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GroEL-GroES Cycling: ATP and Nonnative Polypeptide Direct Alternation of Folding-Active Rings

Hays S. Rye,*† Alan M. Roseman,‡‖ Shaoxia Chen,‡ Krystyna Furtak,*† Wayne A. Fenton,† Helen R. Saibil,‡ and Arthur L. Horwich*†§

*Howard Hughes Medical Institute
†Department of Genetics
Yale School of Medicine
New Haven, Connecticut 06510
‡Department of Crystallography
Birkbeck College
Malet Street
London WC1E 7HX
United Kingdom

Summary

The double-ring chaperonin GroEL mediates protein folding in the central cavity of a ring bound by ATP and GroES, but it is unclear how GroEL cycles from one folding-active complex to the next. We observe that hydrolysis of ATP within the *cis* ring must occur before either nonnative polypeptide or GroES can bind to the *trans* ring, and this is associated with reorientation of the *trans* ring apical domains. Subsequently, formation of a new *cis*-ternary complex proceeds on the open *trans* ring with polypeptide binding first, which stimulates the ATP-dependent dissociation of the *cis* complex (by 20- to 50-fold), followed by GroES binding. These results indicate that, in the presence of nonnative protein, GroEL alternates its rings as folding-active *cis* complexes, expending only one round of seven ATPs per folding cycle.

Introduction

The correct folding of many proteins in prokaryotes and eukaryotes requires the action of large protein structures known as chaperonins (for review, Sigler et al., 1998). GroEL, the chaperonin of *Escherichia coli*, is composed of 14 identical 57 kDa subunits forming two heptamer rings, stacked back to back, each with a large central cavity. Recent structural and functional studies have provided insight into how GroEL assists the folding of a wide variety of proteins. The apical cavity surface of an open GroEL ring is lined with hydrophobic residues, which bind nonnative polypeptides and collapsed protein folding intermediates that expose regions of hydrophobic surface (Fenton et al., 1994; Itzhaki et al., 1995; Lin et al., 1995; Buckle et al., 1997). Subsequent binding of ATP and the cochaperonin GroES (a ring of seven 10 kDa subunits) to the same ring occupied by a nonnative protein produces a dramatic conformational change of the bound ring, forming a *cis*-ternary complex (Chen et al., 1994; Weissman et al., 1995; Roseman et al., 1996). This structural rearrangement moves the apical binding surface up and away from the central cavity, displaces the substrate polypeptide into the enlarged cavity, and initiates folding (Weissman et al., 1996; Rye et al., 1997; Xu et al., 1997). As an additional feature of *cis* complex formation, the walls of the cavity become hydrophilic, favoring burial of nonpolar surface in the substrate protein and promoting folding to the native state.

The *cis*-ternary complex formed in the presence of ATP is very stable and cannot dissociate until the ATP bound by the *cis* GroEL subunits is hydrolyzed. Hydrolysis weakens the interaction between GroES and the GroEL apical domains of the *cis* ring and primes the *cis* chamber for dissociation. Binding of ATP to the opposite ring (the *trans* ring) then triggers disassembly of the weakened *cis* complex, releasing GroES, ADP, and the substrate protein (Rye et al., 1997; Kad et al., 1998), whether folded or not, and completes a single round of the folding cycle (Todd et al., 1994; Weissman et al., 1994). While the events of formation and dissociation of any given *cis* complex have thus been identified, how GroEL cycles from one folding-active state to the next has remained unclear.

Previous studies have indicated that the GroEL-ADP-GroES complex can bind nonnative substrate protein, suggesting that the *trans* ring of this complex might be an entry point for substrate protein into the GroEL reaction cycle (Weissman et al., 1995; Sparrer and Buchner, 1997). These observations were made in the absence of ATP and excess GroES, which would be present under physiological conditions, and do not, for example, eliminate the possibility that GroES might compete for the available ring. Additional studies have suggested that a second GroES molecule binds to the *trans* ring of a folding-active *cis* complex before it is discharged (Llorca et al., 1994; Torok et al., 1996). These studies have also not resolved the order of ligand addition that leads to the next folding-active complex. Finally, the available data do not exclude the possibility that GroEL might completely discharge all ligands before proceeding to the next cycle (see e.g., Corrales and Fersht, 1996; Ranson et al., 1997).

Here, we have addressed the issue of how GroEL cycles from one folding reaction to the next. We used a hydrolysis-deficient mutant of GroEL to determine the point at which nonnative polypeptide enters the GroEL reaction cycle. The structural features of the GroEL-GroES system that define this polypeptide acceptor state were established by cryo-electron microscopy (cryo-EM) and atomic structure fitting. We then employed stopped-flow fluorescence energy transfer to directly observe both association and departure of ligands to and from GroEL. We find that, in the presence of nonnative substrate, GroES, and ATP, the two rings of the chaperonin function in a coordinated and directional manner. Our data indicate that the dissociation of the *cis*-ADP complex is directly and efficiently coupled to

§To whom correspondence should be addressed (e-mail: horwich@csbmet.csb.yale.edu).
‖Present address: MRC Laboratory of Molecular Biology, MRC Centre, Hills Road, Cambridge CB2 2QH, United Kingdom.

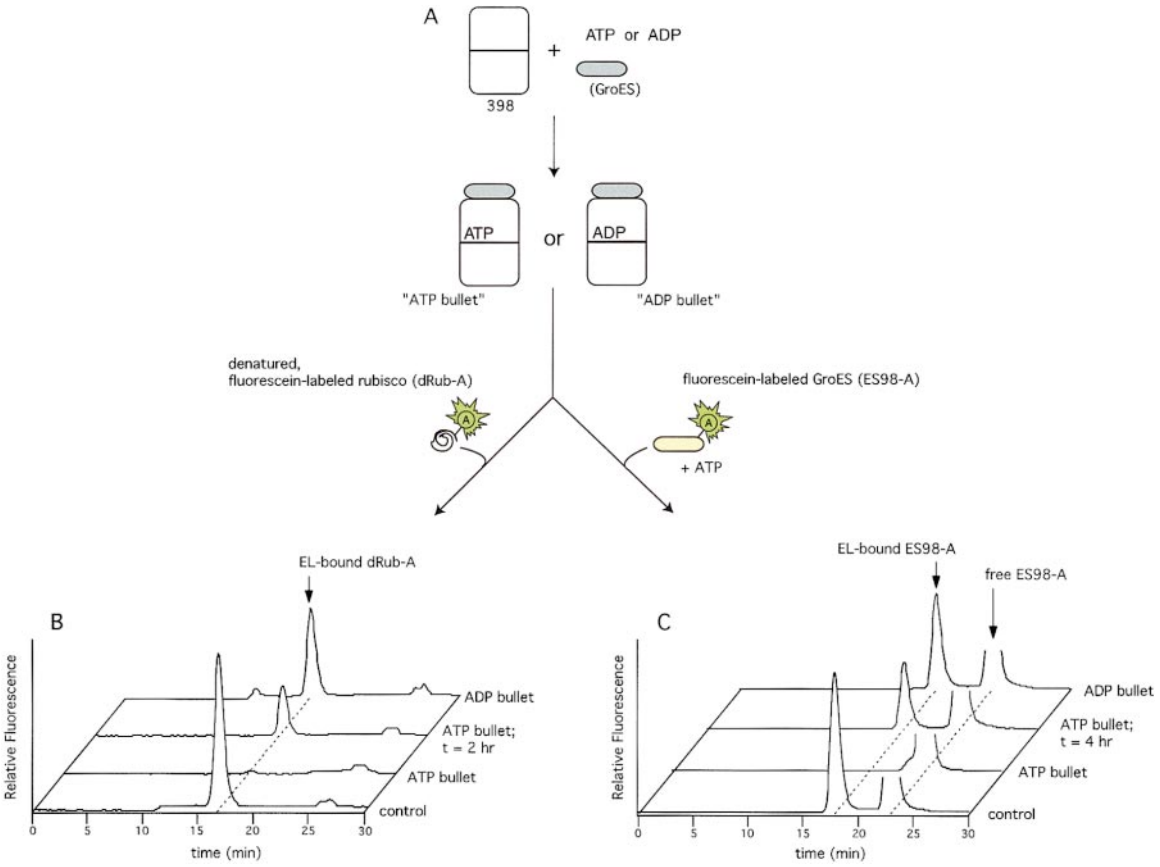

Figure 1. Hydrolysis of ATP in the *cis* Ring Is Required before the *trans* Ring of a GroEL-ATP-GroES Bullet Can Bind Either GroES or Denatured Substrate

(A) Schematic of experiment using the hydrolysis-deficient EL398 mutant of GroEL. By mixing EL398 with ATP and GroES, stable ATP bullet structures can be generated (Rye et al., 1997). ADP bullets can be made either by incubating the ATP bullets for 2–4 hr, to hydrolyze trapped ATP, or by substituting ADP for ATP in the initial assembly reaction. Bullets (1 μM) were mixed with either (B) a denatured, fluorescently labeled Rubisco (dRub-A at 500 nM) or (C) a fluorescently labeled variant of GroES and ATP (2 μM ES98-A and 2 mM ATP) and then separated using gel filtration with fluorescence detection. The trace labeled "control" represents a direct mixing of unliganded EL398 tetradecamers (1 μM) with either 500 nM dRub-A (B) or 2 μM ES98-A and 2 mM ATP (C). The large fluorescence peak due to the excess free ES98-A in (C) has been truncated for clarity. The migration positions of the complexes between EL398 and either ES98-A or dRub-A are shown.

the binding of nonnative substrate protein on the opposite (*trans*) ring and that this event occurs before another molecule of GroES can bind. Thus, the assembly of a new folding chamber is directly linked to the disassembly of the folding complex from the previous cycle, so that the chaperonin alternates folding-active states between rings.

Results

ATP Hydrolysis in the *cis* Ring Must Occur before Nonnative Polypeptide and GroES Bind to the *trans* Ring

As the GroEL-GroES machine proceeds from one folding-active, *cis*-ternary complex to the next, a new nonnative polypeptide must, at some point, form a stable association with a GroEL ring. Because an open GroEL ring (the *trans* ring) is accessible in both the asymmetric GroEL-ATP-GroES and GroEL-ADP-GroES complexes (referred to below as ATP and ADP bullets, respectively, due to their appearance in electron microscope images), we asked whether nonnative polypeptide could be stably bound by the *trans* ring of either a preformed ATP or ADP bullet. To form stable ATP bullets (Figure 1A), we utilized a mutant of GroEL (EL398) that is deficient in ATP hydrolysis but not ATP binding. When mixed with ATP and GroES, EL398 forms stable ATP bullets that are fully capable of initiating and supporting wild-type levels of polypeptide folding inside the GroES-bound ring, but they suffer from an inability to dissociate the *cis* complex in a timely fashion (Rye et al., 1997). This defect is the result of a very slow rate of ATP hydrolysis in the EL398 *cis* ring.

When EL398 ATP bullets are incubated with a denatured, fluorescently labeled version of the substrate protein Rubisco (dRub-A), no stable binding is detected by gel filtration (Figure 1B). On the other hand, when the ATP in the *cis* ring is replaced with ADP, either by waiting a sufficiently long time for the ATP bound in the *cis* chamber to hydrolyze or by substituting ADP in the initial assembly reaction, dRub-A can form a stable association with EL398 (Figure 1B). A similar binding experiment

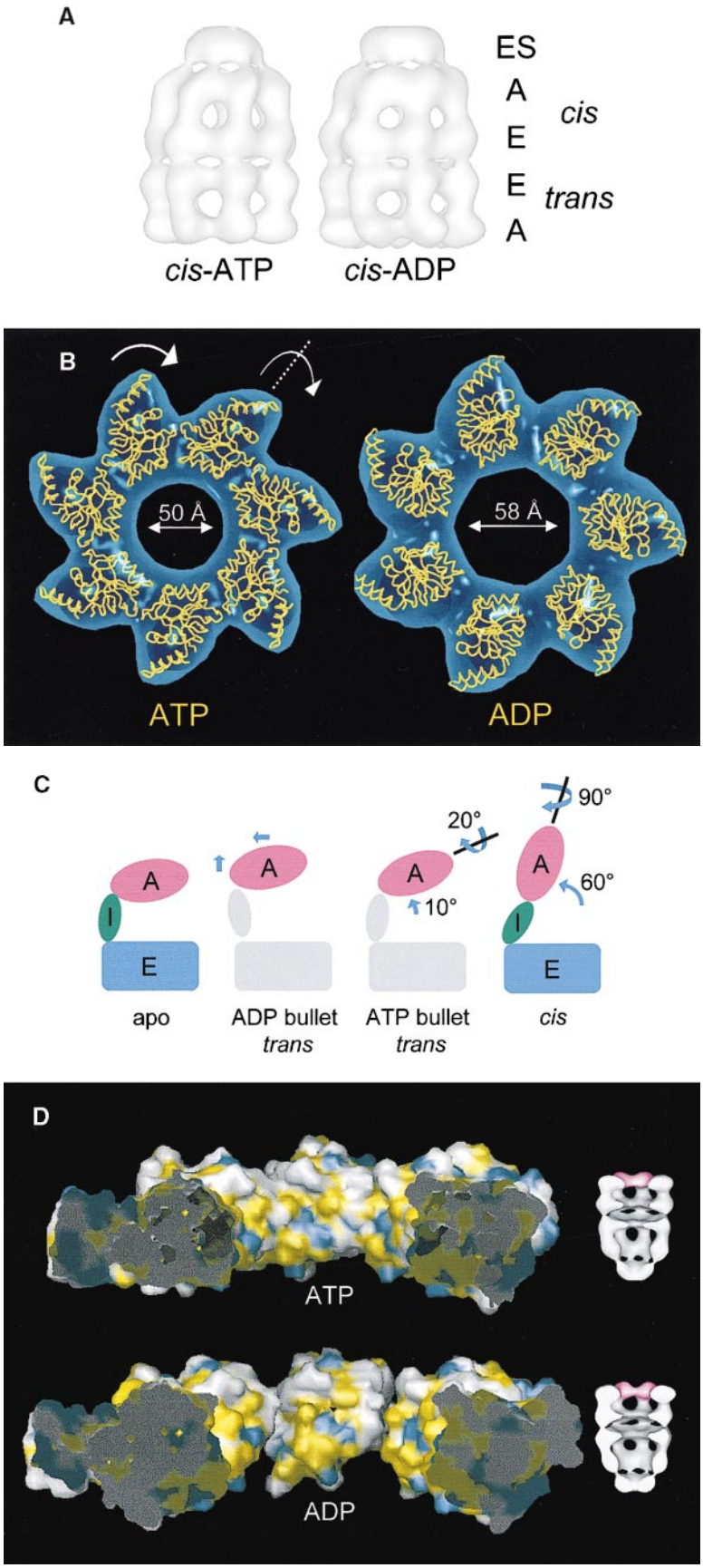

Figure 2. Cryo-EM Structures of the ADP and ATP Bullets Reveal Differences in the Apical Domains of the *trans* Rings

(A) Side views of the ATP and ADP bullets (ES, GroES; A, apical domain; E, equatorial domain). The main difference between the two structures is in the disposition of the apical domains of the *trans* rings.

(B) The cavity of the ADP bullet *trans* ring, which has widely separated apical domains, is signficantly larger than the cavity of the ATP bullet *trans* ring. The cryo-EM maps are shown as blue transparent surfaces, and the backbone structure of the apical domain is yellow. The apical domains of the ATP bullet show in-plane (left arrow) and out-of-plane (right arrow) rotations. The view is from outside the oligomer.

(C) Schematic diagram of the *trans* ring apical domain in the ADP and ATP bullets relative to unliganded GroEL (apo) and the *cis* ring of the ADP bullet (Xu et al., 1997). The apical domains of the ADP bullet *trans* ring have moved outward relative to apo GroEL but remain in a similar orientation, presumably due to hinge movements about the intermediate domain. Orientations of the intermediate (I) and equatorial domains are not determined from the low-resolution cryo-EM maps and are shown in gray. The ATP bullet angles are apparent rotations estimated from different views of the apical domain.

(D) The binding surface of the ATP and ADP bullet *trans* ring apical domains derived from the fitted cryo-EM data. The rings are cut open and viewed as shown by the pink shading in the overviews to the right. Hydrophobic residues are colored yellow and polar ones blue. In this view, the hydrophobic surface of the ATP bullet is rotated downward and to the left relative to the ADP bullet, reducing the accessibility of the hydrophobic binding sites. The *trans* ring apical domains of the ADP bullet display larger radial separation and weaker interdomain contacts than observed in the crystal structure (Xu et al., 1997), but the orientation and hydrophobic accessibility are very similar.

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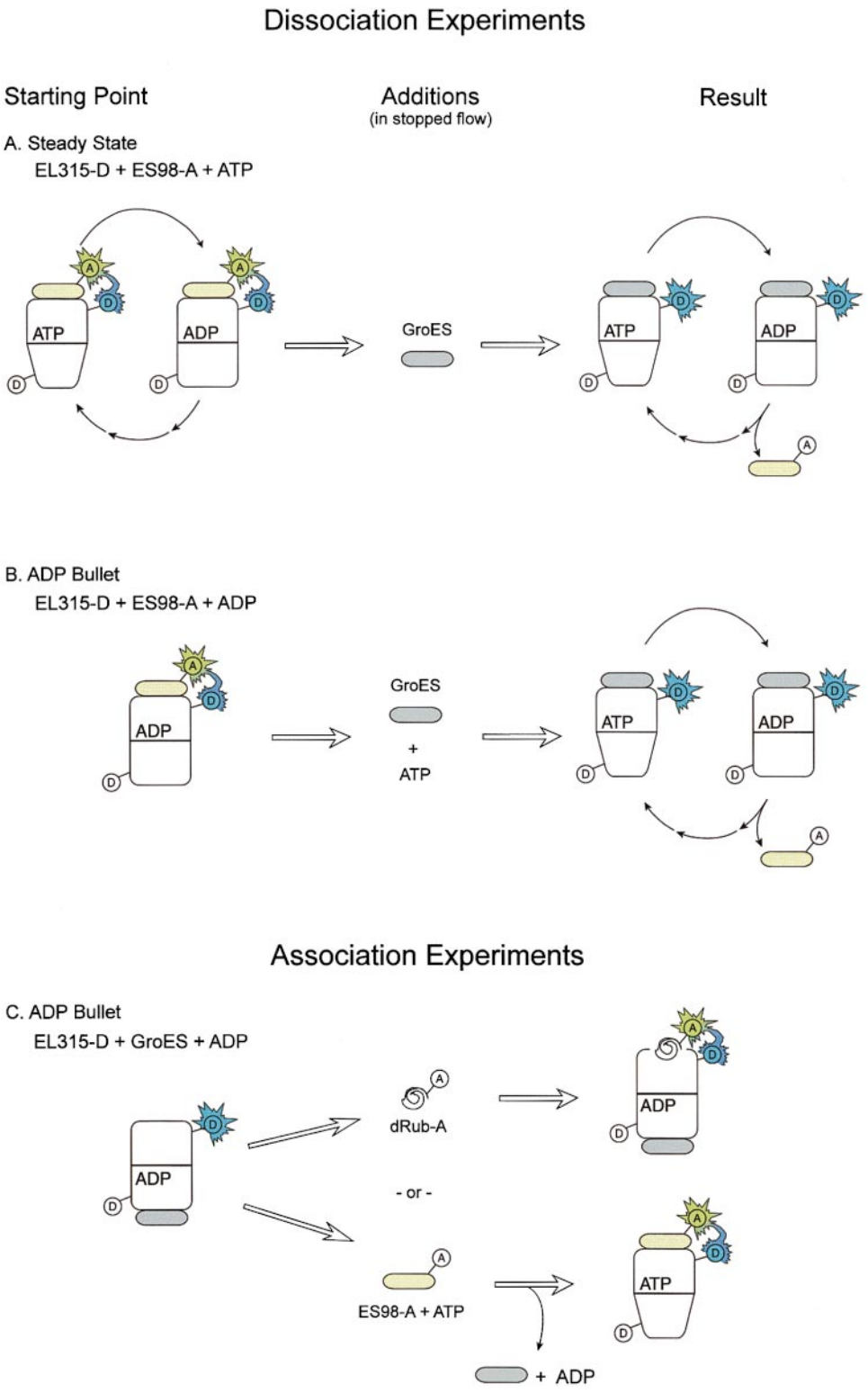

Figure 3. Conceptual Basis for Tracking Chaperonin Dynamics with Fluorescence Resonance Energy Transfer

The transfer of excited state energy between a donor-labeled GroEL (EL315-D) and either an acceptor-labeled GroES (ES98-A) or a nonnative substrate protein (dRub-A) can be used to track both the dissociation (A and B) and association (C) dynamics of the GroEL-GroES system. (A) Schematic of experiment in which EL315-D is first mixed with ES98-A and ATP and allowed to come to a steady state, after which the mixture is rapidly mixed in the stopped flow with an excess of unlabeled GroES (gray disc). The loss of energy transfer as ES98-A is replaced by unlabeled GroES is monitored as an increase in the donor fluorescence intensity and a decrease in the acceptor fluorescence intensity. For clarity the turnover of ATP is shown only in one ring. (B) Simplified dissociation experiment starting with only preformed ADP bullets made

was carried out with a fluorescently labeled GroES (ES98-A). The ES98-A can form stable interactions with either the EL398 tetradecamer alone or the *trans* ring of the ADP bullet, but it does not bind to the *trans* ring of the ATP bullet (Figure 1C). We conclude that the *trans* ring of an ATP bullet cannot accept either nonnative polypeptide or GroES until hydrolysis has taken place in the *cis* ring, transmitting an allosteric change to the open *trans* ring, which then permits ligand binding.

To assess whether there are structural features of the ATP and ADP bullets that might account for the observed differences in ligand binding by the *trans* ring, we compared them by cryo-EM. ATP bullets were produced by adding ATP to a mixture of wild-type GroEL and GroES and vitrifying after 6 s. Images of these samples showed two types of bullets (Figure 2A), as well as complexes with GroES bound at both ends of GroEL and a smaller percentage of unoccupied GroEL. One type of bullet resembled the preformed ADP bullet studied earlier (Roseman et al., 1996), while the other complex was similar to the previously observed steady-state ATP bullet but with sharper features. Because a steady-state GroEL-GroES reaction contains both ADP and ATP bullets, these earlier steady-state ATP images represented an average of the two bullet species. Thus the new, more resolved ATP bullet examined here likely represents a purer population of this structure. When this ATP bullet structure is compared with the preformed ADP bullet, the largest difference is in the configuration of the *trans* rings (Figure 2B). In the ATP bullet, the *trans* ring is constricted with a diameter measuring ~50 Å, whereas in the ADP bullet the *trans* ring is open, with a diameter of ~58 Å. These are larger openings than observed in the *trans* ring of the ADP bullet crystal structure, which measures ~45 Å.

To more fully examine the *trans* ring of the bullet complexes, we assumed that the apical domains moved as rigid bodies and fit the GroEL apical domain atomic coordinates (Xu et al., 1997) into the densities of the ATP and ADP bullet *trans* rings obtained by cryo-EM (Figure 2A). When the *trans* rings of the ATP and ADP bullet complexes are compared, it is apparent that the domains of the ATP bullet move radially inward and are more tightly packed than the domains of the ADP bullet, which are widely separated and barely make contact with each other. In addition, the apical domains of the two complexes occupy significantly different orientations. The *trans* apical domains of the ADP bullet are in a similar orientation to that observed in the crystal structure (Figures 2B and 2D; Xu et al., 1997). However, the domains of the ATP bullet exhibit an ~10° upward movement and an ~20° rotation about their long axes in the same direction as the displacement of the apical domains of a GroES-bound *cis* ring (Figure 2C). Such movements would be expected to partly displace the hydrophobic polypeptide-binding surface of the apical domains away from the central cavity and would result in a structural change similar to a proposed T→R allosteric transition for this ring (Inbar and Horovitz, 1997). This is indeed observed in a surface representation of the fitted rings (Figure 2D). In the ATP bullet, the domains are reoriented to move the hydrophobic sites down and away (leftward in the view shown), placing the main hydrophobic patch adjacent to the intermediate domain and resulting in a less accessible hydrophobic binding surface. Thus, hydrolysis of ATP in the *cis* ring controls the position and orientation of the *trans* ring apical domains, thereby switching the *trans* ring from a collapsed to an open (acceptor) state and signaling the beginning of the next round of substrate interaction.

Fluorescence Resonance Energy Transfer and the Dynamics of the GroEL-GroES Cycle

Starting with the ADP bullet as the most proximal acceptor state for each turn of the GroEL cycle, the pathway of the chaperonin cycle can be mapped by knowing the various rates of ligand association and dissociation. While some of these rate constants have been established or inferred, it has not been possible to directly probe others. Additionally, in many cases, the dynamics of the GroEL-GroES cycle have not been systematically examined in free solution with all of the components (i.e., chaperonin, cochaperonin, nucleotide, and substrate polypeptide) present at one time. To this end, we employed a fluorescence resonance energy transfer (FRET) assay to track the dynamic interactions between GroEL and either GroES or substrate. We placed a donor chromophore, IAEDANS, on the cysteine of an engineered variant of GroEL (E315C) and an acceptor chromophore, fluorescein-5-maleimide, on either the cysteine of an engineered variant of GroES (ES98C; Murai et al., 1996) or an endogenous cysteine in Rubisco (Rye et al., 1997). The acceptor-labeled Rubisco (Rub-A) and ES98-C (ES98-A) behaved like the wild-type proteins by every measure examined, including native size by gel filtration, enzymatic activity, and mediated protein refolding (ES98-A). The donor-labeled EL315-C (EL315-D) behaved exactly like wild-type GroEL as judged by gel filtration and the kinetics and yield of protein refolding, but it showed a modest decrease in steady-state ATPase rate relative to wild-type GroEL (see Experimental Procedures).

Interactions between EL315-D and either ES98-A or Rub-A are monitored as the transfer of excited state energy from the donor to the acceptor when the two chromophores are close in space (10–80 Å; Van der Meer et al., 1994). Dissociation of a complex between EL315-D and ES98-A should lead to a drop in acceptor fluorescence and a rise in donor fluorescence (dequenching). Association should lead to the opposite effect: a rise in acceptor fluorescence and a decrease in donor fluorescence (quenching). Each type of energy transfer experiment was carried out in two ways (Figure 3): dissociation experiments were initiated either from

with ES98-A and EL315-D. The rate of departure of ES98-A from the homogeneous ADP bullet population can be monitored upon rapid mixing of the ADP bullets with unlabeled GroES and ATP. (C) Schematic of binding experiments used to monitor the rate of ES98-A and dRub-A association with the *trans* ring of the ADP bullet. The promotion of energy transfer as the EL315-D *trans* ring binds either ES98-A or dRub-A results in a decrease in the donor fluorescence and an increase in the acceptor fluorescence.

a steady-state mixture of ADP and ATP bullets or from preformed ADP bullets alone, while association experiments were started from either preformed ADP bullets or EL315-D alone. In the steady-state dissociation experiments, EL315-D and ES98-A were first mixed together in the presence of ATP prior to being loaded into the stopped-flow apparatus (Figure 3A). Rapid mixing of this sample with an excess of unlabeled GroES then permitted the observation of the time-dependent decay of the various GroEL-GroES species as the acceptor-labeled GroES was replaced by the much larger pool of unlabeled GroES. Alternately, if EL315-D and ES98-A were first mixed with ADP, they formed a stable population of ADP bullets. Rapid mixing of this sample with ATP and unlabeled GroES resulted in the loss of energy transfer initially created in the ADP bullet (Figure 3B).

An example of a steady-state experiment is shown in Figure 4. The loss of energy transfer upon rapid addition of unlabeled GroES can be readily detected in both the donor (Figure 4A) and acceptor (Figure 4B) channels. The data in both channels are fit by a single exponential model with a rate constant (k_{slow}) of 0.031 s^{-1}. In previous experiments, the release of GroES from the ADP bullet was shown to be the rate-limiting step of the GroEL-GroES steady-state ATPase reaction and to have a rate constant of 0.042 s^{-1} (Burston et al., 1995). The rate constant obtained from Figure 4 is very similar to this previously derived value and, additionally, exactly matches the steady-state ATPase rate of EL315-D in the presence of GroES. Therefore, the FRET signal observed in Figure 4 directly reports the rate of the slowest step in the release of ES98-A from EL315-D. Importantly, utilization of an excess of unlabeled GroEL as a way to sequester the ES98-A, instead of competitor GroES, results in identical decay kinetics (Figure 4C). This indicates that binding of a second molecule of GroES to the *trans* ring of a bullet complex is not required to enable discharge of GroES from the *cis* ring.

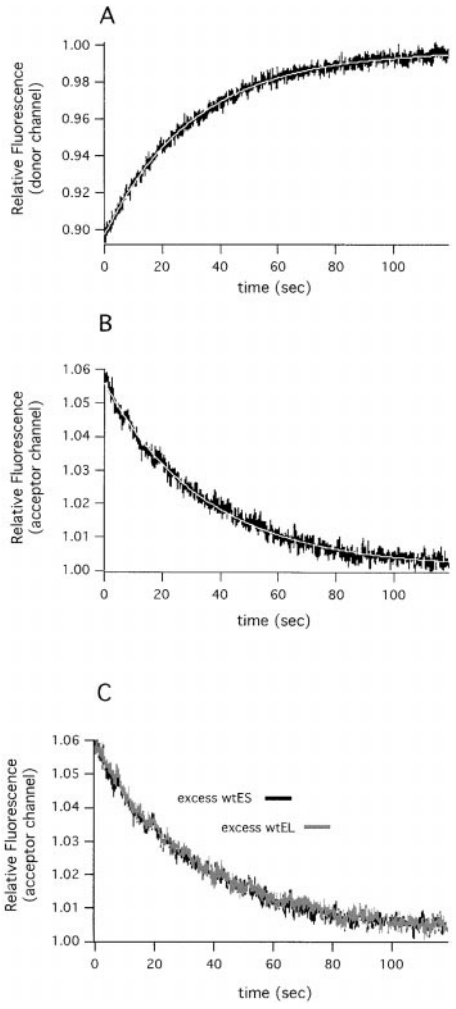

Figure 4. Release of ES98-A from EL315-D during a Steady-State Reaction

For this experiment, 188 nM EL315-D was mixed with 175 nM ES98-A and 5 mM ATP and loaded into one stopped-flow syringe. The steady-state mixture was then mixed (4:1) with 7.5 μM GroES in the stopped flow. The rate of GroES-A release can be monitored as either the dequenching of the donor (A) or loss of fluorescence of the acceptor (B). In both channels, the data are fit by a single exponential model (shown in both [A] and [B] as a thin white line superimposed on each relaxation curve; for [A], $k_{slow} = 0.031 \pm 0.0001\ s^{-1}$ and for [B], $k_{slow} = 0.029 \pm 0.0001\ s^{-1}$). The measured rate constant does not change at different ratios of ES98-A to EL315-D, is not dependent on the salt concentration (up to 250 mM KCl), and is independent of the concentration of competitor GroES used (data not shown). (C) The release kinetics of ES98-A are identical when competitor GroEL (1.5 μM final concentration) is used in place of competitor GroES. Mechanisms other than energy transfer (e.g., spectral shifts and intrinsic quantum yield changes) do not contribute significantly to the fluorescence changes observed, since control experiments in which one of the energy transfer partners was replaced with its unlabeled conjugate show only small signal changes and the emission spectra of the ES98-A and EL315-D do not change upon complex formation.

The Dissociation of GroES from ADP Bullets Identifies a Rapid Step in the Release Reaction

The release of GroES from the *cis* ring following ATP hydrolysis requires the binding of ATP to the *trans* ring and appears to occur in a single slow step ($t_{1/2}$ = 15–20 s; Figure 4 and Burston et al., 1995). These experiments, however, only probe the slowest step that commits GroES to departure and therefore may not fully describe the release pathway of GroES from the *cis* complex. Indeed, when FRET dissociation experiments are initiated from a homogeneous starting population of ADP bullets created with EL315-D and ES98-A (Figure 3B), an additional level of complexity is observed in the GroES release reaction. As shown in Figure 5A, when such ADP bullets are mixed with ATP and competitor GroES, approximately 40% of the bound ES98-A is released much more rapidly than during the steady-state experiments (compare Figure 5A with Figure 4B). The data in Figure 5A can be fit with a two-step (double exponential) model with rate constants of $k_{fast} = 2.3\ s^{-1}$ and $k_{slow} = 0.025\ s^{-1}$. The slower component in Figure 5A essentially matches the slow step measured in the steady-state experiments above for the dissociation of GroES from the *cis*-ADP complex.

A second experiment (Figures 5B and 5C) was carried out to confirm the existence of a fast release step and to control for the possibility that an ADP bullet formed by ATP hydrolysis in situ might be different in kinetic behavior from one formed directly in ADP. It is possible

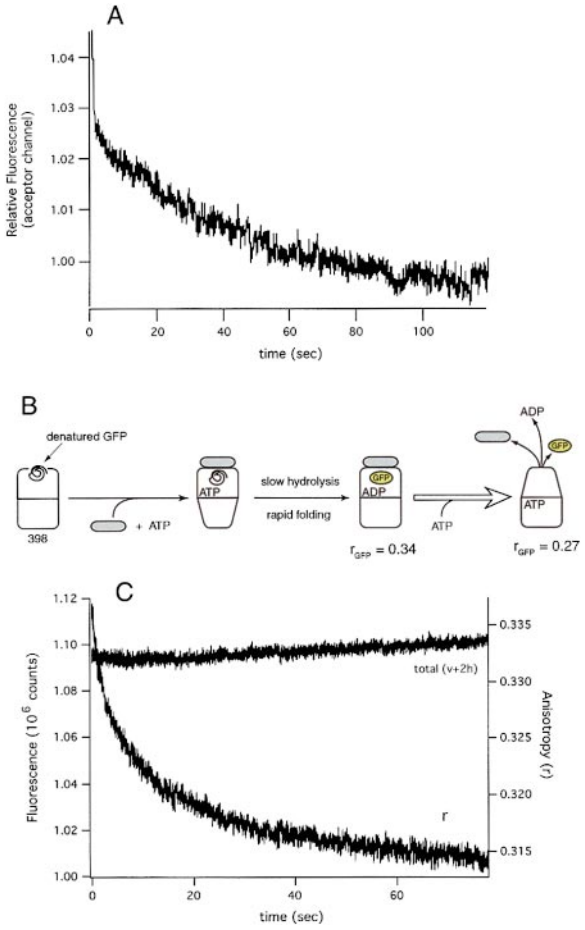

Figure 5. Release of ES98-A from a Preformed ADP Bullet Reveals an Additional Fast Component to the Dissociation of the *cis* Chamber

(A) Release kinetics of ADP bullets monitored as loss of acceptor fluorescence upon mixing with GroES and ATP. A 2 μM sample of ADP bullets made from EL315-D and ES98-A was mixed (1:1) with GroES and ATP (20 μM GroES plus 5 mM ATP). The disassembly of the ADP bullet can be fit by a double exponential model with rate constants of $k_{slow} = 0.025 \pm 0.0002\ s^{-1}$ and $k_{fast} = 2.3 \pm 0.1\ s^{-1}$.

(B) The change in fluorescence anisotropy (r) of folded GFP upon release from an EL398 *cis* chamber into solution can also be used to measure the decay of the ADP bullet.

(C) Plot showing changes in the steady-state fluorescence anisotropy and total intensity (v+2h) of trapped GFP upon addition of ATP. Following formation of an ATP bullet with GFP trapped inside the *cis* space, the sample was incubated for 3 hr to allow the slow hydrolysis of ATP to produce a population of ADP bullets. The ADP bullets (approximately 2 μM) containing trapped and folded GFP were then mixed (1:1) with 5 mM ATP in the stopped flow. The change in anisotropy of the GFP can be fit by a double exponential model with rate constants of $k_{slow} = 0.051 \pm 0.0005\ s^{-1}$ and $k_{fast} = 0.50 \pm 0.0001\ s^{-1}$. Because the total fluorescence intensity remains nearly constant, the drop in anisotropy can be largely attributed to an increase in the rotational motion of the GFP monomers, reflecting release of the GFP from the *cis* cavity into solution as the ADP bullets disassemble.

to trap and fold green fluorescent protein (GFP) within a *cis* complex formed between EL398 and GroES (Rye et al., 1997 and Figure 5B). However, the folded GFP within the *cis* chamber is rotationally constrained and displays a significantly higher fluorescence anisotropy than free GFP (Weissman et al., 1996). Therefore, as the GroES lid of the *cis* complex is released and the folded GFP is set free into solution, the anisotropy of GFP fluorescence reports on the decay of the *cis*-ADP complex. This experiment carries the benefit of examining the decay behavior of the ADP bullet in a fundamentally different manner than the energy transfer assay, providing an independent test of the conclusions from the energy transfer experiment. As shown in Figure 5C, upon rapid mixing of the EL398 ADP bullets with ATP, a large drop in the steady-state fluorescence anisotropy of the trapped GFP is observed. The anisotropy changes require a double exponential fit with rate constants of $k_{slow} = 0.051\ s^{-1}$ and $k_{fast} = 0.5\ s^{-1}$. Again, k_{slow} likely corresponds to the slow release of GroES from the *cis*-ADP complex, as measured above by energy transfer. Further, the observation here of a faster component for GFP release confirms that the decay of the ADP bullet can occur via a step that is at least an order of magnitude faster than that previously observed.

The Fast Release of GroES from ADP Bullets Is Stimulated by Denatured Substrate Protein

The fast phase of GroES release identified in Figure 5 strongly suggests that an additional step must be considered when describing the ADP bullet disassembly pathway. The greater significance of the fast pathway becomes clear when either denatured Rubisco (dRub) or denatured malate dehydrogenase (dMDH) is added to asymmetric complexes in the presence of ATP and competitor GroES. As illustrated in Figure 6, the fast dissociation phase becomes prominent in the presence of the denatured substrate, whether starting from a steady-state mixture (Figures 6A and 6E) or from preformed ADP bullets (Figure 6C). This is particularly noticeable in the steady-state experiment, where the fast decay rate is not detected in the absence of the denatured protein. In both cases, the population of ADP bullets that decay by the faster rate increases in direct proportion to the amount of denatured substrate present (Figures 6B and 6D), but the rate constants of both the slow phase (steady state: $k_{slow} = 0.034\ s^{-1}$; ADP bullet: $k_{slow} = 0.034\ s^{-1}$) and fast phase (steady state: $k_{fast} = 1.3\ s^{-1}$; ADP bullet: $k_{fast} = 1.9\ s^{-1}$) remain unchanged (data not shown).

It should be noted that the effects of ATP and denatured substrate protein on the disassembly of the ADP bullet are synergistic and not simply additive. While ATP binding to the *trans* ring of the ADP bullet is sufficient to initiate *cis* complex dissociation, neither ADP nor ADP plus denatured Rubisco can do so (Figure 6C). Thus, ATP binding in *trans* is an indispensable component of the release trigger, while unfolded substrate polypeptide can enhance this step either by populating an alternate, faster pathway or by catalyzing the passage of the ADP bullet through a slow step.

Ordered Binding of Rubisco and GroES to the *trans* Ring of an ADP Bullet

The most likely manner by which denatured Rubisco could affect the rate of dissociation of the *cis* chamber of an ADP bullet is by binding to the open *trans* ring.

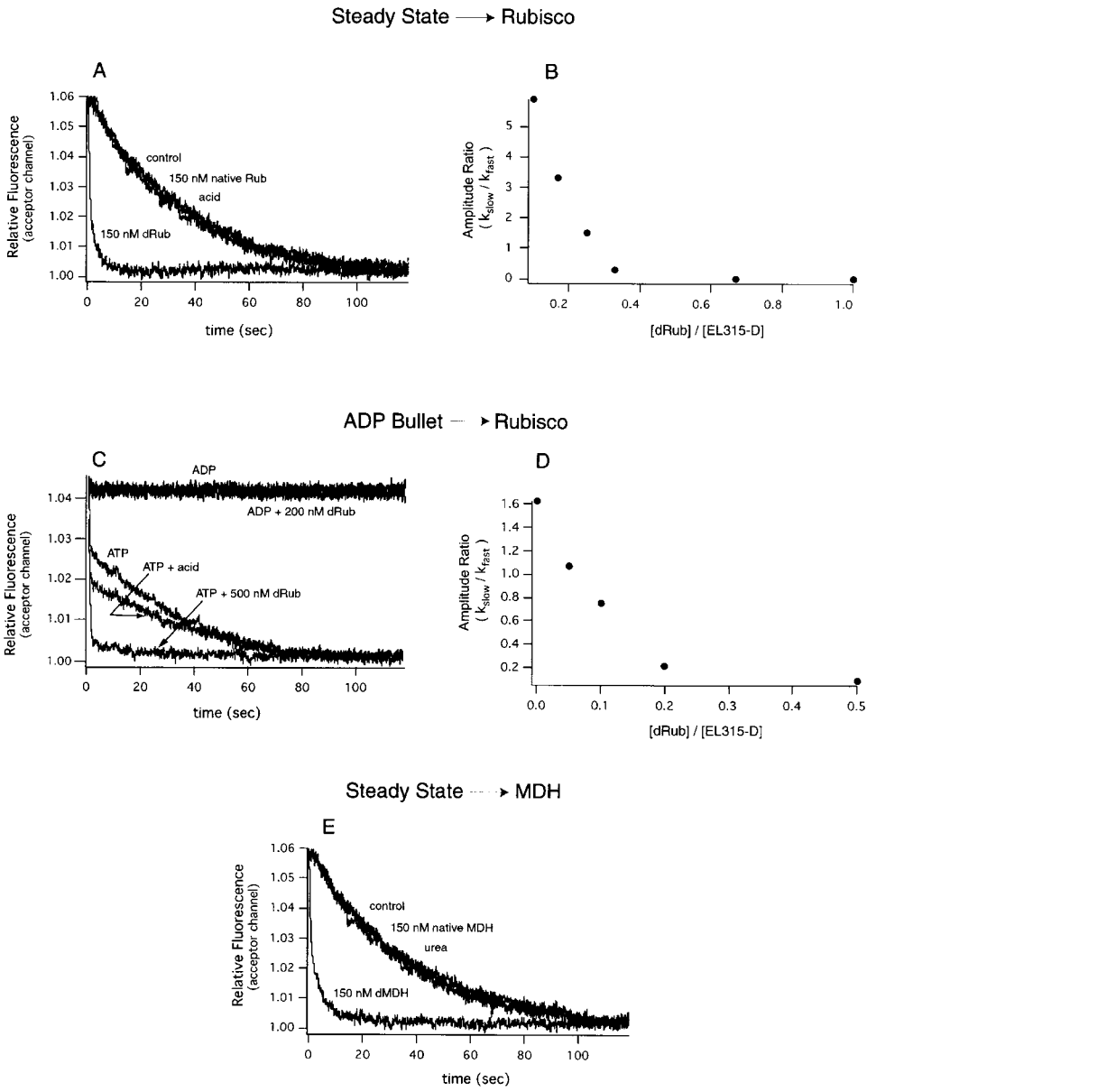

Figure 6. Addition of Denatured Substrate Polypeptide to Either a Steady-State Mixture or a Preformed ADP Bullet Results in a Large Acceleration of the Rate of Dissociation of the *cis* Chamber

(A) Decay of acceptor fluorescence of a steady-state mixture of EL315-D, ES98-A, and ATP upon addition of competitor GroES and dRub. Experimental conditions are the same as Figure 4. Addition of either denaturant (acid) alone or native Rubisco has no effect on the relaxation kinetics. The decay data in the presence of dRub are fit by a model using three exponential components, one likely corresponding to hydrolysis (k_{hydro} = 0.15 ± 0.05 s^{-1}), one corresponding to *cis* chamber dissociation (k_{slow} = 0.034 ± 0.003 s^{-1}), and an additional fast component (k_{fast} = 1.3 ± 0.27 s^{-1}). The values of the rate constants are not dependent on the concentration of dRub present (data not shown). On the other hand, the ratio of the amplitudes of k_{slow} to k_{fast} (B) shows that, as the relative dRub concentration is increased, the fast component dominates the disassembly of the ADP bullets. (C) Decay of acceptor fluorescence from an ADP bullet upon addition of ATP, competitor GroES, and dRub. Addition of either ADP or dRub (with competitor GroES present in both cases) is not sufficient to mediate bullet disassembly, nor does inclusion of denaturant (acid) affect the ATP-mediated kinetics. Addition of dRub along with ATP significantly accelerates the rate of decay of the ADP bullet, however. Decay data in the presence of dRub are fit by a model using two exponential components, one likely corresponding to dissociation of the *cis* chamber as before (k_{slow} = 0.034 ± 0.009 s^{-1}) and an additional fast component (k_{fast} = 1.9 ± 0.5 s^{-1}). As with the steady-state experiment, k_{slow} and k_{fast} are not dependent on the concentration of dRub present (data not shown). Likewise, the ratio of the amplitudes of k_{slow} to k_{fast} (D) shows that, as the dRub concentration is increased, the fast rate dominates the disassembly of the ADP bullets. (E) Stimulation of ES98-A release from a steady-state reaction is also observed in the presence of urea-denatured MDH. The denatured MDH (in 5.5 M urea) is mixed with a steady-state reaction at a ratio of 1:20, resulting in an MDH concentration of 1 μM and a residual urea concentration of 275 μM. This quantity of urea has no effect on the ES98-A release kinetics on its own.

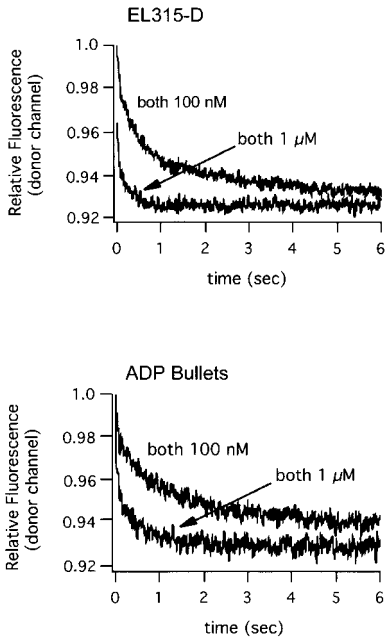

Figure 7. The Rate of dRub-A Binding to the ADP Bullet *trans* Ring Is the Same as the Rate of Binding to Unliganded EL315-D

(A) Binding of dRub-A to EL315-D and (B) to the *trans* ring of ADP bullets made with EL315-D and GroES. In each plot, the upper curve was generated at final concentrations of 100 nM dRub-A and either 100 nM EL315-D or 100 nM ADP bullets made with EL315-D. The lower curve was produced at a concentration of 1 μM in each component. Bimolecular rate constants were estimated from the 100 nM traces only ($k \approx 1–2 \times 10^7\ M^{-1}\ s^{-1}$; see Experimental Procedures), due to a competing aggregation reaction of the dRub-A at higher concentrations. Although the binding curves shown do not exactly fit a single-step binding model, they serve to place an upper limit on the binding rate of the majority of the dRub-A and illustrate that this association to the *trans* ring of the ADP bullet is a fast bimolecular reaction.

On the other hand, binding of GroES to GroEL tetradecamers (Burston et al., 1995) has been estimated to occur at a rate that, at equivalent concentrations, is at least as fast or up to 5–100 times faster than substrates bind to GroEL (Badcoe et al., 1991; Roy et al., 1992; Katsumata et al., 1996; Sparrer et al., 1996; Goldberg et al., 1997). However, ligand binding to the *trans* ring of the ADP bullet might differ significantly from binding to unliganded GroEL tetradecamers. To test this possibility, the rates of association were directly measured by following the induction of energy transfer upon the binding of ES98-A and denatured Rub-A to EL315-D (shown schematically in Figure 3C). The binding of acid-denatured Rub-A (dRub-A) to either unliganded EL315-D or ADP bullets appears to occur at similar rates (Figure 7), and the presence of ATP has less than a 2-fold effect on the rate in either case (data not shown). The binding rate is concentration dependent in all cases, indicating that dRub-A binding to isolated tetradecamers or ADP bullet *trans* rings is a rapid bimolecular process ($k \approx 1–2 \times 10^7\ M^{-1}\ s^{-1}$).

In contrast to dRub-A binding, the kinetics of ES98-A binding to the *trans* ring of the ADP bullet are not identical to those observed for binding to unliganded EL315-D (Figure 8). As expected, binding of ES98-A to EL315-D in the presence of ATP is fast ($k = 5 \times 10^7\ M^{-1}\ s^{-1}$; cf. Burston et al., 1995) and bimolecular. This was confirmed by both an equimolar mixing experiment (Figure 8A) and a limited pseudo first-order experiment (Figure 8B). The kinetics of binding of ES98-A to the *trans* ring of ADP bullets (Figure 8C) is independent of concentration, however. This indicates that a unimolecular process, most likely within the ADP bullet itself, is limiting the rate at which the second molecule of GroES can bind to the ADP bullet *trans* ring. The data in Figure 8C fit to a double exponential model with rate constants of 0.038 s^{-1} and 1.0 s^{-1}. These kinetic parameters are virtually identical to those measured in Figures 5 and 6 for disassembly of the *cis* chamber of the ADP bullet. This strongly suggests that GroES cannot bind to the *trans* ring of the ADP bullet until the previously bound GroES has, at a minimum, begun to depart. Furthermore, the binding rates imply that, regardless of the concentration of GroES, the ADP bullet binds the next molecule of unfolded substrate before binding the next GroES. To test this, we conducted an experiment in which the binding of dRub-A to the *trans* ring of ADP bullets was monitored in the presence or absence of a large excess of GroES. As shown in Figure 8D, the presence of a 10-fold excess of GroES has no effect on the rate or extent of binding of dRub-A to the ADP bullet *trans* ring, demonstrating that the denatured substrate binds before the next GroES.

Discussion

Alternation of the *cis* Ternary Complex Between GroEL Rings Is Coordinated by ATP and Nonnative Protein

The experiments presented here further address how the two rings of GroEL function to drive the chaperonin reaction. The findings from biochemical, cryo-EM, and dynamic FRET studies establish that the progression from one round of protein folding to the next involves a directional and alternating coordination between the two GroEL rings. In particular, we observe that the *trans* ring of the *cis*-ATP bullet can accept neither unfolded polypeptide nor GroES. Once ATP hydrolysis occurs in the *cis* ring to produce an ADP-*cis* complex, however, the *trans* ring can bind both polypeptide and GroES. This toggle of the *trans* ring ligand affinities correlates with a structural transition of the *trans* ring, observed by cryo-EM, in which the *trans* ring apical domains of the ATP bullet are positioned so as to disrupt the ligand binding surface and narrow the ring orifice. Following hydrolysis in the *cis* ring, the binding of nonnative protein and GroES to the ADP bullet *trans* ring is an ordered event, based upon FRET experiments, in which the substrate protein binds before the encapsulating GroES molecule. This sequence of events is ensured because the binding of the next GroES molecule to the ADP bullet *trans* ring is restricted by the same structural transition that controls GroES release from the *cis* ring. Because the binding of nonnative protein to the ADP bullet *trans* ring stimulates this transition (by at least 50-fold) in the presence of ATP, a new *cis*-ternary complex is always assembled on the *trans* ring of each ADP bullet. Thus,

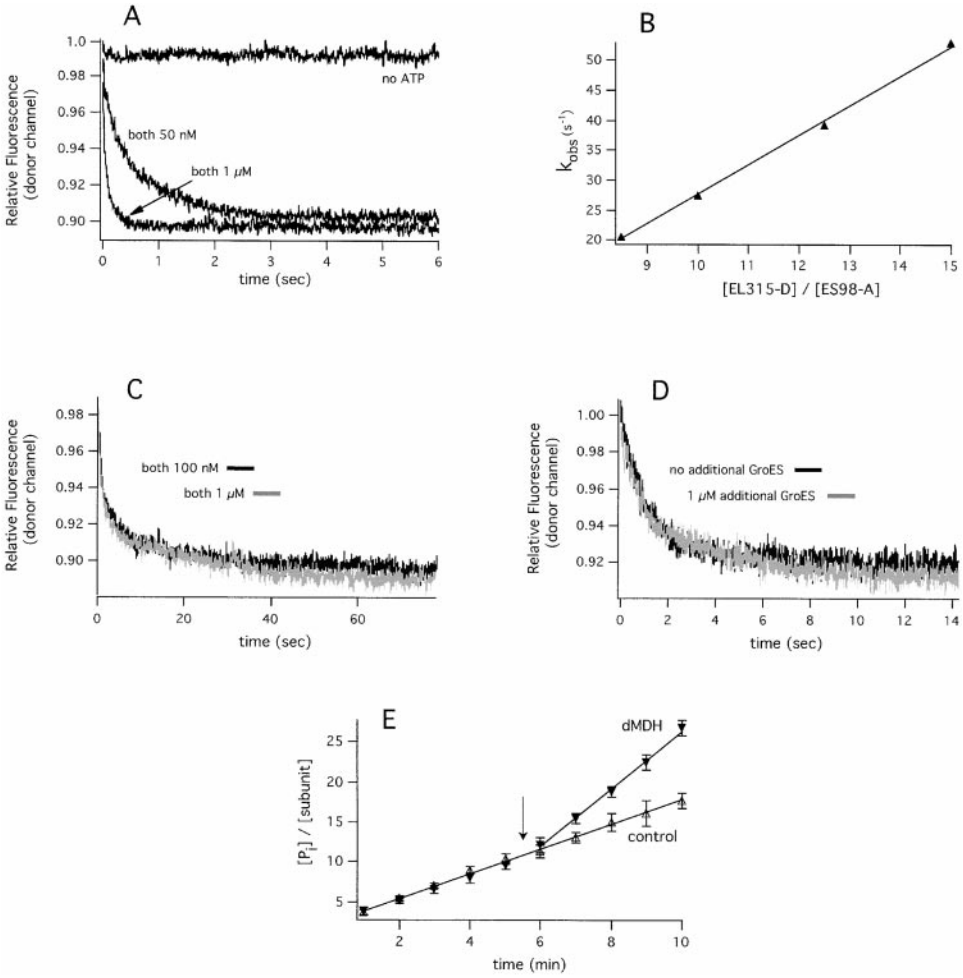

Figure 8. Unfolded Substrate Polypeptide Binds before GroES to the *trans* Ring of an ADP Bullet and Stimulates Steady-State ATP Hydrolysis

(A) Binding of ES98-A to EL315-D at equimolar concentrations. In the upper trace, ES98-A and EL315-D were mixed at final concentrations in the stopped flow of 50 nM each, and in the lower trace at 1 μM; ATP in each case was 2 mM. The bimolecular rate constants derived from these traces are 5.7×10^7 and 2.9×10^7 M^{-1} s^{-1} for the 50 nM and 1 μM traces, respectively.

(B) A limited pseudo first-order experiment was conducted in which the ES98-A and ATP concentrations were maintained at 100 nM and 2 mM, respectively, and the concentration of EL315-D in the binding reaction was varied. A plot of the pseudo first-order rate constants (k_{obs}) versus the ratio of the components is shown. The bimolecular rate constant extracted from this analysis is 5×10^7 M^{-1} s^{-1}.

(C) Binding of ES98-A to the *trans* ring of ADP bullets (in 2 mM ATP) is independent of concentration. The binding data fit to a double exponential model with rate constants of 0.038 ± 0.0006 s^{-1} and 1 ± 0.02 s^{-1}.

(D) The binding of 100 nM dRub-A to the *trans* ring of 100 nM ADP bullets (plus 2 mM ATP) is the same in the absence and presence of 1 μM GroES.

(E) The steady-state ATP hydrolysis rate of GroEL, in the presence of GroES, is stimulated 2.3-fold by denatured MDH. A steady-state hydrolysis reaction was initiated by adding 2 mM ATP to a mixture of GroEL (125 nM) and GroES (500 nM). Either urea-denatured MDH (dMDH) or urea alone was added at 5.5 min (1 μM final monomer concentration, approximately 80 μM residual urea after addition). Free phosphate was assessed as described (Fenton et al., 1994).

given that GroES and polypeptide leave one ring prior to, or simultaneously with, arrival of GroES to form a *cis* complex on the opposite ring, it appears that folding-active states alternate between rings. Such behavior of the GroEL-GroES system as a "two-stroke" machine has been previously proposed (Lorimer, 1997; see also Sparrer and Buchner, 1997; Kad et al., 1998).

These findings have been incorporated into two models of the GroEL reaction cycle (Figure 9): one in the absence of polypeptide substrate (I) and one in its presence (II). The nucleotide cycle of the GroEL-GroES system was initially formulated to involve two hydrolytic steps per cycle, one on each ring. Recent work, however, has shown that ATP binding alone on the *trans* ring is sufficient to initiate discharge of the *cis*-GroEL-GroES complex and complete the reaction (Rye et al., 1997). This finding simplifies the states necessary to describe a full turn of the cycle (Figure 9I; also see Kad et al., 1998). Hydrolysis of ATP in the *cis* complex to form an ADP bullet occurs with a rate constant of 0.12 s^{-1} (Burston et al., 1995). Previous experiments with a pyrene-labeled GroEL strongly suggested that the slowest step in the overall GroEL-GroES cycle in the absence of nonnative polypeptide was the release of GroES from

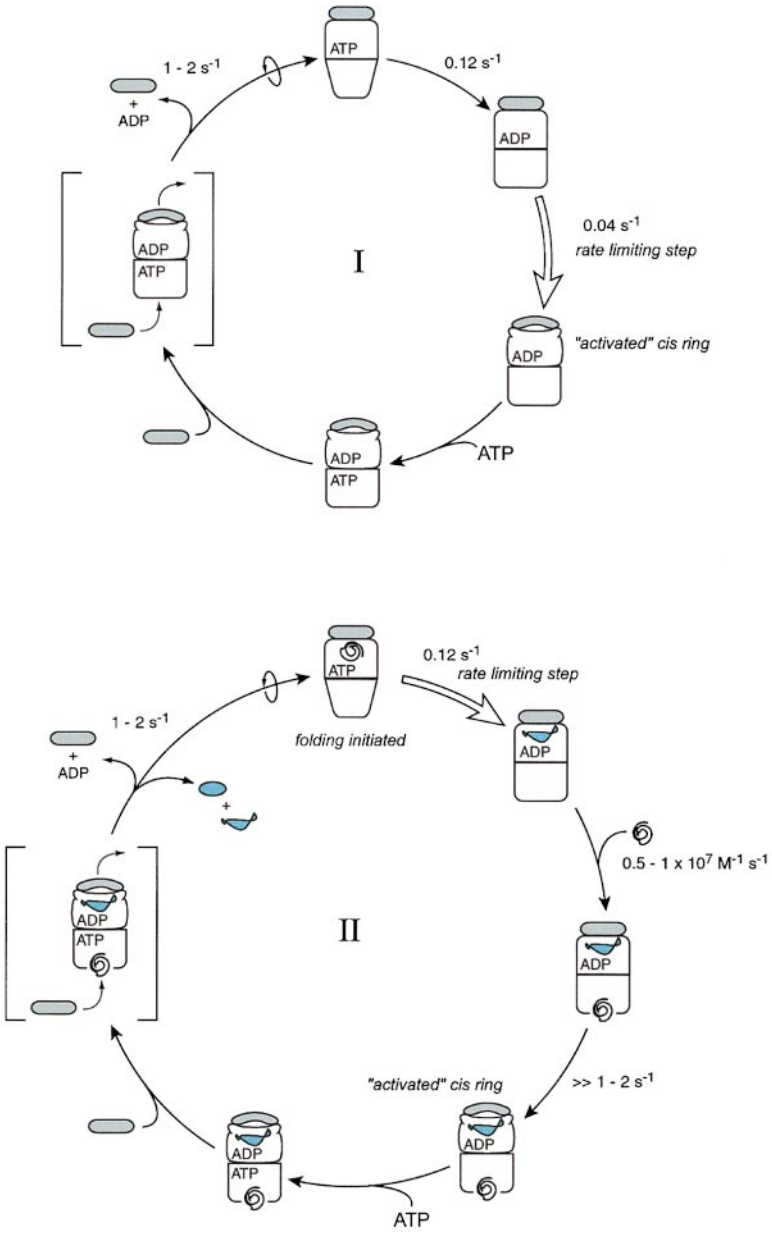

Figure 9. Proposed Models for Cycling of the GroEL-GroES System in the Presence and Absence of Substrate

The GroEL-GroES system can be described with two reaction cycles, one in the absence (cycle I) and one in the presence (cycle II) of denatured substrate polypeptide (see Discussion). Association of ligands with the ATP bullet *trans* ring is blocked by structural transitions induced in the *trans* ring apical domains by the presence of ATP in the *cis*-ternary complex (indicated here as a narrowing of the *trans* ring). Association of the second GroES molecule with the *trans* ring occurs at a rate similar to the rate at which the previous GroES is released and thus appears to be a concerted event (indicated by the brackets). The switching of folding-active states between rings is efficiently coupled to polypeptide binding through a structural transition of the *cis*-ADP complex ("activated" *cis* ring). As a result, the rate-limiting step of the reaction cycle in the presence of nonnative polypeptide becomes *cis* chamber hydrolysis (designated by the open arrow). The blue oval shown here represents proteins that are committed to reaching the native state, while the irregular blue symbol inside the *cis* ring represents proteins that are in the process of folding. The same irregular symbol is used following *cis* complex disassembly to represent proteins that have not reached a committed state and must be rebound for another round of chaperonin-mediated folding.

the *cis* ring, occurring with a rate constant of 0.042 s^{-1} (Burston et al., 1995). The direct examination of this step by FRET (Figure 4) confirms this observation. Moreover, the FRET assay has revealed that there must be an additional fast step ($k_{fast} \approx 2\ s^{-1}$) in the release pathway of GroES from the *cis*-ADP bullet. The simplest incorporation of this fast step places it immediately following the slowest step in the cycle and immediately preceding the formation of a new ATP bullet (Figure 9I). Given these findings, it is attractive to hypothesize that the slow, rate-limiting step is a structural transition of the ADP bullet, indicated schematically in Figure 9I. Passage of the ADP bullet through this slow transition leads to the population of an activated intermediate form of the ADP bullet from which GroES dissociation is rapid. Although a second GroES molecule might bind prior to this dissociation, indicated by the brackets in the model, there is no kinetic requirement in our data for such involvement.

The GroEL cycle in the presence of substrate polypeptide (Figure 9II), likely to be the physiologic state, is also simplified by requiring only one round of ATP hydrolysis per cycle. In addition, the presence of unfolded substrate significantly accelerates the rate at which the *cis* complex dissociates. The rate constant measured for GroES release in the presence of nonnative protein (1–2 s^{-1}) is very similar to that of the fast step described in Figure 9I. This strongly suggests that it corresponds to the same reaction (i.e., the dissociation of GroES from

an activated ADP bullet). In this case, it appears that the unfolded substrate catalyzes the structural rearrangement of the ADP bullet, which is slow in the absence of unfolded protein, so that it is no longer rate limiting in the overall cycle. This directly couples dissociation of one *cis* folding-active complex with the formation of the next. Furthermore, because the binding of unfolded substrate and GroES to the *trans* ring is ordered and sequential, with polypeptide binding first (Figures 6 and 7), the efficient formation of the next *cis* folding chamber is ensured. Once again, the kinetics of the cycle do not suggest the binding of a second GroES before the first departs, but the transient formation of an intermediate with a GroES molecule on each end of the GroEL cylinder cannot be excluded, indicated by the bracketed species.

Effect of Interring Allostery on Polypeptide Release

Following discharge of the *cis*-ADP complex, the vacated ring becomes the *trans* ring of a new ATP bullet complex. As the biochemical and structural studies described above indicate, this ring has no significant affinity for nonnative protein. An incompletely folded substrate protein inside a decaying *cis* complex is, therefore, unlikely to be either retained or immediately recaptured by the apical domains of such a ring. Indeed, not until the slowest step in the reaction cycle (*cis* hydrolysis) has been completed can substrate again bind to this ring. Thus, the allosteric changes transmitted to the opposite ring by formation of a *cis*-ATP complex ensure that the ring from the previous round of folding is completely cleared of substrate protein. Substrate proteins are then ejected into solution and subjected to kinetic partitioning with each turn of the chaperonin cycle (Todd et al., 1994; Weissman et al., 1994; Smith and Fisher, 1995; Taguchi and Yoshida, 1995; Ranson et al., 1997).

Consequences of Polypeptide Binding and Efficiency of the Folding Cycle

The models presented above make a very specific prediction. In the presence of denatured polypeptide, the rate-limiting step of the cycle becomes *cis* hydrolysis (Figure 9II), rather than the slow transition preceding dissociation of the *cis* complex (Figure 9I). Thus the steady-state ATPase rate of the GroEL-GroES system should be stimulated by 2- to 3-fold in the presence of saturating amounts of denatured substrate protein. Stimulation of the ATPase activity of the GroEL-GroES system has indeed been observed in the presence of denatured lactate dehydrogenase (Staniforth et al., 1994). Here also, as shown in Figure 8E, the steady-state ATPase rate of GroEL-GroES is stimulated by 2.3-fold in the presence of saturating quantities of denatured MDH. This result provides specific confirmation of the conclusions drawn from the FRET experiments and demonstrates a significant level of allosteric interaction between the chaperonin system and its substrates.

Whereas the cryo-EM studies presented above provide some indication of the allosteric signal transmitted to the *trans* ring by ATP hydrolysis in the *cis* ring, the structural changes involved in committing GroES to release, a step stimulated by polypeptide binding to the *trans* ring of the ADP bullet, are unknown. However, it seems likely that some of the energy of polypeptide binding to the *trans* ring is employed to accelerate the structural change that is necessary for *cis* complex dissociation. This coupling helps drive the timely disruption of the previous *cis* complex and increases the efficiency of the GroEL machine. Because ATP binding to one ring simultaneously sets up a folding chamber on that ring while discharging the opposite ring, it is apparent that turnover of one ringful of seven ATPs amounts to one cycle of folding. This is at least twice as efficient as a pathway in which GroEL must completely clear its rings of ligands before initiating the next cycle. This efficiency, coupled with the increase in overall rate of the cycle in the presence of nonnative substrate, suggests that GroEL could be responsible for folding a greater fraction of cellular proteins than previously predicted (Lorimer, 1996). It also offers a mechanism by which the effectiveness of GroEL-mediated folding would be increased when the amount of unfolded protein increases, for example, during heat shock.

Experimental Procedures

Proteins

The G315C (EL315C) variant of GroEL places a single cysteine on the exterior of the apical domain of each GroEL subunit, whose endogenous cysteines at positions 138, 458, and 519 have been replaced with alanine. The ES98C version of GroES adds a single cysteine at the COOH terminus of each GroES subunit (Murai et al., 1996). The EL398 mutant of GroEL (D398A) was constructed as previously described (Rye et al., 1997). Wild-type and mutant versions of GroEL and GroES, as well as Rubisco from *Rhodospirillum rubrum*, were expressed in *E. coli* and purified as previously described (Weissman et al., 1995; Rye et al., 1997). Purified pig heart mitochondrial malate dehydrogenase (MDH) was obtained from Boehringer-Mannheim (Indianapolis, IN).

Fluorescent Labeling of EL315C, ES98C, and Rubisco

EL315C was labeled with IAEDANS (Molecular Probes, Inc.) to produce EL315-D (where "D" designates the use of the EDANS fluorophore as an energy transfer donor). ES98C and Rubisco were labeled with fluorescein-5-maleimide (Molecular Probes, Inc.) to produce ES98-A and Rub-A, respectively (where "A" designates fluorescein as an energy transfer acceptor). Labeling reactions were conducted essentially as previously described (Rye et al., 1997). See below for details regarding Supplemental Experimental Procedures.

For Rubisco, a labeling ratio of one fluorescein to one Rubisco monomer was obtained. For EL315-D, an average of 3–4 dye molecules per tetradecamer was achieved, and for ES98-A, approximately 2 dye molecules were added per heptamer. Both labeled and unlabeled EL315C and ES98C were stored in 50 mM Tris (pH 7.4), 100 mM KCl, 0.5 mM EDTA, 4 mM DTT under argon and remained stable for several months. Upon addition of either wild-type GroES or ES98-A, EL315-D refolded Rubisco in a GroES- and ATP-dependent manner that was identical (in both refolding yield and rate) to a wild-type GroEL-GroES reaction. EL315-D did, however, show a 30%–40% reduction in its steady-state ATPase rate. This was not due to the mutation at residue 315 or conjugation with IAEDANS but was the result of changing the endogenous cysteine residues to alanine. Rub-A exhibited wild-type enzymatic activity and refolding.

Gel Filtration of Bullet Complexes

ATP and ADP bullets were formed with EL398 essentially as previously described (Rye et al., 1997). See below for details regarding Supplemental Experimental Procedures. All mixing was done in 50 mM HEPES (pH 7.6), 5 mM KOAc, 10 mM $Mg(OAc)_2$, and 2 mM DTT. Following addition of either denatured Rub-A or ES98-A and ATP to the bullet mixtures, the samples were injected onto a Tosohaas

$G4000SW_{XL}$ HPLC gel filtration column in the same buffer and the elution profile monitored by an in-line fluorescence detector.

Cryo-Electron Microscopy, Three-Dimensional Reconstruction, and Atomic Structure Fitting

Images of GroEL-GroES complexes, vitrified 6 s after addition of 2.5 mM ATP to a mixture of 1 μM each of GroEL and GroES, were collected as previously described (Roseman et al., 1996) and digitized at 6.7 Å/pixel on a Leafscan 45 linear CCD scanner (Ilford Ltd, Cheshire, UK). A data set of 2550 side views contained a mixture of four different structures, identified and separated by a combination of multivariate statistical analysis (van Heel et al., 1996) and angular refinement (Frank et al., 1996; Roseman et al., 1996). The structures were designated as apo, bullet, and football, corresponding to GroEL complexes without GroES, and with one or two GroES molecules, respectively. Two different bullet structures were present, and these were clearly identifiable as *cis*-ADP and *cis*-ATP bullets by their resemblance to preformed *cis*-ADP bullets and to bullet complexes formed in ATP under steady-state conditions, respectively (Roseman et al., 1996).

The positions and orientations of the *trans* apical domains were determined by fitting the apical domain atomic coordinates (*trans* ring from Xu et al., 1997) into the *trans* rings of the *cis*-ATP and *cis*-ADP bullet complexes, using O (Jones et al., 1991) and rigid body refinement in X-PLOR (Brünger, 1992). The fitted structures conformed very well to the density envelope, with the exception of one exposed loop in the ATP bullet (residues 206–214). (See below for details regarding Supplemental Experimental Procedures.)

Stopped-Flow Fluorescence Energy Transfer and Anisotropy

The stopped-flow apparatus has been described (Rye et al., 1997). For fluorescence energy transfer experiments, the excitation wavelength was 336 nm. Both detection channels were fitted with long-pass barrier filters (KV370; Schott). The donor channel was configured with a blue separation filter (SWP filter, Reynard Corporation), and the acceptor channel was configured with a green separation filter (Reynard). All stopped-flow experiments were conducted at 25°C in 50 mM HEPES (pH 7.6), 5 mM KOAc, 10 mM $Mg(OAc)_2$, and 2 mM DTT .

For steady-state dissociation experiments, samples of EL315-D and ES98-A were first mixed together with 5 mM ATP and then mixed in the stopped flow with either excess unlabeled GroES or excess unlabeled GroEL. For ADP bullet dissociation experiments, ADP bullets were formed by first mixing 25 μM EL315-D with 25 μM ES98-A and 1 mM ADP. This mixture was incubated at 23°C in the dark for 1.5–2 hr. Immediately prior to use, the ADP bullet mixture was diluted with buffer and loaded into the stopped flow, then mixed with excess unlabeled GroES and ATP. For control experiments in which ES98-A was replaced with unlabeled GroES in the initial steady-state mix, excess unlabeled GroEL was used to sequester the GroES in the stopped-flow experiments. For control experiments in which EL315-D was replaced with unlabeled GroEL, unlabeled GroES was used as the competitor.

For experiments involving denatured Rubisco or denatured MDH, an additional syringe was employed to hold either acid-denatured Rubisco in 25 mM glycine-phosphate (pH 2.0) or urea-denatured MDH in 5.5 M fresh urea. Mixing was accomplished through the simultaneous push of three syringes, giving the final concentrations of components noted in the figure legends. The final dilution of acid-denatured Rubisco in each case was approximately 10-fold, which was sufficient to neutralize the acid. The final dilution of the urea-denatured MDH was approximately 20-fold.

For experiments involving GFP fluorescence anisotropy, the stopped flow was reconfigured with a pair of crossed polarizers as described previously (Rye et al., 1997). Both detection channels were fitted with long-pass filters (GG495, Schott), and the excitation wavelength was 400 nm. EL398 ATP bullets containing trapped GFP were formed essentially as previously described (Rye et al., 1997). See below for details regarding Supplemental Experimental Procedures. The purified ATP bullets (~2 μM), approximately one-half of which contained a trapped GFP molecule, were then incubated in the dark at 23°C for 1–2 hr in order to permit completion of GFP folding and the hydrolysis of the trapped ATP. This sample was then mixed (1:1) in the stopped flow with 5 mM ATP.

Data Analysis

Data records from the various stopped-flow experiments were first smoothed to remove excess noise and then background corrected. (See below for details regarding Supplemental Experimental Procedures.) Relative fluorescence was then calculated by dividing the data record for the donor plus acceptor by a data record obtained under identical conditions using either the donor only (for donor monitored experiments) or acceptor only (for acceptor monitored experiments). Decay experiments were fit to either single exponentials or sums of exponentials using nonlinear least-squares fitting with IGOR Pro (WaveMetrics Inc). Binding data for the pseudo first-order binding of ES98-A to EL315-D were fit to single exponentials. Binding data obtained for experiments conducted at equivalent concentrations of reactants were fit to the following simplified binding equation (Gutfreund, 1995):

$$\frac{1}{D(t)} - \frac{1}{D(0)} = kt$$

where $D(t)$ is the concentration of unbound EL315-D at time t, $D(0)$ is the concentration of EL315-D at the beginning of the experiment, and k is the bimolecular rate constant for association.

Supplemental Experimental Procedures

For more information on experimental methods, see http://www.cell.com/cgi/content/full/97/3/325/DC1.

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