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Multiple Structural Transitions of the GroEL Subunit Are Sensitive to Intermolecular Interactions with Cochaperonin and Refolding Polypeptide

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In this study we attempted to determine the specific roles of the numerous conformational changes that are observed in the bacterial chaperonin GroEL, by performing stopped-flow experiments on GroEL R231W in the presence of a refolding substrate protein. The apparent rate of one kinetic phase was decreased by ~25% in the presence of prebound unfolded malate dehydrogenase while another phase was suppressed completely under the same conditions, reflecting different effects of the unfolded protein on multiple structural transitions within GroEL. The addition of cochaperonin GroES counteracts the effect of the bound substrate protein in the former case, but had no effect on the latter, more extensive suppression. Using a chemically modified form of GroEL R231W which is incapable of releasing substrate proteins at low temperatures, we identified a conformational transition that is implicated in the release of substrate proteins. Parts of the actual process of substrate protein release were also observed through fluorescence resonance energy transfer experiments involving GroEL and labeled substrate protein. Analysis of the energy transfer data revealed an interesting relationship between substrate protein displacement and a specific structural transition in the GroEL apical domain.

Key words: fluorescence, GroE, molecular chaperone, protein folding, stopped-flow.

Abbreviations: AEDANS-GroEL, wild type GroEL molecules that have been chemically labeled with 1, 5-IAEDANS; FRET, fluorescence resonance energy transfer; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MDH, malate dehydrogenase; 1,5-IAEDANS, *N*-iodoacetylamidoethyl-1-aminonaphthalene-5-sulfonate; NEM, *N*-ethylmaleimide; NEM-RW, GroEL R231W that has been chemically modified with NEM.

The *E. coli* chaperonin GroEL limits the accumulation of unwanted protein aggregates in the cell and assists the recovery of active proteins after cellular stress (*1–3*). Numerous studies have demonstrated that GroEL prevents the irreversible formation of insoluble protein particles by segregating proteins while still in an aggregation-susceptible state, and incubating these protein molecules within a molecular "cage" to encourage reformation of the native structure (*3*). The specific mechanism by which GroEL achieves this feat has been the subject of intensive studies (*4–7*). It has been shown that the structure of GroEL undergoes multiple transitions involving its three distinct domains to bind, encapsulate, and release targeted protein molecules. The binding and subsequent hydrolysis of the nucleotide ATP, as well as interactions with cochaperonin GroES and the target protein molecule itself, modulate these conformational changes.

Very recently, studies have focused on precise elucidation of the mechanistic aspects of GroEL-facilitated protein folding using various experimental methods based on fluorescence analysis (*8–11*). The wild type GroEL subunit lacks fluorescent tryptophan residues, and this fortuitous characteristic allows researchers to introduce unique tryptophan residues as conformational probes through site-directed mutagenesis at various locations in the three domains (apical, equatorial and intermediate) of the GroEL subunit (*8*, *11*, *12*). Taking advantage of this, we recently performed experiments to examine the numerous movements of GroEL using the mutant GroEL R231W protein (*13*). In GroEL R231W the tryptophan probe is situated in the apical domain, near the binding sites for GroES and substrate protein. The study showed that GroEL underwent three major conformational changes upon binding of the nucleotide ATP. A fourth change in fluorescence, observed only in the presence of GroES, was also observed (*13*).

The specific roles of these three conformational changes of GroEL and the characteristics of the transition seen only in the presence of GroES have not been completely elucidated, but such details are necessary to clarify the molecular mechanism underlying GroEL-facilitated protein folding. In this study, we attempted to extend our previous experimental results obtained using GroEL R231W by performing experiments in which a substrate protein was prebound to the chaperonin before the addition of ATP. We found that the apparent rates and amplitudes of two

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conformational changes were selectively affected by the presence of prebound, unfolded malate dehydrogenase (MDH) molecules. The presence of GroES, however, abolished this effect of the bound protein on one of the two phases, while the other remained affected. By utilizing the fact that chemical modification of GroEL using *N*-ethylmaleimide (NEM) results in a chaperonin with temperature-sensitive release characteristics, we found that a key kinetic transition that was triggered in the presence of GroES was essential for successful MDH release and refolding. We also took advantage of an additional experimental technique, fluorescence resonance energy transfer (FRET), to observe the changes in the interaction between GroEL R231W and a substrate protein modified with the fluorescent label *N*-iodoacetylamidoethyl-1-aminonaphthalene-5-sulfonate (1,5-IAEDANS) (*14*, *15*). We detected a change in the FRET signal efficiency that was triggered by the addition of GroES and ATP to a preformed substrate-R231W complex, and this FRET signal change indicated a displacement of the bound substrate protein from the GroEL apical domain. This change in FRET efficiency was observed only at certain temperatures for the NEM-modified chaperonin, in agreement with the tendency of this modified chaperonin to form an arrested ternary complex. Our results highlight multiple molecular events which must occur in a timely fashion to allow the efficient release of bound protein molecules from the GroEL apical domain.

EXPERIMENTAL PROCEDURES

Materials—GroEL proteins (wild type and R231W) were obtained from *E. coli* JM109 cells harboring plasmid pUCESL (*12*) or derivatives. Purification of chaperonin was performed using two anion exchange chromatography sessions in sequence (Q-Sepharose), as outlined by Mizobata *et al*. (*16*); the first elution was performed with a linear KCl gradient, and the second with a linear NaCl gradient.

NEM was purchased from Nacalai Tesque (Kyoto). 1, 5-IAEDANS was purchased from Sigma. Tris-(2-carboxyethyl)phosphine was purchased from Molecular Probes. Bovine rhodanese and nucleotides were obtained from Sigma. Pig heart MDH was obtained from Roche Molecular Biochemicals. All other reagents were obtained commercially from Wako Fine Chemicals (Osaka) and Nacalai Tesque (Kyoto).

Refolding Assays—Refolding assays of rhodanese were performed under the "non-permissive" conditions used by Martin *et al.* (*17*). Refolding assays of MDH were performed according to Hayer-Hartl (*18*).

Chemical Modification of GroEL R231W with NEM—Modification of the cysteine residues of GroEL R231W with NEM was performed according to the method initially reported by Martin (*19*), but modified slightly. GroEL R231W was incubated at a concentration of 10 mg/ml in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM NEM. The samples were incubated for 80 min at 25°C, whereupon 2-mercaptoethanol was added to a concentration of 10 mM to stop the reaction. Each sample was then dialyzed overnight against 50 mM Tris-HCl, pH 7.5, containing 0.5 g/liter acid-washed charcoal. The extent and position of the modified cysteine residue were determined by comparing samples of non-modified GroEL R231W and NEM-modified GroEL R231W (NEM-RW), which were first digested extensively with lysyl endopeptidase and then exhaustively modified with pyrenyl maleimide under reducing conditions. Samples of either GroEL R231W or NEM-RW (6 mg/ml) were incubated overnight with a 1/100 fold concentration (w/w) of *Achromobacter lyticus* AP-1 lysyl endopeptidase at 37°C. Each peptide mixture was then mixed in a 1:1 (v/v) ratio with 2 mM pyrenyl maleimide dissolved in 1,4-dioxane. Labeling with pyrenyl maleimide was performed in 50 mM Tris-HCl, pH 7.5, containing 2 mM tris-(2-carboxyethyl)phosphine, which maintains reducing conditions without quenching of the maleimide reagent. After 30 min incubation at 25°C, the reaction was quenched by the addition of 1.2 mM dithiothreitol (DTT). The two mixtures were separated on a Shiseido Capcell Pak C_{18} reversed-phase HPLC column, using the fluorescence of the pyrenyl group (excitation: 340 nm, emission: 400 nm; Jasco FP-1520S fluorescence detector) to monitor the elution profile. The two profiles were compared, and a single fluorescent peak that markedly decreased for the NEM-modified sample was identified. Fractions of this peak were collected from samples not modified with NEM and subjected to MALDI-TOF mass spectrometry analysis using a Bruker Daltonics Autoflex system and α-cyano-4-hydroxycinnamic acid. Mass spectrometry of the collected peptide peak material revealed that this fraction contained a peptide with a molecular mass of 1,303.574. This value was identical within experimental error to that of a peptide with a sequence corresponding to amino acid residues 133–142 of GroEL with a pyrenyl group attached (theoretical mass: 1,303.39). The efficiency of NEM modification was estimated to be greater than 80% by comparing the relative areas of the identified fluorescent peaks in the elution profiles for GroEL R231W and NEM-RW.

Stopped-Flow Analysis—Stopped-flow analysis of various GroEL proteins was performed on an Applied Biophysics SX-17MV stopped-flow fluorescence spectrophotometer. In experiments to monitor the tryptophan fluorescence, the excitation wavelength was 295 nm and a filter was used to monitor all emission fluorescence above 320 nm. Experiments were performed in stopped flow buffer (50 mM triethanolamine, pH 7.5, containing 20 mM $MgCl_2$ and 50 mM KCl). Protein samples were dialyzed overnight against this buffer containing 0.5 g/liter powdered acid-washed charcoal to remove any fluorescent contaminants. The concentration of GroEL during measurements was set at 0.5 mg/ml (0.62 μM GroEL 14-mer). Experiments were performed at two temperatures, 25°C and 37°C. Twelve to fourteen traces were averaged to obtain traces for kinetic analysis. Analysis of the kinetic traces was performed by non-linear least-squares analysis of selected regions in the traces using the analysis package provided by the manufacturer. Residuals of the analyses were used to confirm that the fits were appropriate.

The effects of the loaded substrate protein (Gdn-HCl denatured MDH) on the fluorescence characteristics of GroEL R231W and NEM-RW were examined as follows. Samples of MDH were denatured by incubation in 3 M Gdn-HCl for 30 min at 25°C. The denatured MDH samples were then diluted in solutions containing either GroEL

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R231W or NEM-RW. The ratio of denatured MDH monomer to native GroEL 14-mer was 1:1 at the time of mixing. This mixture was incubated at the experimental temperature (either 25°C or 37°C depending on the experiment) for 5 min and then immediately used for the stopped-flow experiments.

FRET Analysis—FRET analysis of donor GroEL molecules (GroEL R231W or NEM-RW; tryptophan as the donor) and receptor substrate protein [denatured wild type GroEL modified with 1,5-IAEDANS (AEDANS-GroEL); AEDANS as the acceptor] was performed as follows. AEDANS-GroEL was prepared by mixing samples of purified wild type GroEL (20 mg/ml) with 1 mM 1, 5-IAEDANS for 30 min at 25°C in 50 mM Tris-HCl buffer, pH 7.5. The labeling was quenched after 30 min by the addition of 10 mM 2-mercaptoethanol. Samples were then dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 g/liter acid-washed charcoal overnight. The extent of labeling was estimated using the molar extinction coefficient of AEDANS at 336 nm [5,700 cm^{-1} M^{-1} (*20*)]. The samples of AEDANS-GroEL used in the present study contained 1.7 AEDANS molecules per GroEL subunit.

AEDANS-GroEL was denatured for 2 h in 20 mM Glycine-HCl buffer, pH 2, at 25°C. Unfolding of the protein was confirmed by observing changes in the CD spectra. The unfolded sample was then mixed in a 1:25 (v/v) ratio with stopped flow buffer containing an equimolar concentration (AEDANS-GroEL monomer:native chaperonin 14-mer) of either GroEL R231W or NEM-RW. These samples were incubated for 5 min at the experimental temperature before analysis in the stopped flow apparatus. Experiments were initiated by mixing samples of preformed donor/acceptor complexes with either 1 mM ATP alone or 1 mM ATP and GroES (0.62 μM GroES 7-mer). Experiments were monitored at an excitation wavelength of 295 nm and a filter was used at the emission window to monitor all fluorescence greater than 420 nm. The values for the apparent rates and relative amplitudes with respect to different ATP concentrations were fitted to the Hill equation;

$$y = y_{max}[ATP]^n/(K_{1/2}^n + [ATP]^n)$$

where y denotes either the apparent rate or the relative amplitude, depending upon the analysis performed, y_{max} is the maximum value of the analyzed data, $K_{1/2}$ is the concentration of ATP at which y attains 50% of its value, and n is the Hill constant.

The derived kinetic constants of the major transitions displayed in each figure are summarized in Tables 1 and 2.

RESULTS

Figure 1 shows the changes in fluorescence intensity observed when GroEL R231W was mixed with 1 mM ATP at 25°C. Within the first 0.5 s of observation a total of three distinct kinetic phases may be detected in the fluorescence trace, as initially reported by us: a very rapid increase in fluorescence followed by a large decrease and then a final relatively slow increase in fluorescence. These three phases were respectively denoted as Phase A, Phase B, and Phase C by Taniguchi *et al*. (*13*), and we continue to use this notation in the present article. Of the three kinetic phases, Phase B exhibits the most

Table 1. Analyzed kinetic constants of the various fluorescence traces presented in this study: Tryptophan fluorescence.

Figure number	Sample	Phase B		Phase C		Phase D		Phase S		Other	
		Amp	*k*	Amp	*k*	Amp	*k*	Amp	*k*	Amp	*k*
Fig. 1	GroEL R231W	0.0606	61.4 ± 2.5	−0.0116	1.21 ± 0.054	0.0150 ±	0.125 ±				
	+ ATP	± 0.0017		± 0.0002		0.000114	0.0047				
	GroEL R231W			−0.0130	1.27 ± 4.3	0.0499 ±	0.175 ±	−0.0453 ±	9.43 ± 3.0		
	+ ATP + GroES			± 0.0144		0.00024	0.0020	0.020			
Fig. 2a	GroEL R231W	0.0489	52.2 ± 1.6	-0.0121	1.03 ± 0.046	n.d.	n.d.			−0.00688 ±	0.0826 ±
	+ ATP +MDH	± 0.0011		± 0.00029						0.00012	0.0078
Fig. 3a	GroEL R231W			−0.0139	1.39 ± 0.18	very small	very small	−0.0430 ±	7.45 ± 0.40		
	+ ATP +MDH			± 0.0013				0.0012			
	+ GroES										
Fig. 5a	NEM-RW, 25°C	0.00827	2.53 ± 0.15	n.d.	n.d.	n.d.	n.d.				
		± 0.00023									
	NEM-RW, 37°C	0.0234	3.71 ± 0.11	−0.0153	0.0527	n.d.	n.d.				
		± 0.0034		± 0.00019	± 0.0017						
Fig. 5b	NEM-RW			−0.0261	0.362	n.d.	n.d.	−0.0124 ±	11.3 ± 1.7		
	+ GroES, 25°C			±0.00009	± 0.0047			0.0026			
	NEM-RW + GroES									−0.0179 ±	0.182 ±
	+ MDH, 25°C									0.000072	0.0020

Values are given as derived values ± standard errors. n.d., not detected. Negative values for the relative amplitude (Amp) indicate an increase in fluorescence intensity. Constants are grouped according to the corresponding figures in the article. The "Other" column contains the apparent constants of transitions that could not be uniquely assigned to a certain phase in the base profile for GroEL R231W. See the text for details.

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Table 2. Analyzed kinetic constants of the various fluorescence traces presented in this study: FRET analysis involving AEDANS-GroEL.

Figure number	Sample	Fast phase		Slow phase	
		Amp	*k*	Amp	*k*
Fig. 6a	GroEL R231W+ES	0.00913 ± 0.00071	27.0 ± 2.04	0.00208 ± 0.0000704	1.16 ± 0.0653
Fig. 6b	NEM-RW + GroES, 37°C	0.00251 ± 0.00022	4.24 ± 0.50	0.00352 ± 0.00022	0.839 ± 0.57

Values are given as derived values ± standard errors. Constants are grouped according to the corresponding figures in the article.

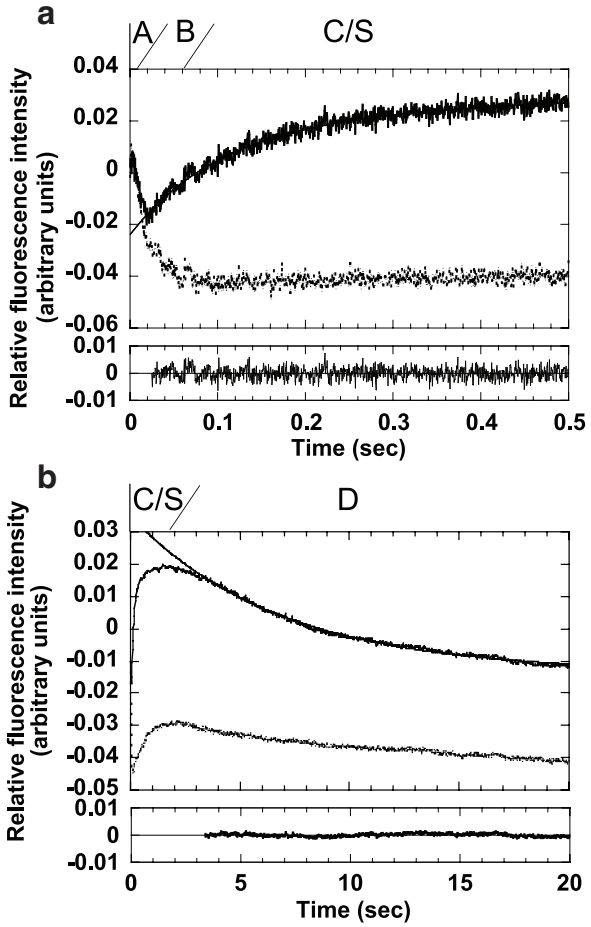

Fig. 1. **Stopped-flow fluorescence traces for GroEL R231W at 25°C.** The dotted line indicates GroEL R231W alone, and the solid line indicates GroEL R231W and an equimolar concentration of GroES. (a) Changes in fluorescence monitored for 0.5 s. The region corresponding to each individual phase is denoted above each panel. Phase A is not immediately apparent in this panel, but has been detected in similar experiments with shorter monitoring times (not shown). The initial sharp decrease in fluorescence corresponds to Phase B, and the following increase (solid line) is the double exponential trace that represents Phases C and S. (b) As in (a), but for a longer monitoring interval (20 s). Details are as in (a). Phase C (dotted line, fluorescence increase) is more prominent in this panel, and we confirmed the appearance of Phase D (fluorescence decrease) in both traces. The residuals below each panel represent deviations of the raw data from a theoretical curve derived from a double exponential fit to Phases C and S in panel a and a single exponential fit to Phase D in panel b, and demonstrate the reliability of the fits.

complex behavior with respect to ATP concentration dependence (*13*). The rate of Phase B exhibits a bisigmoidal dependence on the ATP concentration, a dependence that is retained even for single ring variants of GroEL. The rate of Phase C, on the other hand, exhibits a simple hyperbolic dependence on the ATP concentration indicating a lack of cooperative behavior for this transition (*13*).

When the experiment was monitored for longer intervals, a fourth kinetic phase, detected intermittently in our previous study (*13*) but not characterized in further detail, was detected, as shown in Fig. 1b. This phase consisted of a large relatively slow decrease in fluorescence with an apparent rate constant of 0.12 s^{-1} at 25°C in the presence of 1 mM ATP. Previously (*13*), we reported that the appearance of this phase was rather erratic and thus refrained from characterizing this phase in further detail. We have since found, however, that the appearance of this phase depended on the specific method of GroEL purification. Samples of GroEL R231W, obtained by anion exchange chromatography as described by Mizobata *et al.* (*16*), consistently exhibited this fluorescence decrease (heretofore denoted as Phase D), allowing further characterization. The fact that the detection of Phase D was dependent upon the degree of purification is significant, as discussed below.

An interesting phenomenon was observed during comparison of GroEL R231W samples purified by the two different purification methods, *i.e.*, that of Taniguchi *et al.* (*13*) *versus* that of Mizobata *et al.* (*16*). We found that the apparent rate constants and relative amplitudes of each phase (Phases A, B, and C) changed according to the purification protocol used. The apparent rates were consistently smaller for samples purified according to the protocol described by Mizobata *et al.* (*16*), compared to the samples obtained as described by Taniguchi *et al.* (*13*) [*e.g.*, for Phase B, 61 s^{-1} as opposed to ~180 s^{-1} in Taniguchi *et al.* (*13*), see also Table 1]. We have confirmed that when GroEL R231W samples purified according to Taniguchi *et al.* (*13*) were subjected to additional Q-Sepharose anion exchange column chromatography according to the procedure outlined by Mizobata *et al.* (*16*), we could obtain GroEL samples that exhibited uniformly smaller apparent rate values in the stopped-flow experiments, essentially identical to the behavior of samples purified according to Mizobata *et al.* We are currently probing the cause of this difference in more detail. The kinetic characteristics of each phase were otherwise consistent in both studies and, notably, there were no detectable differences in function between the two chaperonin preparations. Because we have found that we could observe the characteristics of Phase D more consistently, in the present paper, we report the values for samples purified solely by means of anion exchange.

In the presence of an equimolar concentration of GroES, a final additional kinetic phase in the form of a fluorescence increase was observed (heretofore denoted as Phase S, Fig. 1a, solid line). Initially, we reported that this kinetic phase directly reflected the binding of GroES to GroEL,

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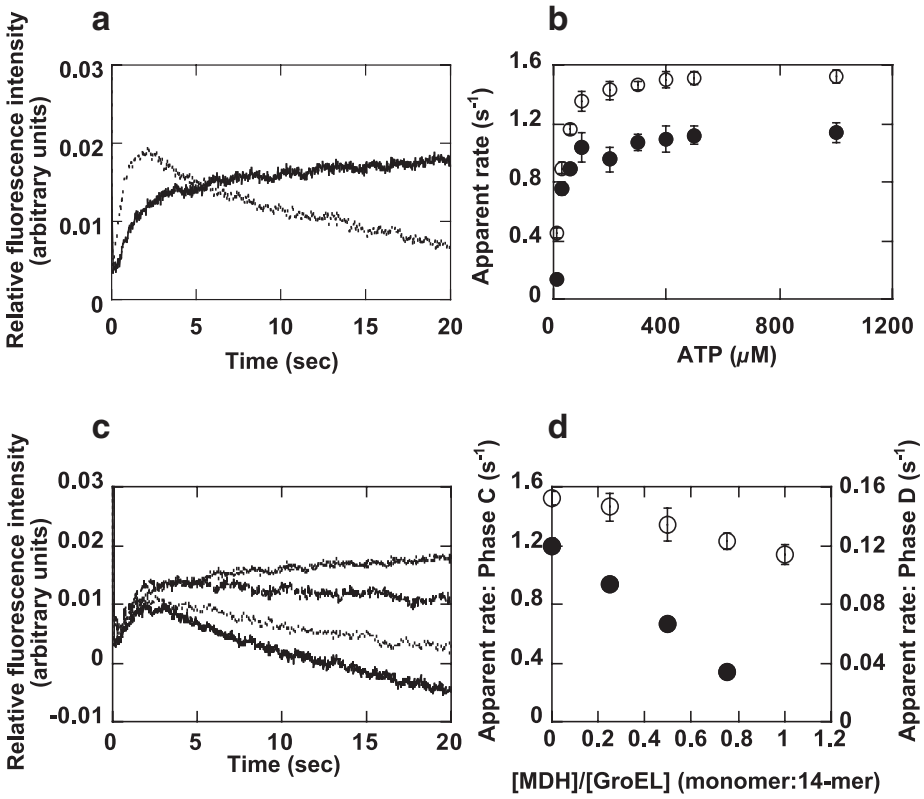

Fig. 2. **Stopped-flow fluorescence traces for GroEL R231W in the presence of prebound unfolded MDH and in the absence of GroES.** (a) Changes in the tryptophan-derived fluorescence of GroEL R231W in the absence (dotted line) and presence (solid line) of an equimolar concentration of MDH prebound. In these experiments, MDH was unfolded using 3 M guanidine hydrochloride. (b) Changes in the apparent rate of the fluorescence increase (Phase C) shown in panel (a) relative to the concentration of ATP. Open circles indicate the changes in the apparent rate of the fluorescence increase in the absence of prebound MDH, and closed circles indicate changes in the apparent rate of the fluorescence increase in the presence of equimolar MDH. (c) Titration of the prebound MDH:chaperonin ratio. The amount of MDH that was prebound to GroEL R231W was titrated toward equimolarity. The fluorescence changes were initiated by the addition of 1 mM ATP. The fluorescence values at t = 20 s, from bottom, solid line, to top; 0.25:1, 0.5:1, 0.75:1, 1:1 MDH to GroEL. The ratios were calculated as [MDH monomer]:[GroEL 14-mer]. (d) Changes in the apparent rates of Phases C (open circles) and D (closed circles) relative to the ratio of prebound MDH to GroEL. The apparent rate of Phase D at MDH:GroEL = 1:1 could not be determined due to suppression of the relative amplitude of this phase.

since the appearance of this phase was dependent on the presence of GroES in the mixture and by the fact that the apparent rate of this kinetic phase was dependent on the GroES concentration. However, we would like to correct our initial conclusion regarding this kinetic phase. Previously (*13*), we reported that the dependence of the apparent rate of this phase on the concentration of GroES was saturated at a certain value under conditions where the ratio of GroES to GroEL was greater than 1 to 1. Such behavior indicates that this kinetic phase is in fact a conformational change that is triggered by GroES binding, rather than a direct reflection of this phenomenon. In the present study, the apparent rate constant of Phase S was estimated to be 9.4 s^{-1} in the presence of an equimolar concentration of GroES at 25°C.

To summarize, for GroEL R231W, a total of five distinct changes in tryptophan fluorescence were observed that were triggered by the binding of ATP to the GroEL subunit. Of these five transitions, one (Phase S) was only observable in the presence of the co-chaperonin GroES. It should also be mentioned here that the apparent rate constants of the other four kinetic phases, A, B, C, and D, were neither increased or decreased as a result of the GroES addition, consistent with our conclusions in Taniguchi *et al.* (*13*).

Monitoring the Movements of the Apical Domain under Substrate Protein Load—Next, as can be seen in Fig. 2, we examined if the fluorescent signals of GroEL R231W were in any way affected when a substrate protein, unfolded MDH, bound to the apical domain. The experiment was facilitated by the fact that porcine MDH, like GroEL, possesses no tryptophan residues in its wild type amino acid sequence.

As can be seen in Fig. 2a, the fluorescence changes in the presence of prebound MDH differed significantly from the trace in the absence of a substrate polypeptide in the time frame corresponding to Phases C and D. Phase D was completely unobservable (Fig. 3a, solid line), and the fluorescence increase was also visibly slower in the presence of MDH. When we analyzed the fluorescence increase to obtain kinetic constants, we found that the change consisted of a fast fluorescence increase with an apparent rate constant of 1.03 s^{-1}, and a slower increase with a rate constant of 0.08 s^{-1}.

In order to determine the nature of the changes in kinetic characteristics between "unloaded" and "loaded" GroEL R231W, we performed titration experiments on the fluorescence change, with respect to the concentration of ATP and the relative proportion of bound, unfolded MDH. First of all, as can be seen in Fig. 2b, ATP titration experiments showed that the ATP concentration dependence of the apparent rate of Phase C (detected in the absence of MDH) and the fast increase in fluorescence seen in the

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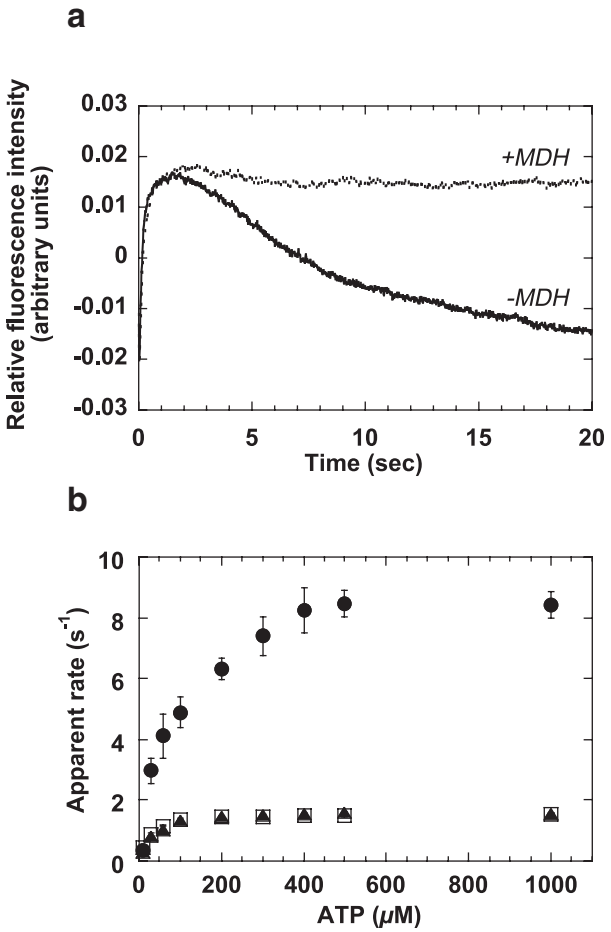

Fig. 3. **Stopped-flow fluorescence analysis of GroEL R231W in the presence of GroES and prebound MDH.** (a) Experiments were performed as in Fig. 2, but an equimolar concentration of GroES relative to GroEL R231W 14-mer was added to the ATP trigger mix. Solid lines indicate fluorescence changes in the absence of prebound MDH, and dotted lines indicate fluorescence changes in the presence of prebound unfolded MDH. (b) Titration of the apparent rates of Phase C and S with regard to the concentration of ATP, and comparison of the changes in the rate of Phase C in GroEL R231W alone. Closed circles indicate the apparent rates of Phase S in the presence of unfolded MDH, and closed triangles indicate the apparent rates of Phase C in the presence of GroES and unfolded MDH. Open squares indicate the apparent rate of Phase C when measured in the absence of both GroES and unfolded MDH. The extensive overlapping of the values (closed triangles to open squares) suggest that the presence of GroES acts to revert the characteristics of Phase C altered in the presence of MDH (Fig. 2b) to those of its original state.

presence of MDH were very similar. Both saturation curves were essentially non-cooperative in nature. The midpoints of saturation were also similar. This similarity suggested that the two changes in fluorescence were not separate events, but represented a change in the apparent rate of a single kinetic phase (Phase C) in response to the presence of unfolded MDH. The amplitude of this fluorescence phase was relatively insensitive to the presence of unfolded MDH (see Table 1).

Figures 2c and d show the differences in the fluorescence change that were observed when the ratio of unfolded MDH to GroEL 14-mer was altered. As shown in Fig. 2c, when increasing concentrations of unfolded MDH were premixed with GroEL, the relative amplitude of Phase D decreased accordingly (Fig. 2c, from bottom to top trace). Figure 2d shows a more detailed view of this titration and we can see that the rate of Phase D decreased linearly with increasing MDH concentrations toward complete suppression at a 1:1 ratio of MDH to GroEL 14-mer. In marked contrast, the rate of Phase C decreased only slightly. The incremental decrease in the apparent rate of Phase C seen in Fig. 2d supported the notion that unfolded MDH decreased the apparent rate of this phase.

Effects of Protein Load on the Conformational Changes of GroEL in the Presence of Cochaperonin—Figure 3a shows the changes in fluorescence of GroEL R231W when an equimolar concentration of MDH is first prebound, and then the chaperonin/substrate protein complex is mixed with ATP in the presence of equimolar GroES. Compared to the traces in Fig. 2, the increase in fluorescence seen at around ~1 s was much stronger in intensity. Analysis of this fluorescence increase showed that it was composed of two phases, a fast phase with an apparent rate of $7.45 \pm 0.4\ s^{-1}$, and a slower phase with an apparent rate of $1.39 \pm 0.18\ s^{-1}$. These values were nearly identical to the values for Phases S and C, respectively, seen for GroEL R231W in the absence of a preloaded protein. To confirm this, we modified the concentration of ATP used in the experiments and we found that the dependence of the apparent rate of the slow phase on ATP concentration was identical to that of Phase C seen in the absence of GroES (Fig. 3b, open squares and closed triangles). From this we believe that in the presence of an equimolar concentration of GroES, the effect of unfolded protein load on Phase C (Fig. 2b) is effectively abolished. In other words, GroES assisted GroEL in completing the molecular movements corresponding to Phase C in the presence of unfolded MDH.

Alteration of Kinetic Behavior of a Chemically Modified Variant of GroEL R231W—The next step of our study was to determine which of the numerous kinetic phases detected for GroEL R231W would be affected if the chaperonin were modified in such a manner as to prevent release of the polypeptide. This prevention of polypeptide release should be performed as delicately as possible, in order to minimize artifacts in the experiments. To this end we utilized a method involving the chemical modification of GroEL R231W with *N*-ethylmaleimide (*19*) that arrested the chaperonin cycle at a restrictive temperature (25°C), but allowed resumption of the cycle upon a temperature increase. Figure 4 summarizes the functional characteristics of this newly derived NEM-RW chaperonin. Prior to the experiments shown in Fig. 4, we confirmed that modification of GroEL R231W with the cysteine modifier NEM occurred satisfactorily, and that cysteine 138 was modified with greater than 80% efficiency (see "EXPERIMENTAL PROCEDURES"). In refolding assays of pig heart MDH and bovine rhodanese initiated at 25°C in the presence of GroEL and GroES without ATP, the recovery of protein activity was strongly suppressed in the presence of wild type GroEL, NEM-RW, and GroEL C138W, a mutant chaperonin that possesses a temperature-sensitive mechanism identical to that of NEM-modified GroEL (*12*). The addition of ATP to these samples resulted in recovery of the substrate protein activity in the case of wild type

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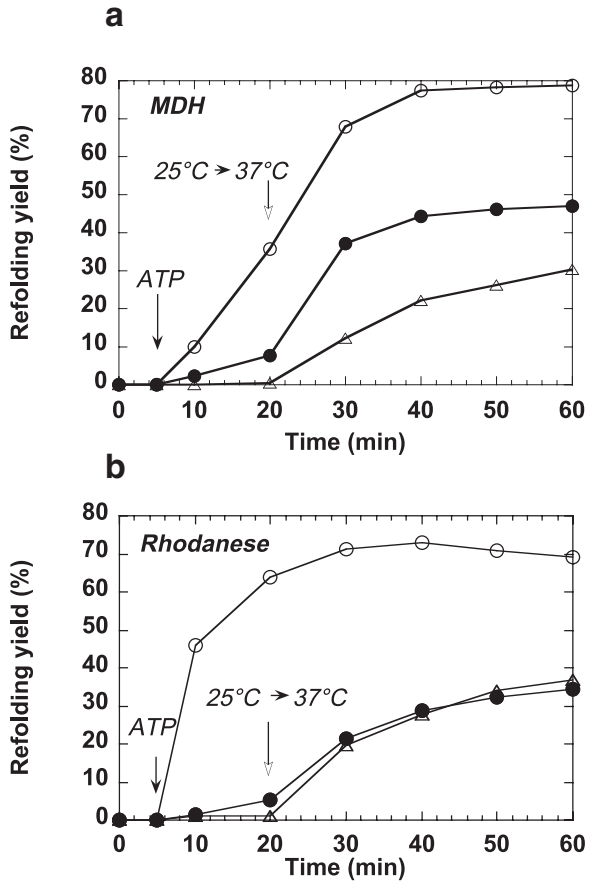

Fig. 4. **Refolding assays of (a) pig heart MDH and (b) bovine rhodanese in the presence of wild type GroEL, GroEL C138W, and NEM-RW.** The initial refolding temperature was 25°C for both proteins. Open circles indicate refolding of each substrate protein in the presence of wild type GroEL, and open triangles indicate refolding in the presence of the temperature-dependent chaperonin GroEL C138W. Closed circles represent refolding in the presence of NEM-RW. At the time indicated by the filled arrow, ATP was added to a concentration of 2 mM. At the time indicated by the open arrow, the temperature of each sample was shifted from 25°C to 37°C.

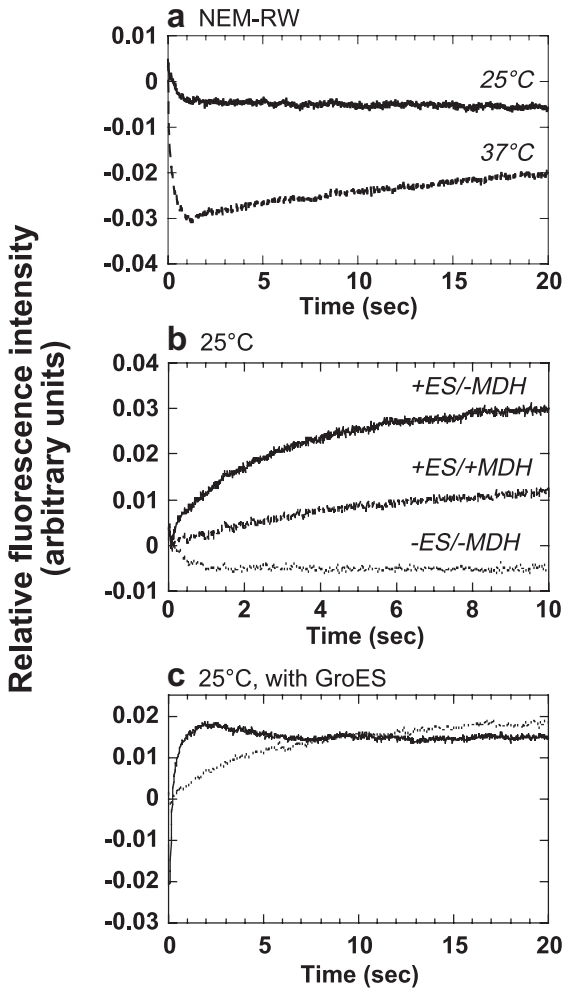

Fig. 5. **Stopped-flow fluorescence traces of NEM-RW at 25°C and 37°C in the presence and absence of GroES.** (a) Fluorescence traces of NEM-RW at 25°C and 37°C. Details are given in the figure. (b) Fluorescence changes of NEM-RW at 25°C in the absence of both GroES and MDH (–ES/–MDH), in the presence of GroES and absence of MDH (+ES/–MDH), and in the presence of both GroES and unfolded MDH (+ES/+MDH). (c) Comparison of the fluorescence traces of GroEL R231W+MDH+GroES at 25°C (solid line) and NEM-RW+MDH+GroES at 25°C (dotted line). Note that GroEL R231W is capable of releasing MDH under these conditions, whereas NEM-RW is not.

GroEL, but refolding was still suppressed for the other two samples. When the experimental temperature was raised to 37°C, the rapid recovery of both rhodanese and MDH activity was observed for the NEM-RW and C138W samples. The temperature-dependent behavior of NEM-RW was very similar to the behavior of the temperature-dependent mutant chaperonin GroEL C138W.

In addition to this experiment, we performed ATPase assays in the presence and absence of a substrate protein to confirm the formation of an arrested ternary complex. The ATPase activity of NEM-RW closely resembled that of GroEL C138W, with wild type-like activity at 37°C but suppression of ATPase activity at 25°C in the presence of an excess amount of refolding substrate protein (data not shown) (*12*). Taking this together with the results of the refolding assays, we conclude that NEM-RW was capable of forming an arrested ternary complex of GroEL, GroES and refolding protein at 25°C.

Next, experiments were performed to examine the effects of NEM modification on the fluorescence changes in GroEL R231W triggered by ATP only, in the absence of protein load. As can be seen in Fig. 5a, we found that chemical modification of GroEL R231W with NEM affected the majority of the fluorescence changes in this chaperonin. At 25°C, where the release and refolding of the unfolded protein is not allowed, the changes in fluorescence intensity consisted of an initial sharp increase corresponding to Phase A, followed by a small decrease in fluorescence. This slight fluorescence decrease may represent a greatly restricted Phase B, although it was difficult to confirm this in further analyses owing to the small amplitude of this phase. When the experimental temperature was increased to 37°C, the amplitude of this decrease in fluorescence increased four-fold. The rate of this fluorescence decrease was $3.71 \pm 0.11\ s^{-1}$, which was more than 10-fold

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smaller than the value for Phase B of the unmodified GroEL R231W chaperonin.

In the presence of GroES, however, an additional strong increase in fluorescence was observed after the initial increase (Phase A) and slight decrease (Phase B?) at 25°C, as can be seen in Fig. 5b (+ES/-MDH). This fluorescence increase was analyzed, and was found to consist of fast and slow phases. The apparent rate of the fast phase was 11.3 ± 1.7 s^{-1}, which was comparable to the rate of Phase S of unmodified GroEL R231W. Considering that this new fluorescence increase was a result of GroES addition, it seems safe to assume that we were observing Phase S in NEM-RW under these conditions as well. The rate of the slower phase was 0.362 ± 0.0047 s^{-1}, which was 1/4 of the value for Phase C.

We next performed stopped-flow fluorescence analyses with NEM-RW prebound to unfolded MDH under conditions identical to those used in Fig. 4, where refolding of MDH was arrested (25°C). As shown in Fig. 5, b and c, the addition of an equimolar concentration of MDH to NEM-RW and GroES resulted in a greatly changed fluorescence trace, compared to the case of GroEL R231W under identical conditions, in the regions corresponding to $t > 1$ s (Fig. 5b, +ES/+MDH). The fluorescence change consisted essentially of a slow fluorescence increase with an apparent rate of 0.182 s^{-1}. This slow increase does not resemble any of the kinetic phases that we have reported in this study. When we compare this trace to the trace observed for GroEL R231W under identical conditions, the differences become much more apparent (Fig. 5c). The additional presence of unfolded MDH therefore affects this phase in a negative manner, in effect suppressing either its rate or amplitude. This suppression is in turn associated with the prevention of MDH release and arrest of recovery.

FRET Analysis of Substrate–Chaperonin Interactions in NEM-RW and GroEL R231W—At what stage is the process of substrate protein release arrested as a result of NEM modification? To answer this question in detail, we surmised, from the results of previous studies by Rye *et al.* (*5*, *21*), that fluorescence resonance energy transfer might be possible between a suitably labeled substrate protein and GroEL R231W subunits. In the present experiment, we used the tryptophan residue in GroEL R231W as the donor fluorescent molecule and protein molecules labeled with AEDANS as the acceptor molecules. It should be noted that in our experiments, since the GroEL molecule consists of seven subunits arranged in a ring, there are potentially seven separate donor tryptophan molecules that are capable of FRET with a given acceptor molecule bound to a substrate protein. Such conditions preclude any quantitative estimation of the donor-acceptor distance. Therefore, the data shown here should be regarded as reflecting large-scale changes in the interactions between a bound substrate protein and the "inner rim" of the GroEL heptameric ring.

The results of experiments involving GroEL R231W as the FRET donor and acid-denatured, AEDANS-GroEL molecules as the FRET acceptor are shown in Fig. 6. When 1 mM ATP was added to preformed complexes of GroEL R231W/AEDANS-GroEL at 25°C, little change in the FRET signal was detected. In the presence of ATP and an equimolar concentration of GroES, however, a prominent decrease in the FRET signal was observed. This

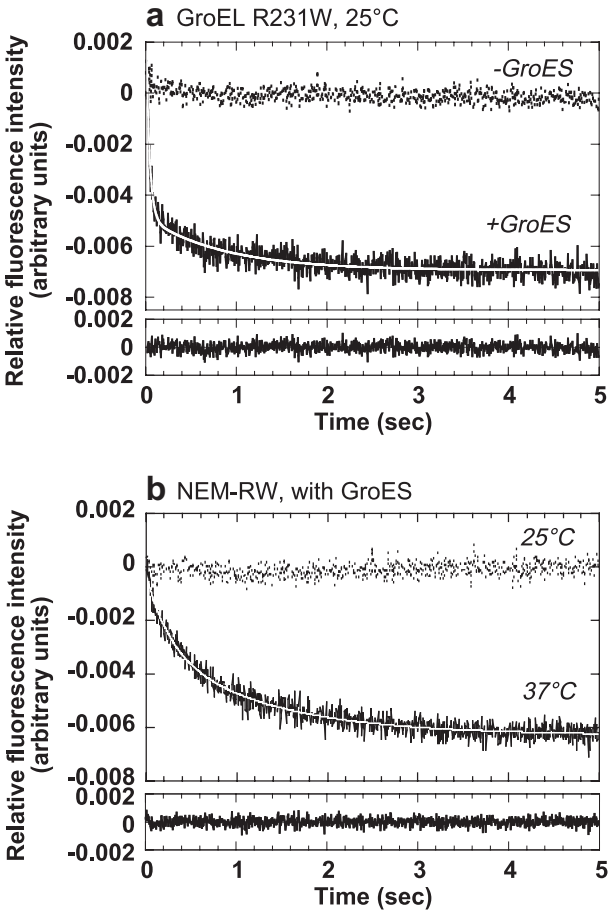

Fig. 6. **Changes in FRET efficiency between GroEL R231W and bound denatured AEDANS-GroEL.** The basic protocol is described under Experimental Procedures. (a) Changes in FRET efficiency between GroEL R231W and AEDANS-GroEL in the presence and absence of GroES at 25°C. The residuals shown below the main panel correspond to deviations from a theoretical fit (shown as a white line) to a double exponential equation for the solid line trace (GroEL R231W•AEDANS-GroEL with GroES). (b) Changes in FRET efficiency between NEM-RW and AEDANS-GroEL at two experimental temperatures, 25°C and 37°C. The residuals correspond to deviations from a theoretical fit (shown as a white line) to a double exponential equation for the solid line trace (NEM-RW•AEDANS-GroEL with GroES at 37°C).

fluorescence decrease consisted of two kinetic phases, a fast phase with an apparent rate constant of 27 s^{-1}, and a slower phase with a rate constant of 1.16 s^{-1}. At 37°C, this behavior was largely unchanged, save for an increase in the apparent rate constant of the slower phase to 7.2 s^{-1} (data not shown).

Similar experiments involving NEM-RW produced very different results. The most interesting difference was that in the case of NEM-RW changes in the FRET efficiency were detected only at 37°C (Fig. 6b), and not at 25°C. This behavior of the FRET signal resembled that of NEM-RW in refolding assays. The change in the FRET signal observed at 37°C was composed of two separate kinetic phases, a fast phase whose apparent rate constant was 4.24 s^{-1}, and a slower phase with an apparent rate constant of 0.839 s^{-1}.

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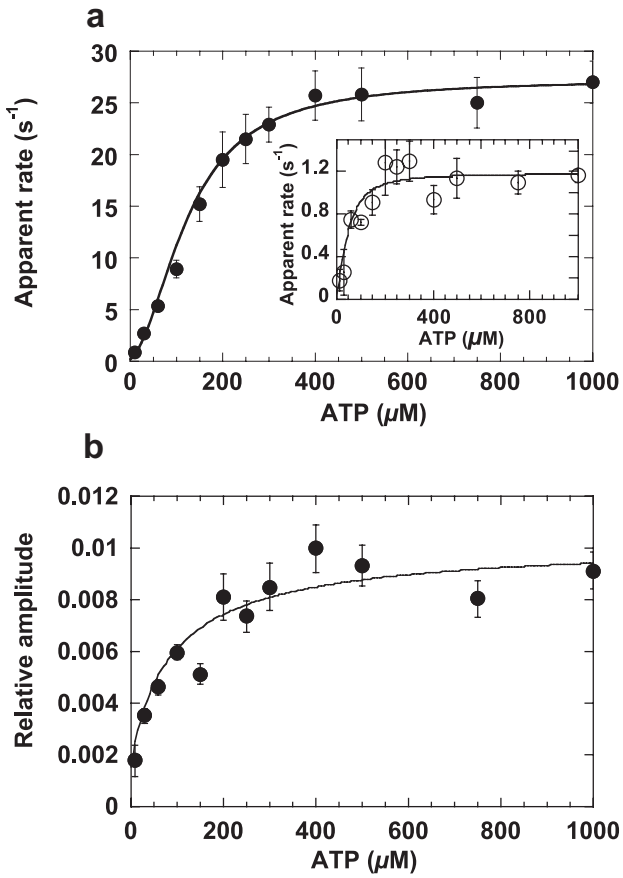

Fig. 7. **(a) Titration with respect to the ATP concentration of the apparent rates of the two FRET signal decreases shown in Fig. 6a for GroEL R231W.** Closed circles, dependence of the apparent rate of the fast FRET signal change on the ATP concentration. The inset shows the dependence of the apparent rates of the slow FRET signal change (open circles) determined simultaneously. (b) Dependence of the relative amplitude of the fast FRET signal change on the ATP concentration. Raw data values with standard errors are shown as well as theoretical curves obtained by fitting the raw data to the Hill equation, as described under "EXPERIMENTAL PROCEDURES."

In order to elucidate the characteristics of the two changes in FRET intensity observed for GroEL R231W, we again modified the concentration of ATP during the experiments. As shown in Fig. 7a, we found that the apparent rate of the fast FRET signal decrease seen in Fig. 6a exhibited a sigmoidal dependence on the ATP concentration. The amplitude, on the other hand, exhibited a simple hyperbolic dependence (Fig. 7b). When we analyzed the data shown in Fig. 7a using the Hill equation, we obtained the following values for $K_{1/2}$ and the Hill constant n: $K_{1/2} = 131 \pm 6.6$ μM, $n = 2.0 \pm 0.2$. Similar values were obtained on analysis of the slower FRET signal change ($K_{1/2} = 53 \pm 11$ μM, $n = 1.8 \pm 0.64$, Fig. 7a, inset). These values were very similar to those obtained in identical experiments involving the analysis of Phase B of GroEL R231W (*13*), more specifically, the values for the first sigmoidal transition seen in the ATP titration experiments on this phase ($K_{1/2} = 154 \pm 42$ μM, $n = 1.6 \pm 0.4$). In contrast, on similar analysis of the relative amplitude shown in Fig. 7b, we obtained values of 72 ± 32 μM for $K_{1/2}$ and 0.88 ± 0.28 for n.

DISCUSSION

Numerous studies have established that the chaperonin GroEL undergoes numerous conformational changes during the course of a single cycle of substrate protein binding, encapsulation, and release (*1*). The results of the present study will facilitate elucidation of the relative roles of individual conformational changes that are detectable using the fluorescence of a tryptophan residue introduced into the apical domain of GroEL R231W.

GroEL R231W has been characterized as a mutant chaperonin with a function that is nearly identical to that of wild type GroEL. Notable differences between the two chaperonins are the slightly increased ATPase activity of GroEL R231W relative to that of the wild type GroEL, and reduced binding affinity toward larger substrate proteins (*13*). With regard to the interactions between GroEL R231W and the substrate proteins utilized in the present study (rhodanese, MDH and wild type GroEL), the behavior of GroEL R231W was indistinguishable from that of the wild type. Therefore, we believe that extending the application of GroEL R231W to include intermolecular interactions is a valid method for deducing the general molecular characteristics of GroEL. Experiments performed in the present study in addition to those reported by Taniguchi *et al*. (*13*) have shown that a total of five conformational changes may be detected using GroEL R231W: Phases A through C were characterized by Taniguchi *et al*. (*13*) and were also detected in this study, and Phase S, previously presumed to reflect GroES binding, is more likely a result of a conformational change that is triggered by the binding of cochaperonin. An additional phase, Phase D, has been characterized in more detail. It is of interest to note here that Phase D was reproducibly detected for samples of GroEL R231W purified solely by means of anion exchange. Additionally, universal reduction of the apparent rates of all of the kinetic transitions detected was also seen for samples purified by means of anion exchange. Although we are not sure at present what causes this reduction in the apparent rates of GroEL conformational changes, we have determined that when samples purified according to Taniguchi *et al.* (*13*) were subjected to an additional anion exchange column purification, Phase D was detected reproducibly and the reduction of the apparent rates was also detected. This finding, together with the behavior of Phase D in the presence of a bound substrate protein, favors the existence of a factor present in purified GroEL samples that accelerates subunit conformational changes and suppress Phase D. However, to date, we have not succeeded in isolating this factor. We have attempted to identify this factor by adding selected fractions from the elution profile on the final anion exchange purification to purified samples of GroEL R231W in the hope of observing additive-dependent acceleration of the kinetic traces. Such experiments have so far not been successful. Attempts to use other purification methods such as dye affinity chromatography and purification in the presence of methanol have met with limited success, mainly because GroEL R231W was incapable of withstanding relatively harsh purification

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procedures (data not shown). We are presently performing further experiments, including experiments to find an explanation other than a novel binding factor to GroEL, to clarify this matter.

Effects of Unfolded Protein Loading on the Conformational Changes in GroEL—Motojima *et al.* recently succeeded in identifying a conformational change within the GroEL subunit whose apparent rate was strongly decreased in the presence of prebound unfolded protein (*22*). Intrigued by the implications of the results of Motojima and coworkers, we performed analogous experiments using GroEL R231W to see if any conformational changes detected in the apical domain were affected in a similar manner. We found that two specific transitions, Phases C and D, were specifically affected by the presence of unfolded MDH molecules. However, the effects of unfolded MDH on the two phases were different; in the case of Phase C, the effects consisted of a small decrease in the apparent rate constant (25% decrease, as determined with 1 mM ATP), and a nearly negligible effect on the relative amplitude of the phase (Table 1). ATP titration experiments showed that other characteristics of Phase C, such as the lack of cooperative behavior with regard to the ATP concentration as well as the ATP concentration at which 50 % of the apparent rate was attained, were also unchanged (Fig. 2b). The overall characteristics of the changes caused by prebound MDH pointed toward specific reduction of the rate of Phase C. In sharp contrast, as can be seen in Fig. 2, c and d, Phase D was drastically altered in the presence of unfolded MDH. Both the rate and amplitude of Phase D were strongly affected by MDH binding, and the effects consisted of outright suppression of this phase in the presence of a stoichiometric concentration of unfolded substrate protein.

The effects of the cochaperonin GroES on these changes caused by unfolded MDH were also different between Phases C and D. Phase D remained suppressed when an equimolar concentration of GroES was added, demonstrating that the effects of MDH on this phase occurred irrespective of the influence of cochaperonin. In the case of Phase C, in contrast, in the presence of GroES at an equimolar concentration the characteristics of this phase reverted to its original state, in effect increasing the rate of the phase that was retarded in the presence of unfolded MDH (Fig. 3b). This effect demonstrated eloquently a postulated function of cochaperonins; *i.e.*, that GroES is capable of assisting certain movements within the GroEL subunit to bring about completion of the chaperonin cycle.

A comparison between Phase C and other previously reported kinetic transitions detected in GroEL reveals a number of additional interesting implications. The relative value of the apparent rate of Phase C in the present study and that for the apical domain movement detected by Motojima *et al*. (*22*) are similar in the presence of unfolded MDH [$\sim$1.1 s^{-1} for Phase C, compared to 1.8 s^{-1} for Motojima *et al.* (*22*)]. The dependence of the rates on the ATP concentration, however, seemed to be slightly different. In the case of Phase C, the apparent rates were saturated at a relatively low concentration of ATP ($\sim$50 μM) compared to the phase reported by Motojima *et al*. In addition, a crucial difference between the two kinetic transitions lay in the fact that the apparent rate of Phase C in the present study recovered to its original value in the presence of GroES (Fig. 3b). This characteristic has not been reported previously, and thus may be a characteristic unique to Phase C in the present study. The characteristics of Phase C are consistent with a subunit movement, which is non-cooperative in nature, and whose rate was decreased in the presence of the bound unfolded protein, but whose completion was facilitated by the binding and influence of the cochaperonin GroES.

With regard to Phase D, the characteristics point toward a different phenomenon. Since the presence of the unfolded protein was sufficient to completely suppress this kinetic phase, it is safe to assume that this phase is intimately related to the initial substrate protein binding. The fact that subsequent GroES addition did not affect this suppression seemed to indicate that this conformational change was related to the initial binding interaction between the substrate protein and the GroEL apical domain, and separate from subsequent interactions with cochaperonin. In the present study, the maximum apparent rate value of Phase D approximated that of ATP hydrolysis [$\sim$0.12 s^{-1} (*23*)], however, ATP hydrolysis occurs irrespective of the presence or absence of Phase D. Therefore, the characteristics of Phase D are consistent with a conformational change that is only peripherally related to the overall mechanism, possibly restricted to only the apo form (no additional factors bound) of the GroEL subunit, and involving intermolecular interactions between the GroEL apical domain and the unfolded polypeptide.

Constricted Movement in a Chemically Modified Chaperonin—Modification of GroEL R231W with NEM resulted in a chaperonin with a temperature-sensitive function, as shown by the refolding assays in Fig. 4. The chemical modification is essentially identical in effect to site-directed mutagenesis of the intermediate domain cysteine 138 (*12*), and experiments confirmed that NEM indeed modified this residue with sufficient specificity (see Experimental Procedures). When NEM-RW was subjected to stopped-flow analysis, we found that essentially all of the kinetic transitions, with the exception of Phases A and S, were strongly affected by the chemical modification. The changes ranged from large decreases in the apparent rates and amplitudes in the cases of Phases B and C, to complete suppression in the case of Phase D. We note, however, that regardless of the fact that conformational changes detected for GroEL R231W were so severely affected by NEM modification, NEM-RW nevertheless exhibited functional characteristics that were nearly identical to those of the wild type chaperonin at 37°C. It may be that in NEM-RW, the environment in the immediate vicinity of tryptophan 231 is decoupled somewhat from the conformational changes that occur at other locations of the GroEL subunit. Experiments involving fluorescent probes located at different positions within the GroEL subunit may shed light upon this matter.

We would like to point out, however, that we did observe a single kinetic phase that changed its characteristics strongly in response to the experimental temperature (when the temperature was shifted from 25°C to 37°C). A fluorescence decrease reminiscent of Phase B in GroEL R231W was seen to increase the relative amplitude four-fold when the temperature was shifted from 25°C to 37°C. In similar experiments using GroEL R231W instead of NEM-RW, we observed at most only a two-fold increase in the amplitude of Phase B as a result of the temperature

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increase, and the large four-fold increase in amplitude seen for NEM-RW could not be explained by a general effect of temperature. We believe that this fluorescence decrease reflects temperature-dependent "switching" between chaperonin cycle arrest and resumption. Although additional experiments need to be performed to prove definitively that this fluorescence decrease corresponds to Phase B in GroEL R231W, circumstantial evidence supports this notion. In addition, an interesting implication regarding this temperature-dependent phase was revealed in the experiments shown in Figs. 6 and 7, which is discussed below.

With regard to Phase S, we determined, as shown in Fig. 5b, that the characteristics of this fluorescence change were at first glance unchanged by NEM modification. However, interestingly, we found that the characteristics of Phase S in the presence of unfolded MDH were significantly changed for NEM-RW. As shown in Fig. 5b, when an equimolar concentration of unfolded MDH was bound to NEM-RW prior to mixing with GroES and ATP, we observed that the fluorescence increase corresponding to Phase S was greatly suppressed. When we compared the fluorescence increase with the corresponding increase for unmodified GroEL R231W, as shown in Fig. 5c, the difference became much more apparent. In Fig. 5c we can see that both the amplitude and the apparent rate of the fluorescence increase were greatly suppressed for NEM-RW. Since MDH remains bound in an arrested ternary complex with NEM-RW and GroES under these conditions, this decreased amplitude and rate of the fluorescence change reflect a certain restriction in the molecular movement of GroEL that leads toward substrate protein release.

Logically, the next step of this study would be to perform experiments identical to those shown in Fig. 5c, but at 37°C, to see if the rate and amplitude of the fluorescence increase recover. When we performed such experiments, however, we found that the fluorescent characteristics of GroEL R231W in the presence of GroES at 37°C exhibited an additional heretofore unsuspected level of complexity (data not shown). At present, we are unable to provide details of the differences in chaperonin-cochaperonin interactions that may occur at 25°C as opposed to 37°C. However, in this context we note that Llorca and coworkers have documented the existence of a thermal switch that functions in the GroEL molecule and is linked to changes in functional capacity (*24*). An exciting possibility is that we may be witnessing such a switch in action in our experiments. Details of this phenomenon await further analysis, however.

Observation of Substrate Protein Release from the Apical Domains of GroEL R231W and NEM-RW Using FRET—The relative position of tryptophan 231 allowed us to perform additional experiments to examine further the process of substrate protein release from the GroEL apical domain, using intermolecular FRET as an experimental tool (*21*). Initially it had been our intention to use MDH, the substrate protein utilized in Figs. 2 and 3, as the substrate protein in the FRET experiments as well. However, preliminary experiments showed that the FRET signal strength for labeled MDH molecules and GroEL R231W was too small to allow detailed characterization. Subsequently, wild type GroEL, a larger polypeptide which also lacks native tryptophan residues, was selected as a suitable substrate protein (albeit slightly confusing from a nomenclature point of view). As can be seen in Fig. 6a, when denatured wild type GroEL molecules fluorescently labeled with AEDANS were bound to GroEL R231W, it was possible to detect the transfer of fluorescence energy from the tryptophan donor to the AEDANS acceptor, and the efficiency of this FRET changed when GroES and ATP were added. The following results were confirmed in our experiments. First of all, the displacement of bound substrate protein from GroEL consisted of both a fast (27 s^{-1}) and a slow (1.16 s^{-1}) transition (Table 2). Second, this change in FRET efficiency was not observed in the absence of cochaperonin, which implied an active role of GroES in the displacement of the bound substrate protein. Third, although this change in FRET was observed for both GroEL R231W and NEM-RW, changes in the FRET intensity for NEM-RW were observed only under conditions where the bound substrate protein was actually released from GroEL and enzymatic activity was recovered (Fig. 6b). Finally, and most interesting of all, titration of the faster ($\sim$27 s^{-1}) FRET signal transition in GroEL R231W with respect to the ATP concentration (Fig. 7) showed that the apparent rate of this transition was dependent on the ATP concentration in a cooperative manner, the estimated Hill constant being 2.0. This value of the Hill constant, n, and also the concentration at which the apparent rate achieved 50% of its maximum value, $K_{1/2}$ (130 μM), suggested a correlation between this intermolecular phenomenon and a specific kinetic transition observed in the GroEL apical domain, Phase B. As described by Taniguchi *et al.* (*13*), Phase B was unique among the detected kinetic transitions in that only Phase B exhibited cooperative behavior with respect to ATP. Also, Phase B was interesting in that this cooperative behavior was bisigmoidal in nature, with two separate values of $K_{1/2}$ and n, each corresponding to a certain maximum rate value. Although the characteristics of this peculiar phase have not been elucidated to satisfaction, we believe that Phase B in GroEL R231W represents a conformational change involving multiple GroEL subunits that rearrange themselves in response to ATP binding. The values for $K_{1/2}$ and n for the curve in Fig. 7a were very similar to the values for the first sigmoidal transition in Phase B (*13*).

Although the actual values of the maximum apparent rate differed, the similarities in these two values seem to indicate the existence of a common structural basis for these molecular events. This notion is entirely reasonable, since cooperative movement of the subunits of the GroEL ring would no doubt significantly alter interactions between the GroEL apical domain and the bound substrate protein. We believe that we have, for the first time, observed these specific changes in interaction. It is interesting to note that in our experiments involving NEM-RW (Figs. 5a and 6b), we found that the changes in FRET signals and the decrease in tryptophan-derived fluorescence putatively assigned to Phase B both exhibited temperature-dependent characteristics. This finding lends support to the idea put forth above, *i.e.*, that these two experimental results were intricately related to each other. Exactly how these two events are related, however, remains to be clarified.

Numerous previous studies have revealed a number of molecular events that occur within this timeframe. In

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addition to the power stroke event documented by Motojima *et al*. (*22*), Rye *et al*. have documented changes in the fluorescence anisotropy of GroEL-bound Rubisco upon the addition of ATP and GroES (*25*). The $t_{1/2}$ of the change in anisotropy observed was ~1 s, corresponding to an apparent rate constant of 0.7 s^{-1}. In addition, Weissman and coworkers detected changes in the fluorescence anisotropy of pyrene-labeled rhodanese bound to GroEL when this complex was mixed with ATP and GroES (*7*). The change in fluorescence anisotropy consisted of an initial very rapid event (that occurred during the 10 ms dead time for the experimental apparatus) and two kinetic transitions with $t_{1/2}$ of ~1 s and ~5 s, respectively. The two transitions that we detected in our FRET experiments correspond to two molecular events with $t_{1/2}$ of 0.03 s and 0.24 s, respectively (for GroEL R231W). Judging from the differences in the half times of the reaction, and also by the cooperative characteristics of the transitions detected in our experiments, we believe that the transitions documented in Figs. 6 and 7 are distinct from previously reported events.

What exactly is happening in the kinetic transitions that are reflected by the FRET efficiency changes between GroEL R231W and the unfolded substrate protein, AEDANS-GroEL? Although we could not address this question by actual determination of the donor-acceptor distance of the two fluorophores, some observations may nevertheless be made. For the reasons noted above it seems reasonable to conclude that this event most likely reflects a change in the interaction between the apical domain of GroEL and the bound polypeptide. In addition we also believe that the actual release of the substrate protein into either the central cavity or the bulk medium does not depend solely on the completion of these two transitions, as other kinetic transitions [*e.g.*, Phase C in the present study, as well as the power stroke of Motojima *et al*. (*22*)] exhibited characteristics that were altered in the presence of the substrate protein. At present we propose that the two transitions detected in the present study reflect displacement of the substrate protein that involves multiple subunits of GroEL and the active participation of cochaperonin GroES, and which precedes the actual discharge of the substrate protein into the GroEL central cavity. The actual release of the protein from the chaperonin ring most likely occurs through a series of molecular events that include both of these kinetic transitions. Additional data regarding this process should be obtained through experiments involving donor probes located at different positions in GroEL (within the chaperonin chamber, for example), followed by correlation of the results to obtain an overall picture. Also, it would be interesting to see if any differences in the fluorescence characteristics occur when substrate proteins with different molecular weights are used in the FRET experiments. Hints of such differences have already been obtained, *e.g.*, the differences in FRET signal strength between MDH and wild type GroEL substrate proteins.

Figure 8 summarizes the results of our experiments, in the form of "grouping" of various individual kinetic transitions based upon their characteristics. Adjacent to the scheme are illustrations that we believe represent plausible conformational movements of GroEL that best fit the characteristics of each kinetic transition identified in this study. Regarding the question of a specific sequence of molecular events that may dictate the execution of these numerous kinetic transitions, our experiments have not provided us with evidence favoring a rigid sequential mechanism, and attempts to fit the raw data to various forms of sequential mechanisms using the program DYNAFIT (*26*) have not yielded satisfactory results to date. However, the experiments involving NEM-RW have shown us that the kinetic transitions may be divided into groups according to their altered characteristics on perturbation, and from this a number of hints regarding this facet of the molecular cycle have been obtained. We hope that through further experiments we will be able to clarify these aspects of the molecular mechanism of GroEL.

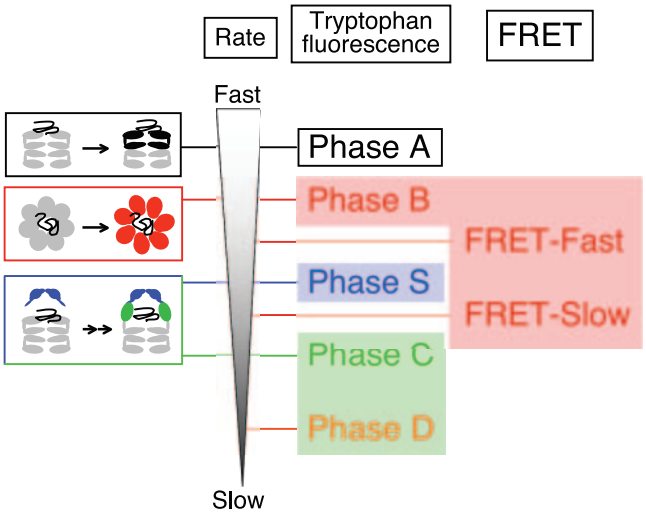

Fig. 8. **Summary of the results presented in this study.** The individual phases are arranged horizontally in descending order of apparent rates, and also arranged in columns according to phases detected by means of tryptophan fluorescence and intermolecular FRET. Shaded boxes indicate groups of phases which share a common characteristic. Phase S, shaded in blue, is correlated with substrate protein release in NEM-RW, and green shaded Phases C and D are sensitive to the presence of bound unfolded protein. The characteristics of Phases C and D differ with regard to the effects of both the unfolded protein (MDH) and GroES, and therefore are thought to reflect different aspects of GroEL. Red shaded phases are phases that exhibit cooperative behavior with respect to the ATP concentration, and share similar $K_{1/2}$ and n values. These last three phases are also correlated with temperature-dependent substrate protein release in NEM-RW. To the left of the scheme, illustrations that represent conformational changes of GroEL that best fit the characteristics of each kinetic transition are shown. The illustrations are colored according to the coloring scheme used for the phase groups shown on the right. Phase A reflects the initial conformational changes brought about by ATP binding (*13*), Phase B and the two transitions detected by means of FRET probably involve rearrangement of subunit interactions within the GroEL ring, owing to their cooperative nature, and Phases S and C are most likely involved in displacement of substrate protein molecules that are initially bound to the apical domain into the central cavity, with the active participation of GroES. Phase D is regarded to be an indirect conformational change that may be due to the apo form of GroEL (see main text), and so is not specifically illustrated here.

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REFERENCES

1. Fenton, W.A. and Horwich, A.L. (2003) Chaperonin-mediated protein folding: fate of substrate polypeptide. *Q. Rev. Biophys.* **36**, 229–256
2. Hartl, F.U. and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**, 1852–1858
3. Saibil, H.R. and Ranson, N.A. (2002) The chaperonin folding machine. *Trends Biochem. Sci.* **27**, 627–632
4. Ranson, N.A., Farr, G.W., Roseman, A.M., Gowen, B., Fenton, W.A., Horwich, A.L., and Saibil, H.R. (2001) ATP-bound states of GroEL captured by cryo-electron microscopy. *Cell* **107**,869–879
5. Rye, H.S., Roseman, A.M., Chen, S., Furtak, K., Fenton, W.A., Saibil, H.R., and Horwich, A.L. (1999) GroEL-GroES cycling: ATP and nonnative polypeptide direct alternation of folding-active rings. *Cell* **97**, 325–338
6. Weissman, J.S., Kashi, Y., Fenton, W.A., and Horwich, A.L. (1994) GroEL-mediated protein folding proceeds by multiple rounds of binding and release of nonnative forms. *Cell* **78**, 693–702
7. Weissman, J.S., Rye, H.S., Fenton, W.A., Beechem, J.M., and Horwich, A.L. (1996) Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. *Cell* **84**, 481–490
8. Cliff, M.J., Kad, N.M., Hay, N., Lund, P.A., Webb, M.R., Burston, S.G., and Clarke, A.R. (1999) A kinetic analysis of the nucleotide-induced allosteric transitions of GroEL. *J. Mol. Biol.* **293**, 667–684
9. Inobe, T., Arai, M., Nakao, M., Ito, K., Kamagata, K., Makio, T., Amemiya, Y., Kihara, H., and Kuwajima, K. (2003) Equilibrium and kinetics of the allosteric transition of GroEL studied by solution X-ray scattering and fluorescence spectroscopy. *J. Mol. Biol.* **327**, 183–191
10. Mizobata, T., Kawagoe, M., Hongo, K., Nagai, J., and Kawata, Y. (2000) Refolding of target proteins from a "rigid" mutant chaperonin demonstrates a minimal mechanism of chaperonin binding and release. *J. Biol. Chem.* **275**, 25600–25607
11. Yifrach, O. and Horovitz, A. (1998) Transient kinetic analysis of adenosine 5′-triphosphate binding-induced conformational changes in the allosteric chaperonin GroEL. *Biochemistry* **37**, 7083–7088
12. Kawata, Y., Kawagoe, M., Hongo, K., Miyazaki, T., Higurashi, T., Mizobata, T., and Nagai, J. (1999) Functional communications between the apical and equatorial domains of GroEL through the intermediate domain. *Biochemistry* **38**, 15731–15740
13. Taniguchi, M., Yoshimi, T., Hongo, K., Mizobata, T., and Kawata, Y. (2004) Stopped-flow fluorescence analysis of the conformational changes in the GroEL apical domain: relationships between movements in the apical domain and the quaternary structure of GroEL. *J. Biol. Chem.* **279**, 16368–16376
14. Hudson, E.N. and Weber, G. (1973) Synthesis and characterization of two fluorescent sulfhydryl reagents. *Biochemistry* **12**, 4154–4161
15. Kawata, Y. and Hamaguchi, K. (1991) Use of fluorescence energy transfer to characterize the compactness of the constant fragment of an immunoglobulin light chain in the early stage of folding. *Biochemistry* **30**, 4367–4373
16. Mizobata, T., Akiyama, Y., Ito, K., Yumoto, N., and Kawata, Y. (1992) Effects of the chaperonin GroE on the refolding of tryptophanase from *Escherichia coli*. Refolding is enhanced in the presence of ADP. *J. Biol. Chem.* **267**, 17773–17779
17. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A.L., and Hartl, F.U. (1991) Chaperonin-mediated protein folding at the surface of groEL through a 'molten globule'-like intermediate. *Nature* **352**, 36–42
18. Hayer-Hartl, M. (2000) Assay of malate dehydrogenase. In *Chaperonin Protocols (Methods in Molecular Biology, vol.140)* (Schneider, C., ed.) Humana Press, Totowa, NJ
19. Martin, J. (1998) Role of the GroEL chaperonin intermediate domain in coupling ATP hydrolysis to polypeptide release. *J. Biol. Chem.* **273**, 7351–7357
20. Haugland, R.P. (2002) *Handbook of Fluorescent Probes and Research Products*, 9th ed.
21. Rye, H.S. (2001) Application of fluorescence resonance energy transfer to the GroEL-GroES chaperonin reaction. *Methods* **24**, 278–288
22. Motojima, F., Chaudhry, C., Fenton, W.A., Farr, G.W., and Horwich, A.L. (2004) Substrate polypeptide presents a load on the apical domains of the chaperonin GroEL. *Proc. Natl. Acad. Sci. USA* **101**, 15005–15012
23. Jackson, G.S., Staniforth, R.A., Halsall, D.J., Atkinson, T., Holbrook, J.J., Clarke, A.R., and Burston, S.G. (1993) Binding and hydrolysis of nucleotides in the chaperonin catalytic cycle: implications for the mechanism of assisted protein folding. *Biochemistry* **32**, 2554–2563
24. Llorca, O., Galan, A., Carrascosa, J.L., Muga, A., and Valpuesta, J.M. (1998) GroEL under heat-shock. Switching from a folding to a storing function. *J. Biol. Chem.* **273**, 32587–32594
25. Rye, H.S., Burston, S.G., Fenton, W.A., Beechem, J.M., Xu, Z., Sigler, P.B., and Horwich, A.L. (1997) Distinct actions of cis and trans ATP within the double ring of the chaperonin GroEL. *Nature* **388**, 792–798
26. Kuzmic, P. (1996) Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV proteinase. *Anal. Biochem.* **237**, 260–273

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