

# Characterization of the Active Intermediate of a GroEL–GroES-Mediated Protein Folding Reaction

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## Summary

Recent studies of GroE-mediated protein folding indicate that substrate proteins are productively released from a *cis* ternary complex in which the nonnative substrate is sequestered within the GroEL channel underneath GroES. Here, we examine whether protein folding can occur in this space. Stopped-flow fluorescence anisotropy of a pyrene–rhodanese–GroEL complex indicates that addition of GroES and ATP (but not ADP) leads to a rapid change in substrate flexibility at GroEL. Strikingly, when GroES release is blocked by the use of either a nonhydrolyzable ATP analog or a single-ring GroEL mutant, substrates complete folding while remaining associated with chaperonin. We conclude that the *cis* ternary complex, in the presence of ATP, is the active state intermediate in the GroE-mediated folding reaction: folding is initiated in this state and for some substrates may be completed prior to the timed release of GroES triggered by ATP hydrolysis.

## Introduction

Chaperonins are large ring structures that use the energy of ATP hydrolysis to increase the efficiency of protein folding in the cell (for reviews, see Gething and Sambrook, 1992; Hendrick and Hartl, 1993). Members of the chaperonin family play an essential role in mediating folding in the cytosol of both prokaryotes and eukaryotes and inside endosymbiotically derived organelles, mitochondria and chloroplasts. The best characterized chaperonin is GroEL, found in the *Escherichia coli* cytoplasm. It is a homo-oligomeric complex composed of 58 kDa subunits arranged in two seven-membered rings stacked back to back. GroEL binds a wide variety of substrate polypeptides in nonnative form in a central channel ~45 Å in diameter (Braig et al., 1994). Substrate binding appears to be mediated by nonpolar interactions between exposed hydrophobic side chains of the nonnative protein and hydrophobic residues in the terminal, apical domains of GroEL that face the central channel (Fenton et al., 1994; Landry and Gierasch, 1994). Addition of ATP significantly weakens the affinity of GroEL for polypeptide and is sufficient to allow the folding in vitro of some substrates. More generally, however,

GroEL-mediated folding is dependent on the cochaperonin GroES (e.g., Schmidt et al., 1994). GroES is an essential protein composed of a single heptameric ring of 10 kDa subunits that can bind to one or both ends of the GroEL cylinder.

A critical question regarding the mechanism of GroEL action is the extent to which a polypeptide can fold while residing in the central cavity of GroEL (Ellis, 1994). Studies of the GroEL-mediated folding of RUBISCO (Todd et al., 1994), rhodanese (Weissman et al., 1994; Smith and Fisher, 1995; Taguchi and Yoshida, 1995), and mitochondrial malate dehydrogenase (mMDH) (Ranson et al., 1995) revealed that proteins could be released from GroEL in predominantly nonnative forms that could be rapidly rebound by another GroEL complex. In the case of rhodanese, it was found that, during each round of release, polypeptide underwent kinetic partitioning in which ~20% of rhodanese molecules reached the native state. The other ~80% of the molecules, however, were released in nonnative conformations that could be rebound by GroEL mutants that act as polypeptide traps, capable of binding but not releasing substrate. Despite the fact that the majority of rhodanese molecules were released in a nonnative form, it remained possible that a portion of the molecules underwent at least partial folding prior to release (see Discussion in Weissman et al., 1994). In support of this proposal, the ~20% of rhodanese molecules that reached the native state during a round of release appeared to have departed GroEL in a conformation committed to fold, because even high concentrations of a GroEL trap could not inhibit productive folding. Similarly, early studies on the GroEL-mediated folding of dihydrofolate reductase (DHFR) found that, in the presence of GroES, high concentrations of GroEL did not slow the rate of folding (Martin et al., 1991). Finally, for two small proteins, kinetic data on GroEL-mediated folding in the absence of GroES suggested that folding could occur on GroEL (Corrales and Fersht, 1995; Itzhaki et al., 1995).

Recently, it has been shown that substrates are productively released from a *cis* ternary GroEL–GroES–polypeptide complex in which polypeptide and GroES are bound to the same ring of the GroEL double toroid (Weissman et al., 1995). The volume of the central channel of the GroEL ring in contact with GroES is approximately 2-fold greater than that of unliganded GroEL (Chen et al., 1994; Weissman et al., 1995). This increase results from an opening of the GroEL apical domains upward and outward. Moreover, mutagenesis studies suggest that GroES interacts directly with the apical polypeptide-binding region of GroEL (Fenton et al., 1994), probably via a mobile hydrophobic loop segment that extends downward from the overarching GroES cochaperonin (Landry et al., 1993). Whether this enlarged space can accommodate steps of polypeptide folding in the presence of ATP is unknown. In the presence of ADP, however, fluorescence anisotropy studies have suggested that nonnative polypeptide inside this space is held rigidly; correspondingly, biochemical studies indicate that even in the presence of GroES, ADP

does not support the renaturation of substrates whose folding is dependent on the full GroEL–GroES chaperonin system (Mendoza et al., 1991; Martin et al., 1993; Todd et al., 1994; Weissman et al., 1995).

Here, we have examined whether protein folding can occur inside the *cis* ternary complexes in the presence of ATP. For these studies, we have used stopped-flow fluorescence anisotropy to monitor early time-dependent changes in polypeptide flexibility. In addition, we have taken advantage of the fact that polypeptide remains confined within the GroEL central cavity in a single-ring mutant of GroEL unable to release GroES and in wild-type GroEL complexed with GroES in the presence of a nonhydrolyzable ATP analog. This has allowed us to determine whether polypeptide folding can be completed while a substrate resides in the GroEL central channel.

## Results

### Addition of GroES and ATP Leads to a Rapid Increase in Flexibility of a GroEL-Bound Polypeptide

To follow the flexibility of a polypeptide during a chaperonin-mediated folding reaction, we examined the time-dependent changes in the fluorescence polarization anisotropy of a fluorescently labeled substrate polypeptide bound to GroEL. We employed the substrate protein rhodanese, a 33 kDa monomeric protein whose efficient folding is dependent on GroEL, GroES, and ATP (Mendoza et al., 1991; Martin et al., 1991). As in previous steady-state experiments (Weissman et al., 1995), rhodanese was fully labeled on its four cysteine residues with pyrene maleimide, which has a relatively long fluorescence lifetime (observed here to be 20 ns). Rhodanese labeled in this manner cannot fold to native form, limiting these experiments to reporting on early events. Binding of pyrene–rhodanese to GroEL appears to be both specific, as it is saturable, and physiologic, as the bound peptide can be sequestered under GroES (data not shown). It is possible, however, that the pyrene also interacts directly with the chaperonin. Stopped-flow mixing of reaction components allows real-time measurements of changes in the rotational properties of the fluorescently labeled protein (Perez-Howard et al., 1995; Rousseau et al., 1995; Otto et al., 1994). In the absence of fluorescence lifetime changes (a condition met in the present study; see legend to Figure 1), a decrease in steady-state anisotropy reflects an increase in fluorophore rotation, suggesting an increase in polypeptide flexibility.

Strikingly, stopped-flow fluorescence anisotropy studies indicated that addition of ATP and GroES resulted in a rapid decrease in anisotropy ( $r$ ) (Figure 1A). A small decrease, from 0.102 to 0.097, occurred in the dead time of the experiment (10 ms), followed by a larger time-dependent change to a final value of 0.084. The kinetics can be approximated as the sum of two exponentials: a major phase with a half-time of  $\sim 1$  s and a minor phase with a half-time of  $\sim 5$  s. These times are considerably shorter than the previously measured half-times of release of GroES and polypeptide of between 15 s and

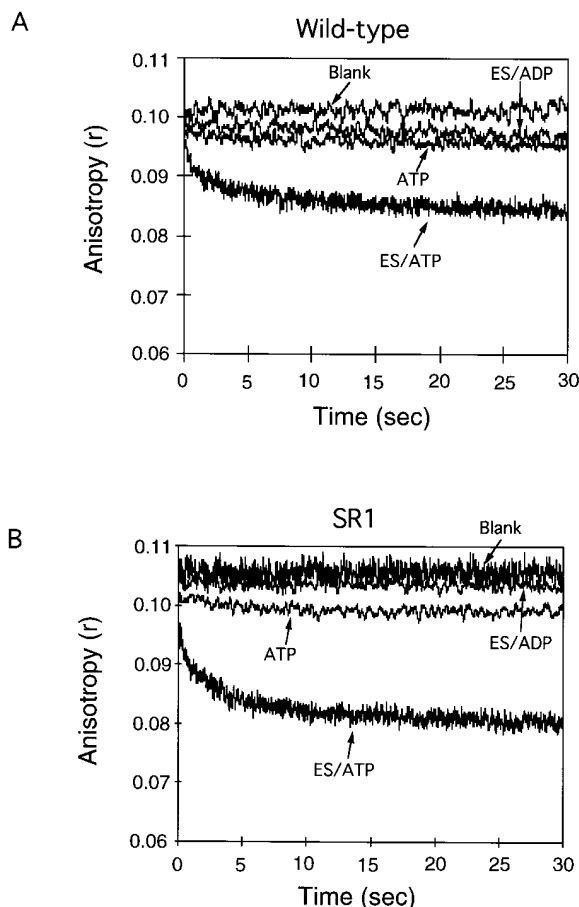


Figure 1. Time Course of Changes in Fluorescence Anisotropy of Pyrene-Labeled Rhodanese Following Addition of Nucleotide and GroES to Binary Complexes

Purified binary complexes were formed between pyrene-labeled rhodanese and either wild-type GroEL (A) or the single-ring mutant (SR1) (B) at a final concentration of 0.5  $\mu$ M GroEL. Samples of these mixtures were reacted by stopped-flow mixing with an equal volume of solutions containing different adenine nucleotides (10 mM) and GroES (1.5  $\mu$ M) or neither (Blank), as indicated. The total fluorescence anisotropy ( $r$ ) is plotted as a function of reaction time. No significant further changes in anisotropy were observed over longer reaction times (5 min). Time-resolved fluorescence anisotropy data collected before addition of ATP/GroES revealed two phases of anisotropy decay, with correlation times of  $\sim 5$  ns (20%) and  $\sim 70$  ns (80%). No significant changes in the fluorescence lifetime occurred under any of the conditions examined.

60 s (Todd et al., 1994; Weissman et al., 1994; Burston et al., 1995; Hayer-Hartl et al., 1995). The rate of change in anisotropy did not depend on the concentration of GroES, suggesting that the decreases reflected conformational changes occurring after GroES binding. Importantly, only a small decrease in anisotropy ( $\sim 0.005$ ) was observed with either ATP alone or GroES and ADP, conditions that are unable to support efficient folding of rhodanese (Mendoza et al., 1991; Martin et al., 1993; see below). These observations argue that the decrease in pyrene anisotropy reflects functionally important conformational changes in polypeptide early in the GroEL-mediated folding reaction.

GroES can bind to a polypeptide–GroEL complex on

either the same GroEL ring as that occupied by polypeptide (*cis*) or the opposite ring (*trans*). Productive folding of a GroES-dependent substrate, however, has been observed only from the *cis* complex (Weissman et al., 1995). Using a designed single-ring mutant of GroEL (Weissman et al., 1995), termed SR1, it was possible to determine whether the increased flexibility observed above could occur in polypeptide bound in the productive *cis* ternary topology, because binding of GroES to SR1 can result only in the formation of *cis* complex. When ATP and GroES were added to a binary complex of pyrene-rhodanese bound to SR1, a rapid decrease in anisotropy was once again observed, with kinetics similar to that seen with the double-ring, wild-type GroEL complex, but with a somewhat larger amplitude (Figure 1B). As with wild-type GroEL, addition of GroES/ADP or ATP alone produced only a small decrease in anisotropy. These data suggest strongly that, in the wild-type GroEL reaction, polypeptide residing under GroES in the productive *cis* topology increased in flexibility in the presence of ATP.

#### Rhodanese Is Trapped in a Sequestered Position under GroES in SR1

The wild-type GroEL-GroES complex is highly dynamic, as hydrolysis of ATP in the *trans* GroEL ring triggers the release of GroES (Todd et al., 1994). By contrast, the SR1 single-ring mutant of GroEL does not readily release GroES, even in the presence of ATP (Weissman et al., 1995). This slow rate of release, which is most pronounced under low salt conditions, is likely to be a consequence of the absence of a ring in *trans* from which to communicate a signal for release. Consistent with this, addition of GroES to SR1 completely inhibited continued ATP hydrolysis (Weissman et al., 1995). We first examined both the stoichiometry of GroES binding and the possibility that SR1 could transiently oligomerize into double rings in the presence of ATP and GroES by carrying out a Hummel-Dreyer experiment (Hummel and Dreyer, 1962), in which metabolically labeled [<sup>35</sup>S]GroES and ATP were present in running buffer during high pressure liquid chromatography (HPLC) gel filtration (Figure 2A). For both SR1 and wild-type GroEL, a stoichiometry of one GroES bound per chaperonin complex was observed (i.e., one GroES heptamer per single ring of SR1 or per double ring of wild-type GroEL). Importantly, these studies also demonstrated that GroES binding to the SR1 mutant did not induce the formation of detectable double-ring structures: the elution times of the SR1-GroES complex and of free SR1 were identical (cf. Todd et al., 1995).

We next examined the release of rhodanese from SR1 in the presence of ATP and GroES by gel filtration and observed that, once such ternary complexes were

(C) Protease sensitivity of rhodanese-SR1 complexes. Solutions containing [<sup>35</sup>S]rhodanese-SR1 complexes and a 2-fold excess of GroES were subjected to trypsin treatment (30 μg/ml) for 30 s. GroES and ATP, which is required for the binding of GroES, was present or absent as indicated. The fraction of full-length, undigested rhodanese remaining, relative to the control reaction without trypsin, is plotted.

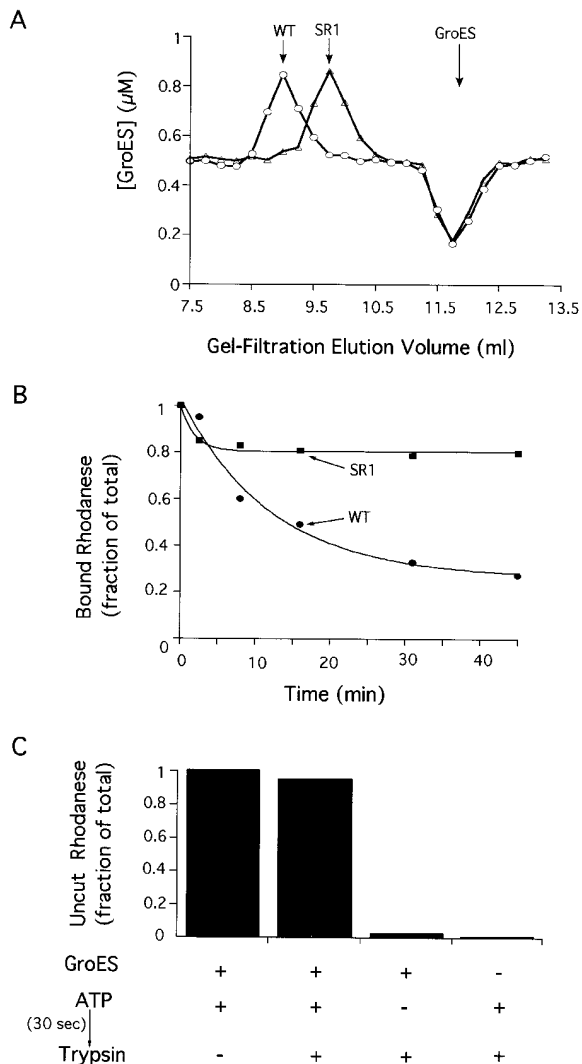


Figure 2. GroES and Rhodanese Form a Stable Ternary Complex with SR1

(A) Determination of stoichiometry and oligomerization state of GroES complexed with SR1 and wild-type GroEL by a Hummel-Dreyer experiment (Hummel and Dreyer, 1962). An HPLC gel filtration column was equilibrated with 0.5 μM [<sup>35</sup>S]GroES and 1 mM ATP in buffer A. Either 1 nmol SR1 heptamer or 1 nmol GroEL tetradecamer, as indicated, was loaded onto the column. The concentration of GroES present, determined by scintillation counting of 0.25 ml fractions, is plotted as a function of elution volume. The total amounts of GroES that coeluted with either SR1 or wild-type GroEL, and missing from the GroES elution position, were equal, and represented 1 ± 0.15 nmol of GroES, establishing a 1:1 stoichiometry for these complexes. The elution positions of wild-type (WT) GroEL, SR1, and GroES are indicated by arrows.

(B) Fraction of rhodanese associated with SR1 and wild-type GroEL as a function of incubation time with GroES and ATP. Binary complexes of [<sup>35</sup>S]rhodanese with SR1 or wild-type GroEL (WT) were incubated with a 2-fold excess of GroES and 5 mM ATP in buffer B. At various times, an aliquot was removed and subjected to gel filtration. The SR1 or GroEL peaks were collected, and the amount of rhodanese bound was determined by scintillation counting. The values are presented as the fraction of the zero timepoint (taken before addition of GroES and ATP). Except for the zero timepoint, each recorded time is the time at which the peak eluted from the column (i.e., the 2.5 min point represents an aliquot removed and loaded on the column immediately after mixing).

formed, the substrate remained trapped underneath GroES (Figures 2B and 2C). For these studies, metabolically labeled [ $^{35}$ S]rhodanese was first bound to SR1, and then ATP and GroES were added. At various times, a portion of the reaction was removed and passed over a gel filtration column to separate SR1-bound rhodanese from unbound rhodanese. About 20% of the rhodanese molecules were released from SR1 by the time of elution of the first timepoint (2.5 min). Longer incubation, however, did not result in further release of polypeptide. This failure of rhodanese to dissociate from SR1 was not the result of a constant release and rebinding process, as GroES binding to SR1 occludes the GroEL central cavity, thereby preventing substrate rebinding. Consistent with the proposal that rhodanese was sequestered in the central channel of SR1 under GroES, ~90% of SR1-bound rhodanese became protected from proteolysis within 30 s after addition of GroES and ATP (Figure 2C).

#### Rhodanese Trapped within the SR1-GroES Complex Reaches the Native State in the Presence of ATP

The failure of SR1 to release rhodanese even in the presence of GroES and ATP allowed us to determine directly the extent to which the polypeptide could fold while sequestered under GroES. Strikingly, we found that addition of ATP and GroES to SR1-rhodanese complexes resulted in efficient reactivation of rhodanese with a  $t_{1/2}$  of ~7 min (Figure 3A). Moreover, the kinetics of rhodanese refolding resembled that from the wild-type GroEL reaction, despite the fact that, unlike SR1, wild type undergoes cycling between high and low affinity states for polypeptide. The similar kinetics may reflect the fact that, in the wild-type reaction, GroEL spends the majority of the time complexed with GroES (Burston et al., 1995).

As with wild-type GroEL, and consistent with the fluorescence anisotropy results, SR1-mediated refolding of rhodanese required both GroES and ATP (Figure 3A). Only very slow production of active rhodanese was observed in the presence of GroES and ADP (Figure 3A), even though SR1 efficiently bound GroES in the presence of ADP. The similarity between SR1 and wild-type GroEL in both the kinetics of folding and the requirement for GroES and ATP suggests that folding mediated by SR1 is physiologically relevant.

Gel filtration analysis confirmed that the native rhodanese generated from SR1-rhodanese complex remained associated with SR1. For this analysis, a folding reaction was initiated as before, and aliquots of the reaction were passed over a gel filtration column, capable of separating 33 kDa native rhodanese from the 400 kDa SR1 complex in ~2 min, before assaying rhodanese. The time course of the generation of rhodanese activity associated with the SR1 peak (Figure 3B) revealed a steady increase in activity that paralleled the regeneration of total rhodanese activity seen in Figure 3A. At the final timepoint (45 min), >80% of the native rhodanese was bound to SR1. By contrast, no more than 3% of the rhodanese activity generated during a folding reaction from wild-type GroEL comigrated with the GroEL peak. The association of active rhodanese with SR1 was not

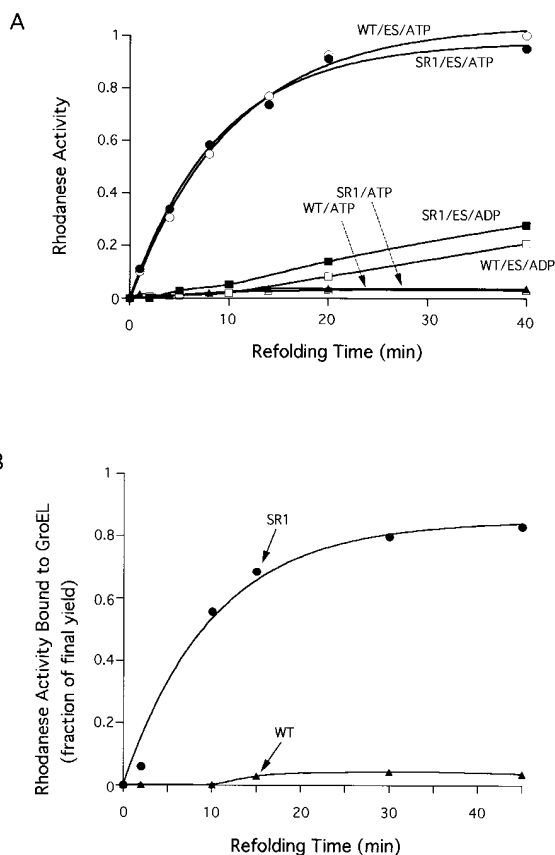


Figure 3. Rhodanese Refolding in Association with SR1-GroES Complexes

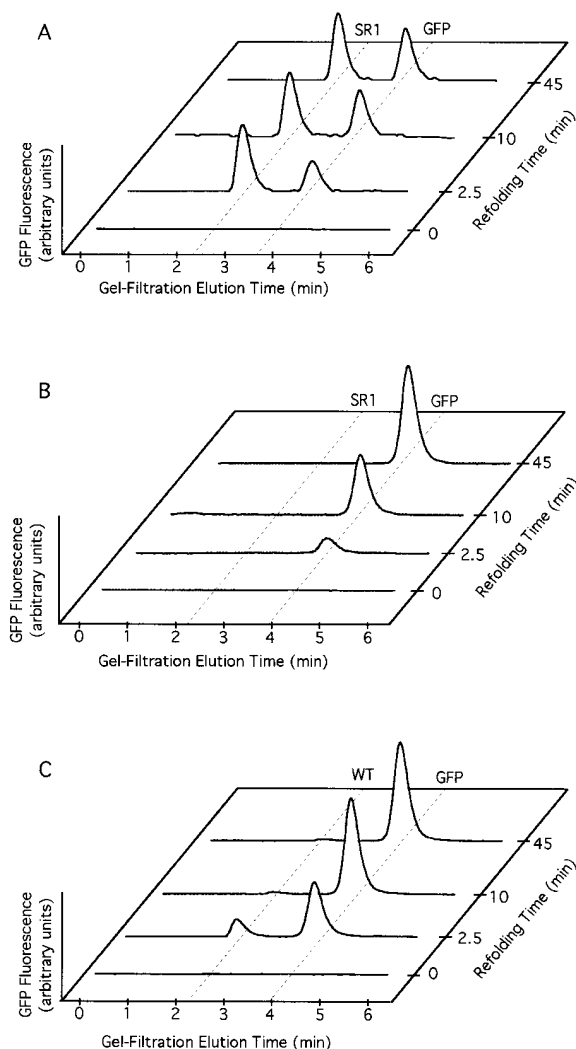
(A) Time course of the total yield of rhodanese activity from wild-type GroEL (WT) or SR1 in the presence of nucleotide plus or minus GroES, as indicated. Activity is expressed as a fraction of the final yield from wild-type GroEL at 45 min, which represents ~70% of the input material.

(B) Determination of rhodanese activity associated with wild-type GroEL (WT) or SR1. Except for the zero timepoint, each recorded time is the time at which the peak eluted from the column. The rhodanese activity coeluting with the GroEL complexes at each timepoint is plotted as a fraction of the total yield of rhodanese activity generated in 45 min as measured directly from the unfractionated reaction mixture.

due to adventitious binding of native rhodanese, because mixing native rhodanese with SR1 resulted in comigration of <1.0% of the rhodanese activity with SR1.

#### GFP in the SR1-GroES Complex Acquires Native Fluorescence but Does Not Tumble Freely

Examination of SR1-mediated refolding of green fluorescent protein (GFP) revealed that, like rhodanese, it was able to reach a native conformation within the central cavity of SR1. GFP is a convenient substrate for these studies, as it is a monomeric protein whose intrinsic fluorophore shows no detectable fluorescence in the denatured state (Ward and Bokman, 1982). In the native state, as shown previously, the fluorophore is buried (Nageswara Rao et al., 1980), and its fluorescence is affected by mutations distant in the primary sequence, indicating that it is sensitive to the tertiary structure of



**Figure 4. Time Course of GFP Folding in Association with GroEL.**  
**(A)** Time course of folding of GFP–SR1 complexes in the presence of GroES and ATP. Folding was initiated by the addition of GroES and ATP to GFP–SR1 complexes. At various times, an aliquot was removed and subjected to gel filtration. The fluorescence intensity as a function of elution time for each folding timepoint is shown. Except for the zero timepoint, each recorded time is the time at which the GroEL peak eluted from the column. The dotted lines mark the elution time of SR1 and free GFP as indicated. Note that at zero time, before addition of GroES and ATP, there was no fluorescence detected. The amount of fluorescence eluting with SR1 remained constant after the 2.5 min timepoint, while the amount eluting with free GFP increased slowly, consistent with a portion of the GFP folding in solution after release from SR1. At the longest timepoint, ~60% of the fluorescence intensity eluted with SR1.  
**(B)** Time course of folding of GFP–SR1 complexes in the presence of ATP only. Experiment was conducted as in (A), except that GroES was omitted.  
**(C)** Time course of folding of GFP–GroEL complex in the presence of ATP and GroES. The dotted lines mark the elution time of wild-type GroEL (WT) and free GFP as indicated.

the protein (Cubitt et al., 1995). Thus, folding can be monitored readily by recovery of fluorescence. Both SR1 and wild-type GroEL efficiently bound acid-denatured, but not native, GFP. The bound GFP was nonnative as judged by both the absence of fluorescence (Figure 4,

zero timepoints) and the high sensitivity of the bound protein to protease, as compared with native GFP (data not shown). Addition of GroES and ATP resulted in a rapid regeneration of GFP fluorescence ( $t_{1/2} \approx 1$  min). In the case of SR1, gel filtration of the refolding mixture indicated that ~60% of the refolded GFP remained associated with SR1 (Figure 4A). Once formed, the complex between SR1, GroES, and refolded GFP was very stable, as there was no detectable loss of SR1-bound fluorescence even after 45 min of incubation (Figure 4A). In contrast with rhodanese, addition of ATP alone to an SR1–GFP complex, in the absence of GroES, promoted GFP refolding, albeit at a reduced rate ( $t_{1/2} \approx 10$  min). In the absence of GroES, however, none of the refolded GFP remained associated with SR1 (Figure 4B), consistent with the proposal that GroES was acting as a cap in preventing the egress of folded GFP from the GroEL central cavity (Agard, 1993). Addition of ATP and GroES to a binary complex between wild-type GroEL and unfolded GFP also resulted in the rapid regeneration of GFP fluorescence (Figure 4C). At the earliest timepoint (2.5 min), a small fraction of the fluorescence comigrated with GroEL. In contrast with SR1, however, almost none of the GFP activity remained associated with wild-type GroEL at later timepoints.

To address whether fluorescent GFP associated with SR1 was fully native, we characterized its fluorescence properties further. The fluorescence excitation (data not shown) and emission (Figure 5A) spectra of SR1-bound refolded GFP were found to be identical to native GFP free in solution. Similarly, the fluorescence lifetimes of refolded, SR1-bound GFP (3.16 ns) and free GFP (3.21 ns) were essentially indistinguishable (Figure 5B). As both fluorescence emission spectra and fluorescence lifetimes are highly sensitive to the local environment of the fluorophore, these observations argued strongly that the SR1-bound refolded GFP had attained a native conformation, at least in the region of the fluorophore.

Examination of the rate of fluorescence anisotropy decay, however, revealed that the native GFP sequestered in the SR1 central cavity was not freely tumbling. In contrast with fluorescence intensity decay, the rate of decay of the fluorescence anisotropy is not generally sensitive to the static environment of the fluorophore but rather reports on the rate at which the molecule is rotating. The rate of anisotropy decay of free native GFP in solution was observed to be considerably greater than that of native GFP bound at SR1 (Figure 5C). In particular, free GFP rotated with a correlation time of 13.2 ns. Assuming the molecule is spherical, this corresponds to a molecular mass of 32 kDa, in excellent agreement with the actual mass of 27 kDa. By contrast, native GFP sequestered under GroES in the SR1 cavity rotated with a correlation time of 54 ns, corresponding to an effective molecular mass of  $120 \pm 30$  kDa. Thus, the movement of refolded GFP within the SR1–GroES complex was significantly hindered.

#### Folding of Rhodanese to Native Form within a Wild-Type GroEL–GroES Complex

While the above studies demonstrated that a polypeptide could efficiently fold within the central cavity of SR1,

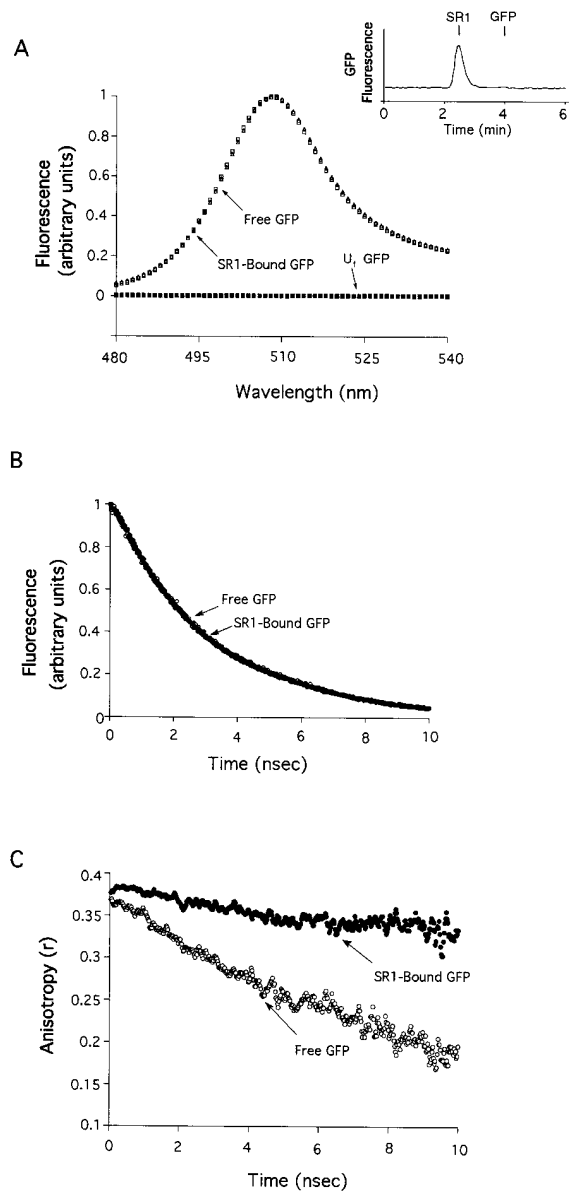


Figure 5. Fluorescence Properties of Chaperonin-Refolded GFP Complexes

(A) Fluorescence emission spectra of SR1-bound and free refolded GFP. SR1-bound, refolded GFP was recovered from HPLC gel filtration. Re-injection of GFP-SR1 complex demonstrated that the fluorescent GFP remained associated with SR1 (inset). Fluorescence emission spectra were recorded with an excitation wavelength of 400 nm. The spectrum of acid-unfolded GFP (U<sub>i</sub> GFP) was also recorded for comparison.

(B) The fluorescence decays of free native GFP and SR1-bound refolded GFP. Free native GFP and SR1-bound refolded GFP were formed as in (A), and their fluorescence lifetimes were determined on a pulsed-laser, time-resolved fluorimeter. The lifetimes were calculated to be 3.16 ns and 3.21 ns for SR1-bound and free GFP, respectively.

(C) The fluorescence anisotropy decay of free native GFP and SR1-bound refolded GFP. Free GFP and SR1-bound refolded GFP were formed as in (A), and the rate of decay of their fluorescence anisotropy (r) was measured on a pulsed-laser, time-resolved fluorimeter. The correlation times of SR1-bound refolded GFP and free GFP are 54 ns and 13.2 ns, respectively. The latter value is appropriate for a 27 kDa protein, such as GFP, while the former value implies that

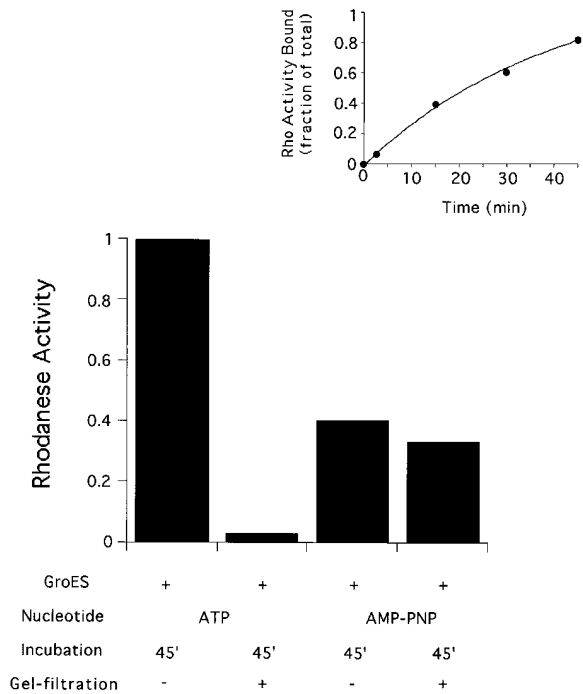


Figure 6. Rhodanese Refolding in Association with Wild-Type GroEL in the Presence of AMP-PNP and GroES

Folding was initiated with the addition of GroES and 5 mM ATP or AMP-PNP, as indicated, to GroEL-rhodanese complex. After 45 min, the samples were either assayed directly or subjected to gel filtration. The bar graph shows the total yield of rhodanese activity or the amount of activity coeluting with GroEL, as indicated. The yield is the fraction of total activity generated after 45 min of incubation with ATP and GroES. The inset shows the time course, in AMP-PNP, of recovery of activity associated with chaperonin following gel filtration relative to the total amount of activity recovered in AMP-PNP at 45 min.

it remained possible that this behavior was a result of the four altered residues in SR1 and did not reflect physiologic behavior of wild-type GroEL. To address this possibility, we carried out a rhodanese folding reaction at wild-type GroEL in the presence of the nonhydrolyzable analog of ATP, AMP-PNP. Because hydrolysis of ATP is specifically required to induce the release of GroES (Todd et al., 1994), folding in the presence of AMP-PNP might result in the trapping of a fraction of bound polypeptide under GroES, thereby recapitulating the behavior of SR1. Accordingly, GroES and AMP-PNP were added to a preformed complex between rhodanese and wild-type GroEL. Under these conditions, roughly half of the bound rhodanese would reside in asymmetric *cis* ternary complexes. Consistent with this and with previous results (Mendoza et al., 1991), we observed an efficiency of folding of ~40% of that seen with GroES and ATP. Protease protection experiments indicated that productive folding occurred exclusively in *cis* complexes (data not shown). Strikingly, in contrast with ATP-mediated folding, in which ~3% of the native rhodanese

the SR1-sequestered GFP is greatly restrained in its tumbling in the central space under GroES.

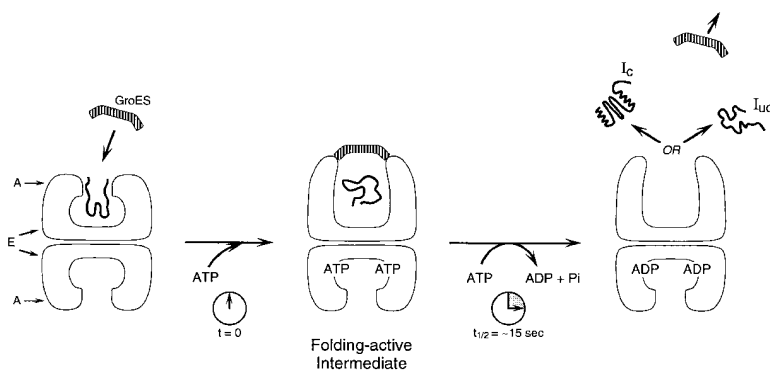


Figure 7. Schematic of Formation and Timed Decay of the Active Folding Intermediate in a GroEL-GroES Folding Reaction

In the presence of nucleotide, GroES can bind in *cis* to the polypeptide-GroEL binary complex (left). Alternatively, this state could be reached through a symmetric, "football," intermediate (e.g., Azem et al., 1995; Llorca et al., 1996). In either case, the polypeptide is sequestered beneath GroES. Conformational changes, which initially require the presence of ATP (see Discussion), occur rapidly in the *cis* ring and initiate polypeptide folding underneath GroES (middle). Simultaneously, binding of ATP in the *trans* ring starts the timer for hydrolysis and release. When ATP

hydrolysis occurs in the *trans* ring ( $t_{1/2} \approx 15$  s), GroES is released (Todd et al., 1994; Burston et al., 1995; Hayer-Hartl et al., 1995), giving polypeptide the opportunity to depart and allowing rebinding of ATP. The released polypeptide is either committed to fold (or already folded) ( $I_c$ ) or in an uncommitted state ( $I_{uc}$ ), which can rebound to the same or a different GroEL complex and undergo another round of folding upon ATP/GroES binding and release (right). The apical (A) and equatorial (E) domains of GroEL are indicated.

was associated with GroEL, in the presence of AMP-PNP, >80% of the regenerated activity remained associated with GroEL (Figure 6). These observations argue strongly that the efficient refolding observed under GroES at the SR1 mutant and at wild-type GroEL in the presence of AMP-PNP reflects a bona fide intermediate state during an ATP-mediated folding reaction.

## Discussion

### Protein Folding in *cis* Ternary Complexes in the Presence of ATP

Recent studies on the mechanism of GroEL-GroES-mediated folding have shown that substrates are productively released from a *cis* ternary complex in which polypeptide is sequestered within an enlarged GroEL central cavity under GroES (Weissman et al., 1995). Evidence presented here indicates that protein folding generally initiates, and can potentially be completed, in this location (Figure 7). First, fluorescence anisotropy measurements indicate that GroEL-bound polypeptide undergoes a rapid increase in flexibility upon addition of ATP and GroES, on a time scale about 10-fold faster than release of GroES and polypeptide (Todd et al., 1994; Weissman et al., 1994; Burston et al., 1995; Hayer-Hartl et al., 1995). The functional significance of this increased flexibility is supported by the finding that it occurs only under conditions where efficient productive folding is observed (i.e., in the presence of both ATP and GroES). Second, similar increases in substrate flexibility are observed with the SR1 mutant. Because SR1 contains only a single ring, GroES binding to an SR1-polypeptide complex necessarily results in the formation of a *cis* ternary conformation in which GroES and polypeptide bind to the same GroEL ring. Third, in the SR1 mutant, which is defective in GroES release, efficient polypeptide folding to native form occurs while the polypeptide remains under GroES. Finally, in wild-type GroEL, folding to native form under GroES is observed when AMP-PNP, a nonhydrolyzable analog of ATP that does not induce release of GroES, is employed.

While *cis* ternary complexes formed in the presence of ATP or a nonhydrolyzable analog are active in promoting polypeptide folding, *cis* ternary complexes formed in

the presence of ADP do not support efficient folding of rhodanese from either wild type or SR1 mutant. Concomitantly, only small changes in flexibility are observed by stopped-flow fluorescence anisotropy in the presence of GroES and ADP. Interestingly, the continued presence of ATP in the *cis* ring does not appear to be required for folding because the SR1 ternary complex catalyzes only a single round of ATP hydrolysis, leaving nonexchangeable ADP in the complex (O. Kovalenko, unpublished data). The nature of the conformational changes as well as the exact nucleotide dependence for the switch from the folding inactive to the folding active *cis* ternary complex remains unresolved.

Several considerations suggest that polypeptide folding reactions initiated within the GroEL-GroES cavity could differ significantly from the corresponding reactions free in solution. In particular, our time-resolved anisotropy experiments indicate that the walls of the cavity are capable of continued interaction with the substrate, as the rate of tumbling of refolded GFP in SR1 is slowed greatly compared with that of native GFP in solution. Such continued interactions are likely to have a far greater impact on relatively labile protein folding intermediates than on the folded protein. Regardless of the nature of interaction with the walls of the cavity, the mere act of confining the polypeptide is likely to alter the spectrum of folding intermediates, because formation of intermediates with an expanded volume will be prevented. In particular, theoretical studies argue that such a confinement of a folding reaction could lead to substantial stabilization of secondary structure (e.g., see Yee et al., 1994).

In addition to preventing aggregation, GroEL appears to be able to alter folding reactions more actively. For example, GroEL can rescue nonnative folding intermediates of both RUBISCO and mMDH that only slowly form irreversible aggregates but nonetheless do not readily reach the native state in the absence of chaperonin (Todd et al., 1994; Peralta et al., 1994). Moreover, GroEL can substantially enhance the folding of mMDH at stoichiometries as low as 1:20 (Ranson et al., 1995). Under such conditions, at least 95% of the substrate resides in solution, so that the effect on the rate of aggregation by sequestration of peptide is negligible. Given such

observations and the present findings, it seems likely that the unique environment provided by the GroEL central cavity, in addition to its ability to capture and perhaps unfold misfolded species (Jackson et al., 1993), allows GroEL to play an active role in a protein folding reaction.

#### ATP Hydrolysis Acts as a Timer Inducing Release from *cis* Complexes

Although both rhodanese and GFP are able to refold to native form while sequestered in the GroEL central cavity under GroES, these observations do not imply that folding is necessarily completed in this topology. Unlike the SR1 mutant, the *cis* ternary active state at wild-type GroEL has a finite half-time of 15–30 s before ATP hydrolysis in the *trans* ring releases GroES (Todd et al., 1994), providing the substrate polypeptide with an opportunity to exit (Figure 7; Agard, 1993). The released substrate will either be rebound or complete folding, oligomerization, or both, in solution.

The significance of the 15–30 s half-time for ATP-induced release of GroES is likely to reflect a compromise among the folding times of the many different GroEL substrates. A faster timer would permit a smaller fraction of bound substrate proteins to reach the native state with each round of ATP binding/hydrolysis: this would either result in a greater fraction of folding in solution, carry a higher ATP cost per mole of native protein generated, or do both. A slower timer, by contrast, would act to restrain proteins that had proceeded fully to native conformation from release to intracellular destinations, e.g., to oligomeric assemblies, resulting in slowing of the overall rates of polypeptide assembly and inefficient utilization of the chaperonin complex.

The nature of the constraints on the ATP timer are illustrated in the GroEL-mediated folding of two well-characterized substrates, OTC and rhodanese. The evolved setpoint allows for commitment of a large fraction of OTC subunits to an assembly-competent state at chaperonin prior to a single event of release: the released subunits occupy a conformation that is no longer recognizable by GroEL but which is competent to assemble into active trimer (Zheng et al., 1993). In contrast, only a small fraction of rhodanese molecules have reached a conformation that is either native or is committed to reach the native state by the time of GroES release (Martin et al., 1991; Mendoza et al., 1991; Weissman et al., 1994). The remainder of rhodanese molecules are fated to rebind to the same or different chaperonin complexes.

#### A Functional Role for the Release of Nonnative Forms

A number of *in vitro* experiments have demonstrated the release of nonnative polypeptides from GroEL, e.g., rhodanese (Weissman et al., 1994; Smith and Fisher, 1995; Taguchi and Yoshida, 1995), RUBISCO (Todd et al., 1994), and mMDH (Ranson et al., 1995). In addition, two proteins that normally do not interact *in vivo* with Hsp60–GroEL, actin and tubulin, are bound and released, but do not reach native form (Tian et al., 1995). That release of nonnative forms also occurs *in vivo* is

supported by a recent experiment observing departure of nonnative rhodanese from a GroEL binary complex in an undiluted *Xenopus* oocyte extract upon addition of GroES and ATP (J. S. W. and A. L. H., unpublished data).

If, as observed here, polypeptides can fold within GroEL, why should there be release into the bulk solution of forms that have not yet reached the native state, placing them at risk for aggregation? Considerations of cell biology suggest that release of nonnative forms is critical to the physiologic function of GroEL. First, many of the *in vivo* substrates of Hsp60–GroEL are subunits of oligomeric complexes (Horwich et al., 1993); not only must such proteins be released for assembly to occur, but often the released subunits must occupy a partially nonnative form to oligomerize efficiently. Second, release of nonnative forms gives substrate proteins the opportunity to interact with other chaperone systems. For example, the 60 kDa protein firefly luciferase forms a stable complex with GroEL *in vitro* but does not reach the native state even in the presence of GroES and ATP (Schroder et al., 1993). By contrast, luciferase is efficiently refolded by the bacterial Hsp70 system, the triad of DnaK, DnaJ, and GrpE, even when initially bound at GroEL (B. Bukau, personal communication). These observations indicate that there can be release of nonnative luciferase from GroEL followed by binding and productive folding by the Hsp70 system. Third, release of mutant or damaged proteins is likely to play an important role in allowing for their proteolytic removal from the cell. Recent studies, for example, have demonstrated delivery of a nonnative protein from GroEL–GroES to the ClpP proteolytic system in *E. coli* (Kandror et al., 1994). For any of the foregoing protein populations, failure to be efficiently released in nonnative form would lead in short order to saturation of all of the GroEL in the cell.

Finally, a significant fraction of cellular proteins are too large to be accommodated under GroES. Such substrates could only be bound in *trans* complexes in which GroES and polypeptide occupy opposite GroEL rings. As such, they would have access to a channel only approximately half the volume of that on the *cis*-bound side under GroES. Thus, volume constraints alone would preclude the possibility of completing folding within the GroEL channel. Whether GroEL can offer such substrates assistance, perhaps through binding and local unfolding, remains to be seen.

#### Experimental Procedures

##### Proteins

Bovine rhodanese, GroEL, GroES, and the single-ring mutant SR1 were expressed in *E. coli* and purified as described previously (Weissman et al., 1995), as were proteins metabolically labeled with [<sup>35</sup>S]methionine. GFP was purchased from Clontech and was judged to be ~90% pure by SDS–PAGE. Unless otherwise stated, all experiments were carried out at 23°C, and all gel filtration separations were effected with tandem Tosohaas “guard” columns (part number 08543) on a Waters HPLC station.

##### GFP Refolding

All GFP refolding experiments were carried out in buffer A (25 mM Tris–HCl [pH 7.4], 12 mM MgCl<sub>2</sub>, 5 mM KCl, 5 mM DTT) at 23°C. Relatively low KCl concentrations were employed to increase the

stability of the SR1–GroES complex. Complex between denatured GFP and GroEL was produced by adding 10  $\mu\text{g}$  of GFP, denatured in 10  $\mu\text{l}$  of 50 mM glycine-phosphate (pH 2.5) to 400  $\mu\text{g}$  of GroEL or SR1 in 200  $\mu\text{l}$  of buffer A at 37°C, followed by centrifugation. GFP–chaperonin complex was then isolated from unbound GFP by gel filtration and concentrated to 500  $\mu\text{g}/\text{ml}$ . Folding reactions were initiated by the addition of >2-fold molar excess of GroES or 5 mM ATP (or both), as indicated. At various times, an aliquot was removed and subjected to gel filtration analysis. GFP fluorescence was monitored by an on-line fluorescence flow detector (Applied Biosystems 980) with an excitation wavelength of 370 nm and a 480 nm longpass emission filter. For the fluorescence spectra, intensity lifetimes, and anisotropy decay measurements, refolded GFP bound to SR1 was generated by initiating a folding reaction of SR1-bound GFP with GroES and ATP, as above, followed by gel filtration after 5 min to remove unbound folded GFP. Fluorescence spectra were taken on a Quanta Master Steady State Spectrophotometer (Photon Technology International) with an excitation wavelength of 400 nm.

#### Rhodanese Folding

All rhodanese folding studies were carried out at 23°C in buffer B (25 mM Tris–HCl [pH 7.4], 12 mM  $\text{MgCl}_2$ , 5 mM KCl, 5 mM DTT, 20 mM sodium thiosulfate). The kinetics of refolding at SR1 and wild type without thiosulfate were similar to those in its presence, except that the yields in both reactions were reduced to a similar extent. Complexes between denatured rhodanese and GroEL or SR1 were produced as described previously (Weissman et al., 1994) with the exception that the rhodanese was denatured in 7 M urea rather than guanidine–HCl. Reactions were initiated by adding GroES (>2-fold molar excess with respect to total GroEL concentration) and 5 mM ATP, AMP–PNP, or ADP, as indicated. At various times, reactions were quenched by addition of CDTA to 20 mM, and rhodanese enzyme activity was determined (Tandon and Horowitz, 1989).

To detect refolded rhodanese associated with wild-type GroEL or SR1, aliquots of reaction mixtures were passed over a gel filtration column equilibrated in buffer B. The GroEL-containing fraction was collected and assayed directly for rhodanese activity. Native rhodanese (elution time 3.9 min) was well resolved from both wild-type GroEL (elution time 2.15 min) and SR1 (elution time 2.3 min). To determine the total rhodanese activity generated, an aliquot of the folding mixture was diluted directly into buffer B and assayed. The fraction of rhodanese activity bound to GroEL was corrected for the efficiency of recovery of GroEL from the column (~75%).

The association of rhodanese with GroEL during a folding reaction was determined by gel filtration. [ $^{35}\text{S}$ ]rhodanese was used, and the fraction of bound rhodanese was determined by scintillation counting.

Protease protection of rhodanese in SR1 ternary complex was assessed by adding GroES (1  $\mu\text{M}$ ) or ATP (5 mM) to a 0.5  $\mu\text{M}$  solution of [ $^{35}\text{S}$ ]rhodanese–SR1 complex in buffer A. After 30 s, trypsin was added to a final concentration of 30  $\mu\text{g}/\text{ml}$ . The protease reaction was stopped after 30 s with the addition of 300  $\mu\text{g}/\text{ml}$  soybean trypsin inhibitor (Worthington). The samples were subjected to SDS–PAGE, and the fraction of undigested rhodanese was determined by phosphorimager analysis (Molecular Dynamics).

#### GroES Binding

For the Hummel–Dreyer experiments, separations were carried out on a Tosoh Haas G4000SW $_{\mu}$  column equilibrated with buffer A supplemented with [ $^{35}\text{S}$ ]GroES (0.5  $\mu\text{M}$ , 20,000 cpm/ $\mu\text{l}$ ). GroEL (1 nmol) or SR1 complex (1 nmol) was incubated in 500  $\mu\text{l}$  of running buffer for 5 min prior to injection on the column. Fractions of 250  $\mu\text{l}$  were collected, and the concentration of GroES present was determined by scintillation counting.

#### Stopped-Flow Fluorescence Anisotropy

Measurement of fluorescence anisotropy changes by stopped flow was performed with an SFM-3 stopped-flow unit (Molecular Kinetics, Pullman, WA) with a 50  $\mu\text{l}$  FC.15 fluorescence cuvette and a fluorescence detection system (Otto et al., 1994). For the pyrene-labeled rhodanese (Weissman et al., 1995), the excitation monochromator was set to 347 nm, and 380 nm longpass glass filters (Hoya Optics, Fremont, CA) were utilized in each detection channel. Complexes

between pyrene-labeled rhodanese and either wild-type GroEL or SR1 were purified by anion exchange chromatography (Mono Q, Pharmacia), exchanged into buffer A, concentrated to 0.5  $\mu\text{M}$ , and loaded into one syringe of the stopped-flow device. A solution of GroES (0.75–5  $\mu\text{M}$ ) and ADP or ATP (10 mM) in buffer A was loaded into the second syringe. Reactions were initiated by mixing equal volumes from each syringe (typically 100  $\mu\text{l}$ ). Fluorescence emission was simultaneously monitored in both the parallel ( $I_{\text{parallel}}$ ) and perpendicular orientations ( $I_{\text{perpendicular}}$ ) at 10 ms intervals, and the anisotropy ( $r$ ) was calculated as  $(I_{\text{parallel}} - I_{\text{perpendicular}})/(I_{\text{parallel}} + 2 I_{\text{perpendicular}})$ . G factor correction of the instrument and data analysis were conducted as previously outlined (Otto et al., 1994). Each experiment involved the summation of 25–50 individual runs.

#### Time-Resolved Fluorescence Anisotropy Decay

The measurement of the fluorescence anisotropy decay of free GFP and SR1-bound GFP was conducted essentially as previously described (Perez-Howard et al., 1995), using 30 nM free native GFP in buffer A or refolded GFP–SR1–GroES ternary complex, generated as described above. The laser was a Coherent Mira 900F titanium sapphire laser pumped by a Coherent Innova 304 argon ion laser operating on all lines at 8 W. The short wavelength mirror set was installed, and the wavelength was set to 800 nm. The second harmonic of the Mira emission was generated by a Coherent 4500 BBO frequency doubler. The average power at 400 nm was approximately 3 mW. The emission monochromator was at 515 nm; the emission filter was a Hoya Y46; the TAC was set for 0.1  $\mu\text{s}$  full scale, and the digitizer was at 11 ps per channel. The correlation times were determined using the Globals software package (Beechem, 1992).

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