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# Toward a mechanism for GroEL·GroES chaperone activity: An ATPase-gated and -pulsed folding and annealing cage

**(protein folding/hsp60/cpn60/barnase)**

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ABSTRACT **Free GroEL binds denatured proteins very tightly: it retards the folding of barnase 400-fold and catalyzes unfolding fluctuations in native barnase and its folding intermediate. GroEL undergoes an allosteric transition from its tight-binding T-state to a weaker binding R-state on the cooperative binding of nucleotides (ATP/ADP) and GroES. The preformed GroEL·GroES·nucleotide complex retards the folding of barnase by only a factor of 4, and the folding rate is much higher than the ATPase activity that releases GroES from the complex. Binding of GroES and nucleotides to a preformed GroEL·denatured-barnase complex forms an intermediately fast-folding complex. We propose the following mechanism for the molecular chaperone. Denatured proteins bind to the resting GroEL·GroES·nucleotide complex. Fast-folding proteins are ejected as native structures before ATP hydrolysis. Slow-folding proteins enter chaperoning cycles of annealing and folding after the initial ATP hydrolysis. This step causes transient release of GroES and formation of the GroEL·denatured-protein complexes with higher annealing potential. The intermediately fast-folding complex is formed on subsequent rebinding of GroES. The ATPase activity of GroEL·GroES is thus the gatekeeper that selects for initial entry of slow-folding proteins to the chaperone action and then pumps successive transitions from the faster-folding R-states to the tighter-binding/stronger annealing T-states. The molecular chaperone acts as a combination of folding cage and an annealing machine.**

The molecular chaperone GroEL of *Escherichia coli* is essential for the folding of many proteins *in vivo* and *in vitro* (1, 2). It is a typical member of the cpn60 class of chaperonins and the hsp60 class of heat shock proteins, consisting of two stacked heptameric rings with a large central cavity (3, 4). The cochaperonin, GroES, a member of the heptameric cpn10 family, is an essential adjunct for the GroEL-mediated folding of some proteins *in vitro* (5). The functional form of the complex appears to be asymmetric, with one ring of seven GroES subunits at one end of GroEL (6–8), although this is controversial (9). The structural and biophysical properties of GroEL, its complex with nucleotides (ATP and ADP), and GroES have been probed by a number of highly ingenious experiments (for the most recent general review, see ref. 10, and for specific biophysical details, see ref. 7). But, its mechanism of action is still controversial because of insufficient of information about individual steps in the folding of substrates. Two different mechanistic roles have been proposed for the GroE complex. One is that it acts as a cage in which a newly synthesized protein can fold in isolation (11), thus avoiding problems of aggregating with other partly folded chains (12–15). The other is that it functions as an ATP-driven unfolding machine that actively catalyses the unfolding of misfolded intermediates (16) that are "kinetically trapped" and would otherwise fold too slowly or aggregate (17–20). Correctly folded structures would snap back and misfolded parts may refold correctly. It is also proposed for this mechanism that folding takes place after the release of proteins from GroEL (18, 19). The precise role of the ATPase activity in the chaperone function is not clear.

Small proteins, whose mechanisms of folding *in vitro* have been well characterized, can be used to probe the details of individual steps in the folding in the presence of chaperones. Both the 110-residue ribonuclease barnase (21–23) and the 64-residue chymotrypsin inhibitor 2 (CI2) (24) fold while complexed with GroEL *in vitro*, consistent with the "folding cage" hypothesis. It has been suggested that barnase (and CI2) are models for the folding of domains in larger proteins (23). Experiments on barnase and CI2 have also provided evidence for an annealing function of GroEL. GroEL slows down the rate of folding of denatured CI2 because bonds between them are broken during the folding transition (24). Conversely, unfolding should be catalysed because those bonds are formed during the unfolding transition. There is direct evidence for barnase being transiently unfolded by GroEL from H/$^2$H-exchange studies which show that GroEL catalyzes the exchange of deeply buried protons that require full unfolding for exchange to occur (25).

We use the term "annealing" to mean the transient melting of (mis)folded structures, followed by their refolding. Annealing does not imply that the major GroEL-bound species is the fully unfolded species, although it could be so in some cases. Indeed, kinetic data indicate that the predominant state of denatured barnase that is bound to GroEL is a compact folding intermediate (22), so that the fully unfolded form that is detected by H/$^2$H-exchange is a minor component. GroEL does not cause the observable unfolding of a compact folding intermediate in the folding pathway of an antibody fragment (26), but annealing could still be occurring in the complex. It is sufficient for the correction of misfolding that parts of the protein become only transiently fully unfolded.

We have now investigated the effects of GroES and nucleotides and the order of addition of reagents on the rate of folding of barnase in the presence of GroEL. These results, together with published data from elsewhere, point toward a mechanism of action that explains the role of the ATPase activity and reconciles some of the conflicting proposals.

## MATERIALS AND METHODS

The mutant W94Y (27) was used as the wild-type protein in this study because of its more convenient changes in fluorescence on folding and unfolding (23). GroEL and the mutants W94Y(S91A) and W94Y(D8A/D12A/R110A) of barnase were obtained as described (28). The concentration of W94Y

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Abbreviation: CI2, chymotrypsin inhibitor 2.

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mutants of barnase was determined from their $A_{280}$, using $\varepsilon_{280}$ = 23200 (calculated according to ref. 29. GroES was expressed and purified from *E. coli* cells by a modification of a described procedure (30). The concentrations of GroEL (always expressed as that of the 14-mer) and GroES (always expressed as that of the 7-mer) were determined by quantitative amino acid analysis, which gave identical results to the BioRad protein assay kit [based on the Bradford assay (31)].

The kinetics of folding of barnase was measured as described (23) using an Applied Photophysics (Leatherhead, Surrey, U.K.) SX-17MV stopped-flow spectrofluorimeter fitted with both excitation and emission monochromators, and with additional mixers, delay loops, and electronics so that two solutions could be mixed together, allowed to incubate for a predetermined time interval and then mixed with a third (as illustrated in ref. 32). The excitation wavelength was 290 nm (10-nm bandpass) and the emission wavelength was 315 nm (10-nm bandpass). Folding was initiated by mixing barnase that had been denatured in 32 mM HCl (pH 1.5) with a folding buffer (containing various concentrations of GroEL or GroES and nucleotides, where appropriate) to give final concentrations of 100 mM Mes (pH 6.3), 2 mM KCl, 2 mM $MgCl_2$, and 1 $\mu$M barnase at 25°C.

## RESULTS

**Kinetics of Barnase Folding.** We showed previously that wild-type barnase (in this case, wild type is the mutant W94Y, which has enhanced fluorescence changes) folds in solution from a compact intermediate state with a rate constant of 10 $s^{-1}$ (23). As described in detail (23), the denatured state of barnase binds rapidly to GroEL, followed by a rearrangement step. At very high ratios of barnase to GroEL, up to 4 mol of barnase binds per 14-mer of GroEL, and the multiply bound molecules fold at 0.24 $s^{-1}$. At high ratios of GroEL to barnase, the singly bound molecule of barnase, which occupies the highest affinity site, folds at 0.025 $s^{-1}$. ATP speeds up the folding reaction by up to 15-fold (23).

**Addition of ATP or ADP to GroEL·Denatured Barnase.** We have now examined the effect of ATP and GroEL on the folding of barnase by mixing ATP with GroEL in a stopped-flow fluorimeter, allowing them to incubate for 100 ms at pH 6.3, and then mixing with acid-denatured barnase. The rate of folding of a singly bound molecule of barnase (1 $\mu$M) with GroEL (2 $\mu$M) has a sigmoid dependence on [ATP] with a Hill constant ($n_H$) of 2 and the concentration of ATP for 50% of the increase in rate ($[ATP]_{50\%}$) is 110 $\mu$M, the maximum folding rate constant being 0.35 $s^{-1}$ (Fig. 1). The results are independent of the time of preincubation of ATP and GroEL between 50 and 1000 ms. ADP has only a weak effect on rate, causing a two-fold increase to 0.08 $s^{-1}$. The binding of ADP is weak ($[ADP]_{50\%}$ = 880 $\mu$M). The binding is so weakly cooperative ($n_H$ = 1.4) that it is difficult to distinguish from noncooperative binding, especially as relatively high concentrations of ADP have to be employed.

**Addition of GroES to GroEL·Denatured Barnase.** The effect of GroES on a preformed GroEL-denatured barnase complex was measured by mixing denatured barnase with GroEL and allowing them to incubate for 100 ms at pH 6.3 before adding GroES (1 equivalent of 7-mer per 14-mer of GroEL). The results are identical to those in the presence of GroEL alone (23), consistent with earlier demonstrations that GroES does not bind to GroEL in the absence of ATP or ADP (6, 8).

**Addition of GroES and Nucleotides to GroEL·Denatured Barnase.** The previous experiment was repeated, but nucleotides were included in the solution containing GroES. The presence of 500–2500 $\mu$M ADP increased the folding rate constant to 0.22 $s^{-1}$ and the presence of 500–2500 $\mu$M ATP increased the folding rate constant to 0.5 $s^{-1}$ for 1 $\mu$M barnase, 2 $\mu$M GroEL, and 2 $\mu$M GroES. The rate of refolding at physiological concentrations of ADP and ATP [0.38 mM and 2.7 mM, respectively (33)] added to the buffer is 0.5 $s^{-1}$. The results are independent of the time of preincubation of nucleotides and GroEL between 50 and 1000 ms.

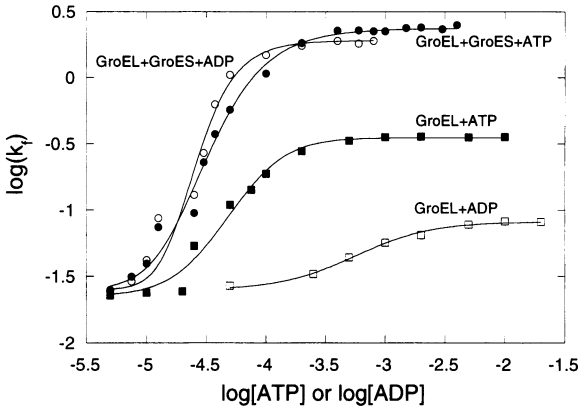


FIG. 1. Effects of nucleotides and GroES on the refolding of GroEL-bound denatured barnase. Refolding rate constants ($k_f$) are in $s^{-1}$ and concentrations are in M. The Hill equation is derived for the reaction $P + n_H L \rightleftarrows PL_{n_H}$, which assumes that $n_H$ mol of ligand L bind simultaneously to protein P. The dissociation constant $K$ is defined by $K = [P][L]^{n_H}/[P{\cdot}L_{n_H}]$. The fractional saturation $Y$ is defined by $Y = [P{\cdot}L_{n_H}]/([P] + [P{\cdot}L_{n_H}])$. The Hill plot is then $\log(Y/(1 - Y)) = n_H \log[L] - \log K$. If the reaction is followed by kinetics (e.g., that of an ATPase activity or of a folding rate constant) such that a rate constant $k$ is found at a particular concentration of L, $k_0$ is the basal rate constant in the absence of L, and $k_{max}$ is that at saturating L, then the plot translates into $\log((k - k_0)/(k_{max} - k)) = n_H \log[L] - \log K$. This equation may be applied to the binding of ADP and ATP to GroEL in the absence of GroES. The situation is more complicated in the presence of GroES because GroES binds to GroEL only weakly in the absence of ATP or ADP and so the association equilibrium between GroEL and GroES must be included. However, a simple equation may be derived for the case of equal concentrations of GroEL 14-mer and GroES 7-mer that bind in 1:1 stoichiometry and where their binding is negligible in the absence of nucleotide, and nucleotides bind more strongly to the complex than to the separate components. If the dissociation constant of the GroEL·GroES complex is $K_{diss}$ in the absence of nucleotides, then $\log(Y/(1 - Y)^2) = n_H \log[L] - \log K{\cdot}K_{diss} + \log[GroEL]_0$, where $[GroEL]_0$ the total concentration of 14-mer and $Y = [GroEL{\cdot}GroES{\cdot}L_{n_H}]/[GroEL]_0$. $(k - k_0){\cdot}(k_{max} - k_0)/(k_{max} - k)^2$ may be substituted for $Y/(1 - Y)^2$. The concentration of nucleotide for 50% saturation, $[L]_{50\%}$, varies with $[GroEL]_0$ and is given by $[L]_{50\%} = (2K{\cdot}K_{diss}/[GroEL]_0)^{1/n_H}$. The solid curves are those calculated from theoretical fits to the Hill or modified binding equations.

**Addition of Denatured Barnase to GroES·GroEL·Nucleotide Complexes.** The kinetics of folding of barnase in the presence of a preformed complex of GroEL/GroES and either ADP or ATP is both simpler and much faster than in the presence of GroEL alone. Only two phases are seen: a binding phase, in which up to 3 mol of barnase bind per mol of complex [stoichiometry determined as described (23)], followed by a single folding phase that has the same rate constant irrespective of stoichiometry of binding. The rate constant for the folding of denatured barnase is lowered only by a factor of four or five, being 2.4 $s^{-1}$ in the presence of saturating ATP or 1.9 $s^{-1}$ with ADP. The rate of refolding of 1 $\mu$M barnase at physiological concentrations of ADP (0.38 mM) and ATP (2.7 mM), 2 $\mu$M GroEL, and 2 $\mu$M GroES is 2.1 $s^{-1}$. The results are independent of the time of preincubation of nucleotides and GroEL between 50 and 1000 ms.

**Effects of Mutation of Barnase on Refolding Rates.** It was shown previously that fluorescence changes observed on mixing denatured barnase with GroEL correspond to the refolding process since they occur with the same rate constants as does the rate of regain of RNase activity and are sensitive to the effects of mutations that are known to affect folding rates (23). The rate constants for the folding in solution of the mutants of barnase W94Y(S91A), W94Y, and W94Y(DA8/D12A/

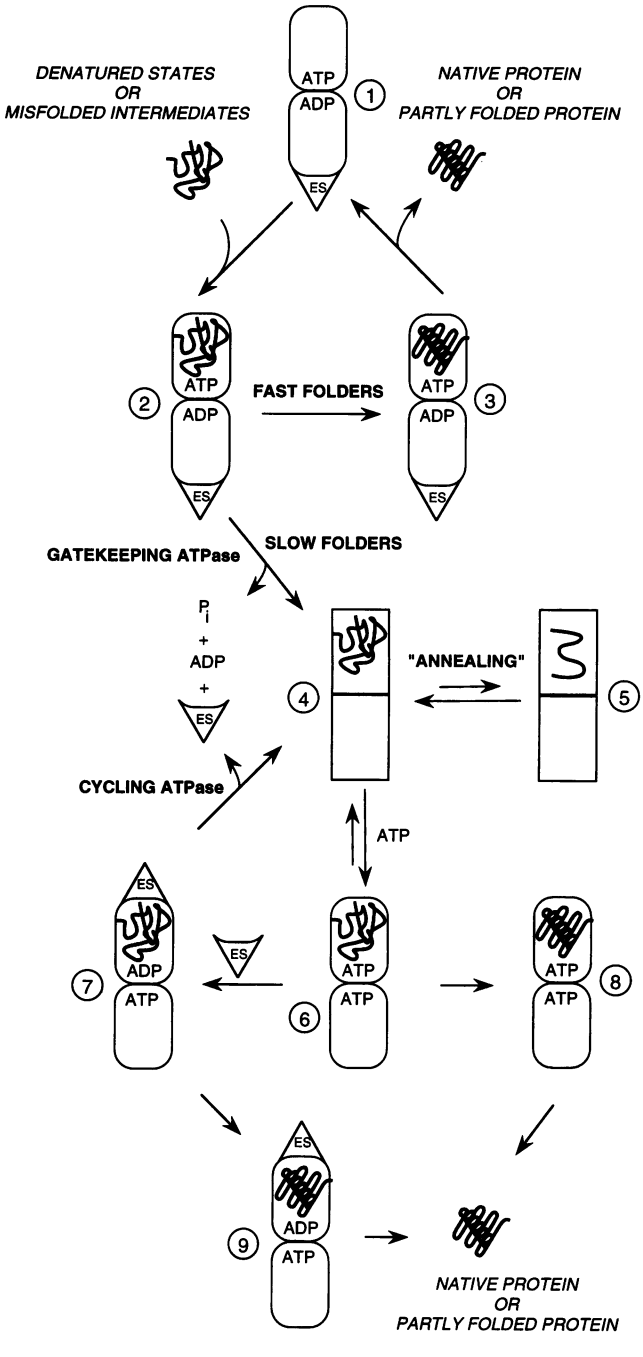


FIG. 2. Minimal scheme for GroE mechanism. Other states, involving additional conformations of proteins (36), different combinations of ATP and ADP, or binding of denatured proteins to different rings, may be involved (7). The rectangles represent the **T**-state, which has strong affinity for peptides and weak affinity for GroES and ATP. Rectangles with rounded corners represent the **R**-state, which has weak affinity for peptides and strong affinity for GroES and ATP. A denatured state binds to **1**, the GroEL·GroES·ATP·ADP complex. Fast folders, such as barnase, rapidly fold to give **3** and release folded protein. (Fast folding parts of larger proteins may also fold rapidly to give partly folded proteins on **3**, where they may translocate or dissociate.) For slow folders, GroES dissociates from **2** on ATP hydrolysis to give **4**, the **T**-state. There is an equilibrium with the **R**-state **6**, induced by the binding of ATP. GroES does not directly give **2** on binding to **6**. The **T**-state **4** has the potential for unfolding a compact denatured state, as evidenced from the catalysis of $H/^2H$-exchange in barnase (25). The top cycle (**1** → **2** → **3**) is a selection step which uses ATP hydrolysis as a gate keeping action to allow access of slow folders to the bottom cycle which encompasses rounds of annealing, if necessary, and any folding or translocation. This cycle uses ATP hydrolysis to pump the conversion of **R**-state to **T**-state. The R110A) are 2.27, 9.8, and 13.2 $s^{-1}$, respectively (23). The changes in fluorescence, which are attributed to folding, in the presence of 2 $\mu$M GroEL, 2 $\mu$M GroES, and 1000 $\mu$M ATP occur at 0.32, 2.4, and 2.9 $s^{-1}$, respectively.

## DISCUSSION

**Allosteric Behavior of GroEL.** Key to the action of GroEL is its allosteric behavior. It switches, on the cooperative binding of ATP, between a state that binds proteins the most tightly, the **T**-state, to a weaker binding **R**-state (7, 17, 30), the cooperative binding transition having been detected by a sigmoid dependence of ATP-hydrolysis on [ATP] with a Hill constant ($n_H$) of 2–3 (30, 34). Sophisticated analyses have shown that the cooperativity is complex, having nested components of mixed **R** and **T** forms (35–37). The transitions have been directly observed by electron microscopy (38). The cooperativity of binding of ATP increases on the addition of GroES (30); the formation of the GroEL·GroES complex requires the presence of either ATP or ADP (6, 8).

The importance of the data on barnase is that its folding pathway is so well characterized by kinetic and biophysical studies on it and its mutants that individual steps in folding and unfolding may be analyzed, and it folds fast enough that individual steps may be resolved in the presence of chaperones. The results here show that its rate of folding follows allosteric changes in GroEL, consistent with earlier work on slower folding proteins. Importantly, we find that the folding of barnase is so rapid in the presence of the preformed GroEL·GroES·nucleotide complex (the rate constant for folding of barnase, $k_f = 2.4\ s^{-1}$) that it is far faster than the ATPase activity. The addition of GroES and nucleotides to a preformed GroEL·denatured-barnase complex gives only a slower folding form ($k_f = 0.5\ s^{-1}$). Experiments on CI2 show directly that the more the hydrophobic bonds (and interactions from positively charged groups) between the denatured protein and GroEL, the slower the rate of folding (24). These rate constants will thus be scaled down for larger proteins.

**Proposed Mechanism for Chaperoning Action.** We synthesize from these results and published data from elsewhere a mechanism, illustrated in Fig. 2, that encompasses the two opposing views on the mechanism (that the chaperone is a folding cage and an annealing machine), accounts for the formation of different types of complexes of GroEL and GroES, and proposes precise roles for the ATPase activity.

*The gate.* There is some discussion about the nature of the resting state of the GroEL complex (7). We find that the GroES·GroEL·nucleotide complex which is present at physiological concentrations of ADP (0.38 mM) and ATP (2.7 mM) is the very fast folding form ($k_f = 2.1\ s^{-1}$). The resting form of GroEL in the cell in the absence of denatured proteins should, therefore, be the very fast folding form (species **1** in Fig. 2). Thus, fast folding proteins that fold within a second or so when bound to **1** will become native before appreciable quantities of ATP are hydrolyzed by GroEL since its ATPase activity is low [turnover number = 0.04 $s^{-1}$ in the absence of denatured protein (7, 40)] and is stimulated 4-fold (37) to 20-fold (17) in the presence of denatured protein. Thus, fast-folding proteins are ejected by the GroES·GroEL·nucleotide complex before ATP hydrolysis whereas slow-folding proteins enter the next cycle via the ATPase activity. We postulate, therefore, that the ATPase activity of GroES·GroEL·nucleotide complex is a gatekeeping function that allows only slow folding proteins to

experiments on barnase have shown that it folds in the GroEL complexes **4** and **6** (21–23) and in GroEL·GroES complexes that are likely to be (39) **2** and **7**, and that annealing processes occur (**4** → **5**) and the complex **8** exists (25). All the illustrated allosteric changes are observed in these reactions.

be subjected to later chaperoning events. The choice between **2** → **3** and **2** → **4** in Fig. 2 may be described as a kinetic partitioning step (41).

*Nature of the complexes.* ATP binds in the ring of GroEL that is opposite to GroES (trans) (7). Peptides initially bind to the trans ring, but GroES dissociates on the hydrolysis of ATP and rebinds to the opposite end of GroEL so that the peptide ends up in the cavity cis to GroES (i.e. in the cavity contiguous with GroES (39). It is the hydrolysis of ATP in the trans ring that causes the expulsion of GroES from the complex (19).

**The Annealing/Folding Cycle.** Barnase folds too rapidly to enter the chaperoning cycle directly (**4** to **9** in Fig. 2), (apart from the 20–30% of the protein that folds slowly because its peptidyl–prolyl bonds are in the wrong conformation). We have reconstructed events in the cycle by measuring the individual steps directly by mixing the reagents in a suitable order. The ATPase reaction (**2** → **4**) causes the concomitant dissociation of the GroEL·GroES complex (7, 19, 42), a process that is also promoted by bound peptides (17). GroES will recombine with the GroEL·nucleotide·denatured-protein complex after exchange of nucleotide (7, 19, 42). We mimicked this step by adding GroES to a preformed GroEL·denatured-barnase complex in the presence of physiological concentrations of ADP (0.38 mM) and ATP (2.7 mM). This gave a slower folding form of the GroEL·GroES·nucleotide·denatured-protein complex ($k_f$ = 0.5 $s^{-1}$). Proteins that fold much more slowly than barnase will enter the chaperoning cycle after the first round of ATPase activity. As those proteins slowly fold, there will be many rounds of ATP hydrolysis that cause the expulsion of GroES, which is followed by its rebinding. We have studied directly the GroEL·denatured-barnase complexes that should be present when GroES is not bound. We (R. Zahn, S. Perrett, G. Stenberg, and A.R.F.) have shown that an annealing step (**4** → **5**, Fig. 2) takes place in the presence and absence of ADP, and that GroEL·native-barnase complexes do occur with dissociation constants in the mM range (25). Here, we have shown the allosteric conversions occur on the binding of ATP to the GroEL·denatured-barnase complexes. Annealing is most efficient when denatured barnase is bound to the **T**-state, which binds the denatured state most tightly [i.e., free GroEL or GroEL·ADP complexes: the binding of denatured proteins enhances the dissociation of nucleotides (37)]. Folding takes place most rapidly from the **R**-states (**6** and **7**) and slowly from **4**. There will be a pulse of annealing every time ATP is hydrolyzed and causes the transient release of GroES and formation of the **T**-state. Thus ATP hydrolysis pumps GroEL from the weaker binding/better folding **R**-state, which is the predominant species, to the stronger binding/stronger annealing **T**-state. The protein can leave in a partly folded state from the cycle.

We speculate, although it is not necessary for the mechanism, that the slower folding GroES·GroEL·nucleotide·denatured barnase complex (**7**) that is formed on binding GroES to the GroEL·nucleotide·denatured barnase complex (**6**) has GroES and barnase cis because it has been shown that protein folding takes place from the cis complex in other systems (10, 39). The distribution of ATP and ADP in the GroEL complexes is not clear, and there will be a mixture of bound states. We find ADP binds weakly ($[ADP]_{50\%}$ = 0.88 mM), but Burston *et al.* (7) report a tightly bound set of ADP molecules, which, if they are present here, do not affect the refolding rate. There is enough ATP present in the cell (2.7 mM) to saturate ($[ATP]_{50\%}$ = 110 μM). But, the **T**-state complex (**4**) must be continually formed as ATP is hydrolysed. Annealing still takes place when ADP is bound to **4** (25).

**Note Added in Proof.** We have now found that the addition of excess GroES to complex 7 in Fig. 2 leads to some formation of the fast folding complex 2 via a transient $(GroES_7)_2GroEL_{14}$ intermediate.

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