroEL at each time point. For refolding yields, native rhodanese activity at 25 °C was set as 100%. 2 mM ATP was added at the time indicated by the arrow, and the temperature was shifted at 20 min. Wild-type GroEL with no temperature shift (diamonds); C138W with no temperature shift (circles); temperature shift to 30 °C (triangles) and 37 °C (squares). With the exception of the temperature, all assay conditions were the same as described under Materials and Methods.

teristics of C138W seen at 25 °C were not an artifact due to the mutation, but that a transient conformation of the ternary complex was observed temporarily at 25 °C by the disruption of communications between the apical and equatorial domains at the mutation site of the intermediate domain. Similar characteristics of GroEL function were also reported for GroEL chemically modified at cysteine residue(s) (*34*, *35*). However, in the NEM-modified GroEL study (*35*), the ATPase activity of the GroEL−rhodanese complex was only partially suppressed, suggesting an incompletely arrested cycle. In our experiments, the suppression observed was complete, which allowed us to characterize this previously undetected ternary complex in more detail.

DISCUSSION

The mechanism of chaperonin function that facilitates protein folding of various proteins is very sophisticated (*2*, *3*). The action involves binding of folding intermediates that are prone to aggregation and a controlled release which is regulated precisely by ATP binding- and ATP hydrolysis-dependent association and dissociation of GroES. Previous studies (*2*, *3*, *6*, *28*) have shown that numerous conformational changes in each domain of GroEL are involved in the expression of chaperonin activity and also that changes in one domain affect the function of the other two domains. In the present study, we have tried to clarify the role of each domain of GroEL, especially of the intermediate domain, in more detail, and also probed for any new evidence of communication between the domains.

Signals from the Equatorial Domain to the Apical Domain Initiate Peptide Release. Each mutant displayed significant characteristics: changing Tyr203 at the apical domain to Cys (Y203C) resulted in a decrease in affinity for refolding intermediates (Figures 3 and 4) and GroES protein (Figure 2); changing Thr89 at the equatorial domain to Trp (T89W) caused the loss of ATP hydrolysis and GroES binding abilities at the apical domain (Figures 2 and 5); and changing Cys138 at the intermediate domain to Trp (C138W) caused a conditional defect in ATPase activity such that the ATPase activity was arrested in both rings of the chaperonin when refolding intermediates were bound to the apical domain (Figure 6). These results allow various conclusions to be put forth regarding the binding of refolding protein to the apical domains of the GroEL cis-ring and the subsequent release of refolding protein into the central cavity.

First of all, from the results obtained in experiments using GroEL T89W, we deduced that for certain proteins, the binding of various nucleotides to the GroEL equatorial domain results in the release of refolding protein intermediates from the GroEL central cavity. This suggested that the binding of various nucleotides induces a signal which is transferred from the equatorial domain to the apical domain, and as a result, changes in the apical domain result in the release of protein folding intermediates. The specific requirements for the release of various folding proteins naturally differ for each substrate protein (*17*, *36*); nevertheless, we believe that the signal induced in the equatorial domain, and the subsequent changes in the apical domain, may be essentially similar for each nucleotide, with differences only in the extent of the changes induced.

Supporting evidence for this conclusion was obtained in the experiments using GroEL Y203C. Mutation of Tyr203 to a cysteine residue resulted in a GroEL subunit with a reduced affinity for GroES and refolding substrate proteins, as demonstrated in the refolding arrest experiments in Figures 3 and 4. More interesting, however, was the fact that as a result of this mutation, the nucleotide specificities regarding the release of various proteins from the GroEL cylinder were subtly altered. In the case of LDH, addition of ADP to the LDH−GroEL complex was sufficient in the mutant to allow a significant fraction of LDH molecules to be released, whereas for wild-type GroEL, protein release was observed only upon addition of ATP. Once released, a portion of the molecules were able to recover their intrinsic activities, at a yield which was significantly better than the spontaneous yield. In the case of rhodanese under permissive conditions, again the incorporation of the Tyr to Cys mutation resulted in an alteration of the conditions required for polypeptide release; addition of only ATP was sufficient to initiate refolding of about 15% of the total population of rhodanese molecules. These results strongly suggest that the conformational changes induced by the binding and/or hydrolysis of various nucleotides to GroEL are similar in nature; for instance, for wild-type GroEL, the changes induced by ATP only are insufficient to allow the effective release of rhodanese folding intermediates. Changes in the apical domain which cause a reduction in the affinity for protein folding intermediates change this situation, and now a significant portion of the refolding protein molecules may be released.

To summarize, we propose that the results obtained in the experiments on T89W and Y203C outline the nature of the nucleotide-induced release trigger of GroEL; that it is induced by nucleotide binding, as previously demonstrated elegantly by Rye et al. (*8*), and that the conformational changes triggered are essentially similar in nature for each nucleotide and different only in extent (*8*, *10*, *17*, *36*−*41*). Alteration of the substrate protein binding affinity of the apical domain is, in the case of LDH and rhodanese, sufficient to alter the nucleotide requirements for the successful refolding of each substrate protein.

Signals from the Apical Domain to the Equatorial Domain Trigger ATP Hydrolysis. A reverse transfer of signals was suggested from the results obtained by the experiments on C138W (Figure 6). Changing the cysteine residue at position 138 to a more bulky tryptophan residue resulted in a novel conditional defect in ATPase activity. In the absence of refolding protein, the characteristics of C138W in ATPase activity and GroES binding were indistinguishable from those of wild-type GroEL (Figure 2). In the presence of refolding intermediates, however, the chaperonin function was altered drastically; i.e., the mutant bound refolding intermediates at the apical domain and did not display any chaperonin activities in this state. In other words, when the refolding intermediates bound at the apical domain, ATP hydrolysis activity at the equatorial domain was completely suppressed. The fact that a mutation in the intermediate domain resulted in such a suppression clearly suggests that the transfer of a signal from the apical to the equatorial domains via the intermediate domain exists.

When we performed further experiments to determine the reasons for ATPase suppression as outlined above, we discovered a previously undetected intermediate of the GroEL−folding intermediate−GroES cis-ternary complex

(Figure 6). This intermediate was characterized by the simultaneous binding of both refolding protein and GroES to the same toroid of the GroEL 14-mer. Experiments using proteinase K demonstrated that in this ternary complex, the refolding protein was localized inside the GroEL cylinder and encapsulated by GroES. Interestingly, no ATPase activity could be detected for this cis-ternary complex, which indicated that it was locked in this static conformation at 25 °C, and, also, by raising the temperature to 37 °C, this intermediate resumed functioning to produce active refolded protein, indicating that it was not an irreversible byproduct of the mutation. We suggest that this novel intermediate is an integral part of the overall chaperonin mechanism, and represents a view of the conditions immediately prior to polypeptide release into the central cavity. Such a conformation was created previously by Martin (*35*) using chemical modification of the same Cys138 residue. In our case, the formation of the intermediate form was more efficient, and allowed us to perform some additional characterization experiments. Previously, existence of a functional communication between the Cys138 residue and the Cys519 residue that is located in the equatorial domain was also reported by single and double mutations on the residue(s) to serine residue(s) (*42*). This result also indicated that Cys138 at the intermediate domain is a critical residue for interdomain communication.

It is tempting to speculate on the reasons why such an intermediate state may be formed as a result of the C138W mutation. As determined by Xu et al. (*6*), the mutated Cys138 residue is located at the loop just before the F-helix and forms van der Waals contacts with Ala406 that is present at the end of the M-helix (Figure 1c). At the mid-position of this M-helix, Asp398, which participates in ATP hydrolysis (*8*), exists closely (within about 5 Å distance) with other important catalytic residues such as Asp52 (*43*) and Asp87 (*28*). Thus, it may be that changing Cys138 to a more bulky tryptophan residue pushes away the M-helix, resulting in a perturbation of the conformation and an arrested ATPase activity. Since the *B*-factor of the hinge region supporting the highly flexible apical domain is also rather high (*44*), the flexibility around the hinge region (Trp138 and Ala406) would be more increased at higher temperature in the mutant, and then the ATPase activity and the chaperonin cycle coupled with it would recover (Figure 6e).

Significance of the GroEL−Folding Intermediate−GroES cis-Ternary Complex. Another interesting point for discussion is the specific conformation of the apical domain in this cis-ternary complex. In the GroEL−GroES−$(ADP)_7$ structure, the accessible surface of the inside of the enlarged central cavity was changed from a hydrophobic to a hydrophilic environment by opening upward and twisting the apical domain about 90°, and this environment change at the inside of the cavity is considered to be a trigger for the dissociation of the trapped folding intermediates from the wall of the cavity (*6*). The fact that recovery of rhodanese activity was not observed for the ternary complex indicates that GroEL is still maintaining the refolding rhodanese molecule in a state of folding arrest in the central cavity; however, GroES is also bound to this form, suggesting that the apical domain is accommodating both refolding protein and the co-chaperonin in this transient state. Considering that the interactions between the apical domain of GroEL and

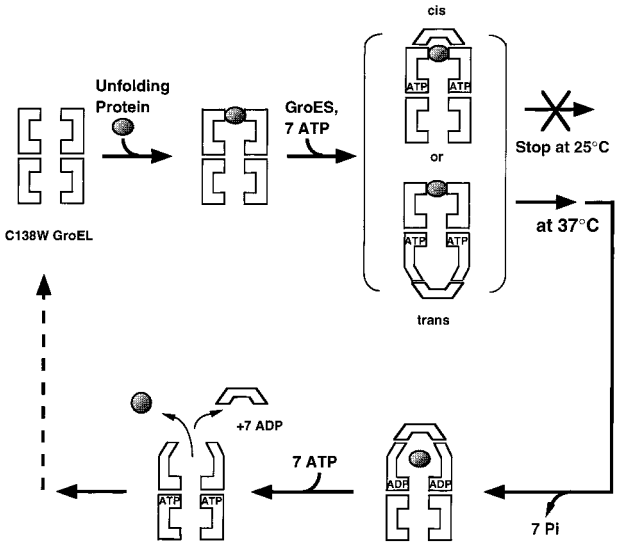

FIGURE 7: Schematic model of the formation of C138W−refolding intermediate−GroES complex at 25 °C, and a normal productive chaperonin cycle at 37 °C. The cis-complex that bound both the refolding intermediate and GroES proteins simultaneously was isolated stably only at 25 °C, and was identified to be a transient step in the chaperonin functional cycle. At 37 °C, the productive chaperonin cycle after the novel cis-ternary complex state proceeds as outlined in the introduction. It should be noted that both release of trapped intermediate and ATPase activity are resumed upon an increase in temperature.

refolding polypeptides are mediated mainly by hydrophobic interactions (*26*), it is likely that the ternary complex still has the hydrophobic environment partially localized at the inside wall; in other words, the central cavity probably has not completed its transition from a hydrophobic to a hydrophilic environment. A detailed examination of this ternary complex would, in theory, yield numerous details regarding the transfer of affinities from refolding polypeptide to GroES during the crucial process of polypeptide release.

Chaperonin Cycle. According to the results regarding C138W, the mechanism of chaperonin action that was outlined in the introduction can now be illustrated schematically in more detail by Figure 7. In the mechanism, it should be noted that in the cis-complex, both refolding intermediates and GroES protein are bound to the same apical domain of GroEL at the same time, and refolding of the intermediates is arrested in this state. The presence of this intermediate state in the general chaperonin cycle was also reported by Rye et al. (*8*) although it was detected only transiently in kinetic study. In this chaperonin cycle, it should be stressed that the binding of refolding intermediates at the apical domain of GroEL affected ATPase activity at the equatorial domain through the intermediate domain; in other words, a communication signal mediated by conformational changes that occurred at the apical domain upon binding of refolding intermediates is transmitted to the equatorial domain through the intermediate domain, and vice versa. It is the communication network mediated by the intermediate domain that is very important for the overall chaperonin mechanism.

CONCLUSIONS

We have studied the functional role of each domain of chaperonin GroEL by using three different mutants, in which a mutation is located in the apical, equatorial, and intermedi-

ate domains. With three refolding substrate enzymes having different refolding characteristics, it was found that Tyr203 at the apical domain is involved in the interactions with the refolding intermediates and GroES protein, and Thr89 at the equatorial domain is closely involved with nucleotide binding and ATP hydrolysis. A most interesting conformation was detected in experiments involving the mutation of Cys138, located at the hinge region of the intermediate domain. The C138W mutant showed normal ATPase activity and GroES binding in the absence of refolding intermediates, but once the intermediates bind to the apical domain, ATP hydrolysis reaction in the equatorial domain is halted at 25 °C. In the presence of GroES, a stable ternary complex of GroEL−refolding intermediate−GroES was formed. This novel ternary complex was reincorporated into the productive refolding cycle when the temperature was shifted to 37 °C. This disruption of the productive chaperonin cycle caused by a mutation in the intermediate domain demonstrated the presence of functional communications between the apical domain and the equatorial domain via the intermediate domain.

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