



COMMUNICATION

A Thermodynamic Coupling Mechanism Can Explain the GroEL-mediated Acceleration of the Folding of Barstar

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Despite extensive structural and kinetic studies, the mechanism by which the *Escherichia coli* chaperonin GroEL assists protein folding has remained somewhat elusive. It appears that GroEL might play an active role in facilitating folding, in addition to its role in restricting protein aggregation by secluding folding intermediates. We have investigated the kinetic mechanism of GroEL-mediated refolding of the small protein barstar. GroEL accelerates the observed fast (millisecond) refolding rate, but it does not affect the slow refolding kinetics. A thermodynamic coupling mechanism, in which the concentration of exchange-competent states is increased by the law of mass action, can explain the enhancement of the fast refolding rates. It is not necessary to invoke a catalytic role for GroEL, whereby either the intrinsic refolding rate of a productive folding transition or the unfolding rate of a kinetically trapped off-pathway intermediate is increased by the chaperonin.

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The Escherichia coli chaperonin GroEL, in association with its co-chaperonin GroES, is involved in the folding of 10-15% of all proteins in the cytosol under normal growth conditions (Ewalt et al., 1997). GroEL is composed of two seven-membered rings of 57 kDa subunits, each having a central cavity of 45 Å diameter (Braig et al., 1994), and stacked back to back to form a double toroid. GroES is made up of seven 10 kDa subunits arranged as a ring (Hunt et al., 1996). In vitro studies have shown that chaperonins can facilitate the folding of a large range of polypeptides under conditions where the spontaneous folding reaction is non-productive or inefficient (Fenton & Horwich, 1997). Although the interplay of GroEL, GroES and nucleotide in chaperonin-assisted folding has been well delineated structurally, the kinetic and molecular events at the level of the refolding polypeptide remain poorly understood.

It has long been presumed that the principal role of the chaperonins is to prevent aggregation of partly folded intermediate forms of proteins by sequestering them inside their cavity, thus providing a favorable micro-environment for the polypeptide to fold (Buchner et al., 1991). More recently, there has also been evidence that GroEL might play a more active role in the folding process, by partially or completely unfolding misfolded conformers, thus allowing them to fold productively (Todd et al., 1996; Weissman et al., 1994; Zahn et al., 1996a). The "unfoldase" activity of GroEL alone has been demonstrated in the case of barnase, which undergoes global unfolding while bound to the GroEL surface (Zahn et al., 1996a,b), and cyclophilin whose secondary structure gets destabilized in the presence of the chaperonin (Zahn et al., 1994). It has been suggested that GroEL accelerates the folding of lysozyme by actively promoting reorganization of misfolded structures (Coyle et al., 1999). The complete chaperonin machinery i.e. GroEL, GroES and ATP, has been reported to actively catalyze the partial unfolding of RuBis-CO trapped in a misfolded condition (Shilterman et al.,1999).

Abbreviations used: RuBisCO, ribulose-1,5bisphosphate carboxylase-oxygenase; BSA, bovine serum albumin; ANS, 8-anilino-1-naphthalene sulfonic acid; CD, circular dichroism.

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It is difficult to study the role of GroEL in facilitating the folding of its natural substrate proteins, because their folding mechanisms are poorly understood, and the partly folded forms of these proteins, to which GroEL binds, are very aggregation prone. Thus, many of the studies aimed at understanding what transpires to the substrate protein, during GroEL-mediated folding, utilize proteins whose folding pathways have been well characterized. One such model protein is barstar, an 89 amino acid residue, single-domain protein that functions as the natural inhibitor of the ribonuclease, barnase in Bacillus amyloliquifaciens, and whose mechanism of folding has been studied extensively (Agashe et al., 1995; Bhuyan & Udgaonkar, 1999; Nolting et al., 1995, 1997; Schreiber & Fersht, 1993; Shastry et al., 1994; Shastry & Udgaonkar, 1995).

Folding mechanism of barstar

Equilibrium unfolded barstar at 25 °C consists of 30 % fast refolding molecules, U_F , and of 70 % slow refolding molecules, U_S . The Tyr47-Pro48 peptide bond is in the native-like *cis* conformation in the former, while it is in the alternate *trans* conformation in the latter. In strongly stabilizing conditions, such as those used in the present study, folding has been shown (Shastry & Udgaonkar, 1995) to occur according to mechanism 1 *via* parallel pathways:

$$\begin{array}{cccc} & I_{M1} & \rightarrow & I_{N} \\ & & & & \\ U_{S} & fast & & slow & N \end{array}$$

$$\sim$$
 I_{M2} \rightarrow I_{S2}

Mechanism 1.

 I_{M1} , I_{M2} and I_{F1} are early intermediates that form within the initial six milliseconds after commencement of refolding (Agashe *et al.*, 1995; Nolting *et al.*, 1995, 1997; Shastry *et al.*, 1994; Shastry & Udgaonkar, 1995), whose fluorescence and circular dichroism (CD) properties resemble those of U. I_N and I_{S2} are late, structured intermediates that differ in their fluorescence and CD properties from the early intermediates. The fast steps in mechanism 1 are the major structural transitions, and occur with very similar rate constants (Schreiber & Fersht, 1993; Shastry & Udgaonkar, 1995). The kinetics of the slow steps are dominated by proline isomerization.

Thus, the folding of barstar in strongly stabilizing conditions can be represented more simply as:

$$U \stackrel{K_{\text{UI}}}{\rightleftharpoons} I_{\text{E}} \stackrel{k}{\rightarrow} I_{\text{L}} \stackrel{\text{slow}}{\rightarrow} N$$

Mechanism 2.

 I_E represents an ensemble of rapidly formed intermediates that equilibrate with U prior to the major structural transition to I_L , which in turn represents an ensemble of late structured intermediates. I_E includes I_{M1} , I_{M2} and I_{F1} , while I_L includes I_N and I_{S2} , and it is likely that both ensembles consist, in addition, of many more intermediates (Bhuyan & Udgaonkar, 1999). According to mechanism 2, a rapid pre-equilibrium is established between U and I_E before further transformation to I_L , and the observed fast rate of folding is given by $\lambda_2 = k [I_E]/([U] + [I_E])$. Since the slow rate is unaffected by the presence of GroEL, it is not considered further.

GroEL-mediated refolding of barstar

Here, the folding of barstar has been monitored by measurement of the accompanying change in intrinsic tryptophan fluorescence. This is a useful optical probe because GroEL has no tryptophan residues, while barstar has three at positions 38, 44 and 53, with Trp53 making the predominant contribution to the fluorescence (Nath & Udgaonkar, 1997). As seen in Figure 1(a), the folding kinetics of barstar are biphasic, ~90% of the fluorescence change occurs with an apparent rate of $30(\pm 4)$ s⁻¹, while the remaining $\sim 10^{10}$ % occurs with an apparent rate of 0.018 s⁻¹. The faster rate, λ_2 , represents the apparent rate of formation of I_L (see above). The slower rate is dominated by the proline isomerization reaction that accompanies the complete folding of I_L to N (Schreiber & Fersht, 1993; Shastry & Udgaonkar, 1995). No change in intrinsic tryptophan fluorescence accompanies the formation of I_E, which can be monitored only by the capacity of I_E to bind ANS, or by a fluorescence energy transfer method that measures compaction of U to I_E (Agashe et al., 1995; Shastry & Udgaonkar, 1995).

In the presence of GroEL, the two characteristic phases are retained, suggesting that there is no drastic perturbation of the folding pathway of barstar by GroEL. There is, however, an increase in the apparent rate of the fast phase with increasing concentrations of GroEL (Figure 1(b)) while its relative amplitude is unaltered. Figure 2 shows that this increase in rate is quite significant, with nearly a twofold increase in the presence of fourfold





Figure 1. Refolding kinetics of barstar at 25 °C, pH 7 in the absence and presence of GroEL. (a) Refolding in 0.6 M urea was initiated by 12-fold dilution of 12 µM barstar in 7.2 M urea into refolding buffer. The continuous horizontal line represents the fluorescence of 1 µM unfolded barstar. The continuous line through the data represents a non-linear least-squares fit of the data to the sum of two exponentials, which are characterized apparent rate constants of $30(\pm 4)$ s⁻¹ bv and $0.018(\pm 0.004)$ s⁻¹. (b) Fast refolding in the absence and presence of GroEL: 0.5 µM barstar was refolded in 0.6 M urea in the absence (right curve) and presence (left curve) of 3 µM GroEL. Only the fast phase of folding is shown. The data have been normalized such that the signal of the native barstar is identical in both cases. The procedure for purification of barstar has been described (Shastry et al., 1994). Concentrations of barstar were determined using a molar extinction coefficient of 23,000 M^{-1} cm⁻¹. Kapid kinetic experiments were carried out using a Biologic SFM-4 stopped-flow module, with a dead time of 6 ms. Intrinsic tryptophan fluorescence emission above 320 nm was monitored with the excitation set at 295 nm. Refolding experiments were performed by a 12-fold dilution of the denatured barstar, 6 µM barstar in unfolding buffer (8 M urea in refolding buffer), into refolding buffer (50 mM sodium phosphate, 0.1 M KCl, 0.25 mM DTT, 0.25 mM EDTA at

excess of GroEL. The dependence of the observed fast folding rate on GroEL concentration is, however, not linear, as would be expected if GroEL plays the role of a catalyst. Instead, the folding rate clearly appears to saturate at higher concentrations of GroEL, as would be expected if the acceleration is a consequence of the simple binding of barstar to GroEL. Since the magnitude of the fluorescence change associated with the fast phase is unaffected by the presence of GroEL, there does not appear to be any fluorescence change associated with the binding of barstar to GroEL. The presence of GroEL does not have any effect on either the rate or relative amplitude of the slow phase (Figure 3).

Barstar is not a natural substrate of GroEL, and it was important to ascertain whether the acceleration in the fast rate of refolding was due to the specific binding of the protein in the GroEL cavity. If this were the case, then prior blocking of the specific binding sites in the central cavity by another substrate known to bind there, should abolish the accelerating effect. This was indeed found to be the case when a known stringent substrate of GroEL, rhodanese, was used for this purpose. A GroEL-rhodanese complex formed by refolding denatured rhodanese in the presence of GroEL was unable to enhance the rate of refolding of barstar (Figure 4). The apical domains of GroEL to which substrates bind constitute a hydrophobic surface (Braig et al., 1994; Fenton et al., 1994), and it was possible that the enhancement in rate might be due to non-specific binding of barstar to any available protein surface. Such a possibility could be ruled out by showing that when barstar was refolded in the presence of bovine serum albumin (BSA), there was no enhancement in rates.

pH 7.0) to give a final barstar concentration during folding of 0.5 µM in 0.6 M urea. GroEL was purified from a GroE-overproducing strain of E.coli harboring the plasmid pOFX6 (Fayet et al., 1986; Viitanen et al., 1990). To remove small, tryptophan-containing contaminating peptides, an extra step involving the reactive-red resin was added, and the eluted protein was impurity-free as judged by tryptophan fluorescence emission. The GroEL preparations were checked for purity and activity as described elsewhere (Clark et al., 1998). The concentrations of GroEL that refer to the 14-mer were determined using an extinction coefficient of ε (0.1%, 1 cm) = 0.2 at 280 nm (Tsurupa et al., 1998). A Pharmacia PD-10 column was used to buffer-exchange GroEL into the refolding buffer immediately before use. Refolding experiments were carried out using increasing concentrations of GroEL in the refolding buffer, keeping the final barstar concentration fixed at 0.5 µM. The small contribution of GroEL to the fluorescence signal, which was mainly due to scattering effects, was determined by 12-fold dilution of unfolding buffer not containing barstar into GroEL-containing refolding buffer, and was subtracted appropriately from the refolding traces.



Figure 2. Dependence of the apparent fast folding rate, λ_2 , of barstar on GroEL concentration. The concentration of barstar is 0.5 μ M. The continuous line represents a non-linear, least-squares fit of the data to either equation (1) or (2). Values for the parameters obtained by the use of either equation are given in the text, along with the standard deviations from two repetitions of the experiment.

Figure 4 also demonstrates that in the presence of MgATP, GroEL does not mediate acceleration of the fast rate of barstar refolding. MgATP is known to induce an allosteric transition in GroEL to a form with lower affinity for substrate proteins (Sparrer et al., 1996; Staniforth et al., 1994). Thus, it appears that GroEL cannot bind barstar in the presence of ATP and, consequently, the enhancement of the folding rate of barstar does not occur. This result also suggests that ATP hydrolysis and GroES binding are not required for GroELmediated folding of barstar. GroEL-mediated acceleration of folding occurs, however, in the presence of MgADP, to an extent similar to that seen in the absence of any nucleotide, suggesting that the ADP-bound form of GroEL and free GroEL have similar affinities for barstar. These observations are in accord with electron-microscopy studies that have shown that GroEL, GroEL-ADP and GroEL-ATP have distinct conformations (Roseman et al., 1996).

Reaction mechanism for the increase in refolding rates

A simple explanation for the GroEL-mediated acceleration of the folding of barstar is based on several previous observations: (1) GroEL may bind to many different partly folded forms of proteins



Figure 3. Effect of GroEL on the relative amplitudes, α_1 , and rates, λ_1 , of the slow phase of barstar refolding. The final concentration of barstar is 0.5 μ M. The continuous lines have been drawn by inspection only. Manual-mixing experiments were carried out using a SPEX fluorimeter (SPEX 320); the mixing dead time was ten seconds.

ranging from early collapsed intermediates (Badcoe *et al.*, 1991; Katsumata *et al.*, 1996; Staniforth *et al.*, 1994) to molten globule states (Martin *et al.*, 1991; Katsumata *et al.*, 1996) to late structured folding intermediates (Goldberg *et al.*, 1997; Sparrer *et al.*, 1996) with varying dissociation constants in the 10^{-6} - 10^{-11} M range. (2) GroEL may bind substrate proteins at diffusion-controlled rates with a bimolecular rate constant greater than 10^8 M⁻¹ s⁻¹ (Gray & Fersht, 1993; Perret *et al.*, 1997). (3) A protein may fold while bound to GroEL (Corrales & Fersht, 1995; Gray & Fersht, 1993; Tsurupa *et al.*, 1998). (4) The early inter-



Figure 4. Acceleration of barstar refolding takes place within the GroEL cavity. The rate of the fast phase of barstar refolding, λ_2 , under the conditions described is depicted in the histogram: 0.5 µM barstar was refolded in the absence of GroEL (-EL), in the presence of fourfold molar excess of GroEL (+EL), in the presence of fourfold excess of GroEL whose central cavity was blocked with rhodanese (EL-RHO), in the presence of milligram equivalent of BSA (final concentration of 1.68 mg/ml (BSA), in the presence of fourfold excess of ATP-bound GroEL (ATP-EL), and in the presence of fourfold excess of ADP-bound GroEL (ADP-EL). To presaturate the GroEL cavity with the stringent substrate, rhodanese, a fourfold molar excess of denatured rhodanese in unfolding buffer was refolded in the presence of GroEL, and allowed to incubate at 25 °C for 15 minutes. The complex was separated from free rhodanese using a Sephacryl S-300 column; fractions containing the complex were pooled and concentrated. Refolding of 0.5 µM barstar in the presence of BSA was carried out using a final BSA concentration that was the weight equivalent of a fourfold molar excess of GroEL, i.e. ~1.68 mg/ml final concentration. Refolding experiments in the presence of nucleotides were performed by diluting denatured barstar (final concentration 0.5 µM) into refolding buffer containing GroEL (final concentration during refolding = 2 μ M), which had been mixed 15 ms earlier with a solution containing either magnesium acetate and ADP or Mg-ATP, to give final concentrations of either 5 mM magnesium acetate and 1 mM ADP, or 5 mM MgATP.

mediate ensemble, I_{E} , on the folding pathway of barstar (mechanism 2) has exposed hydrophobic patches, as seen in its ability to bind ANS strongly (Shastry & Udgaonkar, 1995), and is therefore likely to bind GroEL. It is therefore proposed that GroEL binds I_{E} , and that both GroEL-bound and unbound I_{E} can refold to I_{L} with the same microscopic rate constant *k*. Such a mechanism can be depicted as:



In this mechanism, the binding of chaperonin, G, to I_E is characterized by the dissociation constant K_D . It is assumed that rate of binding is rapid compared to k, and that a pre-equilibrium is established between U, I_E and I_EG before further transformation to I_L . Then the observed rate of folding, λ_2 is given by $\lambda_2 = k$ ($[I_E] + [I_EG]$)/($([U] + [I_E] + [I_EG])$. Since [U], $[I_E]$ and $[I_EG]$ are the free concentrations in solution, and with the assumption that each GroEL molecule has only one binding site for barstar, λ_2 is given by:

$$\lambda_{2} = k \times \left[\frac{2P_{T}K_{UI} + (P_{T} + G_{T} + K_{D}(1 + 1/K_{UI}))}{2P_{T}(1 + K_{UI})} + \frac{\sqrt{(P_{T} + G_{T} + K_{D}(1 + 1/K_{UI}))^{2} - 4P_{T}G_{T}}}{2P_{T}(1 + K_{UI})} \right]$$
(1)

 $P_{\rm T}$ and $G_{\rm T}$ are the total concentrations of barstar and GroEL, respectively. Figure 2 shows that the dependence of λ_2 on GroEL concentration fits well to equation (1), indicating that mechanism 3 accounts well for the data. The values obtained for k, $K_{\rm D}$, and $K_{\rm UI}$ are 116(±12) s⁻¹, 1(±0.2) μ M and 0.34(±0.1), respectively.

Distinguishing kinetically whether early folding intermediates, such as I_E , are productive on-pathway intermediates or are, alternately, unproductive off-pathway intermediates is a major challenge in the study of protein folding pathways (Baldwin, 1996). In mechanisms 1 and 2, I_E is assumed to be on-pathway, and therefore productive. It has not been possible to rule out the possibility that I_E instead represents an off-pathway, kinetically trapped intermediate ensemble, whose formation slows the overall rate of folding, as shown in mechanism 4:

$$I_E \stackrel{K_{\text{UI}}}{\rightleftharpoons} U \stackrel{k}{\rightarrow} I_L \stackrel{\text{slow}}{\rightarrow} N$$

Mechanism 4.

According to mechanism 4, the observed fast rate of folding is given by $\lambda_2 = k [U]/([U] + [I_E])$.

Even if I_E is indeed a dead-end ensemble as in mechanism 4, a thermodynamic coupling mechanism, similar to mechanism 3, will account for the acceleration of folding rates. It is only necessary that GroEL preferentially bind U and not I_E as in

mechanism 3, and that the GroEL-bound protein again be capable of folding. Indeed, GroEL is known to bind to the unfolded states of other proteins (Viitanen *et al.*, 1991; Zahn & Pluckthun, 1994; Zahn *et al.*, 1996b). If I_E is an off-pathway intermediate formed by a rapid non-specific hydrophobic collapse, in which hydrophobic surfaces with which GroEL might interact get largely buried, then it is conceivable that GroEL might bind preferentially to U rather than to I_{E_i} and the following mechanism would account for the data:

The observed fast rate of folding, $\lambda_2 = k$ ([U] +[UG])/([U] + [I_E] + [UG]) is then given by:

$$\lambda_{2} = k \times \left[\frac{2P_{\rm T} + K_{\rm UI}(P_{\rm T} + G_{\rm T} + K_{\rm D}(1 + 1/K_{\rm UI}))}{2P_{\rm T}(1 + K_{\rm UI})} \right]$$
$$\pm \frac{K_{\rm UI}\sqrt{((P_{\rm T} + G_{\rm T} + K_{\rm D}(1 + K_{\rm UI}))^{2} - 4P_{\rm T}G_{\rm T})}}{2P_{\rm T}(1 + K_{\rm UI})} \right] (2)$$

The data in Figure 2 fit equally well to equation (2) as they do to equation (1). The values obtained for k, K_D , and K_{UI} from the use of equation (2) are 116(±12) s⁻¹, 1(±0.2) µM and 3(±0.6), respectively.

The salient feature of both mechanisms 3 and 5, is that neither invokes catalysis of folding. The microscopic rate constant of folding is the same, whether barstar folds free in solution or while bound to GroEL. This suggests that the binding of GroEL to the transition state of folding is not any tighter than its binding to I_E in mechanism 3, or to U in mechanism 5. Moreover, its relatively weak $(K_{\rm D} = 1 \ \mu \text{M})$ binding implies that the binding energy of GroEL (~ -0.5 kcal/mol) in the maximum concentration used here (\sim 3.5 μ M), is too small to otherwise perturb the activation free energy of folding and, hence, the folding rate. The binding energy is, however, comparable in magnitude to the free energy of formation of I_E from U. Thus, in both mechanisms, the acceleration in folding is the consequence of the binding event shifting the equilibrium away from the species that does not fold directly: U in mechanism 3, and I_E in mechanism 5. Folding is accelerated because rapid coupling of the binding equilibrium to the $U \rightleftharpoons I_E$ equilibrium increases the concentration of species that can fold directly: I_E and I_EG in mechanism 3, U and UG in mechanism 5.

It is particularly pertinent that even in the scenario (mechanisms 4 and 5) where I_E is a kinetically

trapped misfolded ensemble, it is unnecessary to postulate that GroEL binds I_E , catalyzes the rate of the $I_E \rightarrow U$ unfolding transition that otherwise limits the overall folding rate, and thereby accelerates folding. Such an active role for GroEL in promoting the unfolding of a trapped intermediate and thereby facilitating the folding reaction has been suggested previously (Todd *et al.*, 1996; Weissman *et al.*, 1994; Zahn *et al.*, 1996a).

In both mechanisms 3 and 5, it is assumed that GroEL does not bind the fully folded protein, N. In fact, both native gel electrophoresis and steadystate fluorescence studies have been unable to detect any interaction of GroEL with N (unpublished observations). While the possible interaction of I_L and N with GroEL is still under study, it should be noted that in both mechanisms 3 and 5, it is optional whether to exclude (or include) binding of GroEL to I_L or to N. Attempts were made to detect directly any interaction of GroEL with U. The highest concentration of urea that GroEL can tolerate is, however, only 1.5 M (Lissin, 1995), in which barstar remains completely folded; hence, it has not been possible to determine if GroEL binds unfolded U. It should be noted, however, that even if GroEL interacts with U, it does not necessarily mean that mechanism 5 is more appropriate to describe the data. GroEL might also bind $I_{E'}$ and if it does so tighter than it binds U, then mechanism 3 with an on-pathway I_E might still be more appropriate.

Since the $U \rightleftharpoons I_E$ transition is silent to fluorescence and CD change, it had not been possible to obtain the value of $K_{\rm UI}$ in previous studies (Agashe et al., 1995; Shastry & Udgaonkar, 1995). The thermodynamic coupling mechanisms 3 and 5, have now allowed values for $K_{\rm UI}$ to be determined: 0.33 for the former mechanism, and 3 for the latter. Thus, in the absence of GroEL, the pre-equilibrium mixture of U and I_E will consist of $\approx 25\%$ I_E or \approx 75% I_E, depending on whether mechanism 2 or 4, respectively, is more appropriate. Previous monitoring of the $U \to I_E$ reaction by a fluorescence energy transfer method (Agashe et al., 1995) had suggested that 70% of the energy transfer efficiency is restored within 6 ms, indicating that at least 70 % of all molecules form $I_{\rm E}$ in the pre-equilibrium mixture. This extent of formation of I_E appears to be compatible only with mechanism 4, in which I_E is an off-pathway intermediate ensemble.

The only other protein whose refolding has been reported to be accelerated in the presence of GroEL alone, without the involvement of GroES and ATP, is lysozyme (Coyle *et al.*, 1999). In that case it was suggested that GroEL plays an active role in the reorganization of non-native tertiary interactions, so that the rate of a slow folding transition involving domain docking is enhanced through catalysis. Here, it is shown that it is not necessary to invoke a catalytic mechanism to explain the enhancement of the folding rate by GroEL, but that a simple thermodynamic coupling mechanism suffices. Previously, it has also been suggested that it is unnecessary to invoke an active catalytic role for GroEL to account for its unfolding activity (Zahn et al., 1994, 1996a,b), because a thermodynamic coupling mechanism, in which GroEL does not alter the microscopic rate constant of unfolding (Walter et al., 1996) is adequate. It is not implausible that the principal role of GroEL is to prevent aggregation by seclusion of folding intermediates, and that its apparent ability to catalyze folding or unfolding transitions is merely a reflection of its discriminating ability to differentially bind the multiple unstructured and structured forms that populate a protein folding pathway. From the data in Figures 2 and 4, it is estimated that the affinity of GroEL-ATP for barstar must be approximately five- to tenfold lower than that of GroEL alone. ATP and also co-chaperonin GroES may therefore play the roles of allosteric ligands in modulating the binding ability of GroEL.

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References

- Agashe, V. R., Shastry, M. & Udgaonkar, J. B. (1995). Initial hydrophobic collapse in the folding of barstar. *Nature*, 377, 754-757.
- Badcoe, I. G., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., Lund, P. & Clarke, A. R. (1991). Binding of a chaperonin to the folding intermediates of lactate dehydrogenase. *Biochemistry*, **30**, 9195-9200.
- Baldwin, R. L. (1996). On-pathway versus off-pathway folding intermediates. Fold. Des. 1, R1-8.
- Bhuyan, A. K. & Udgaonkar, J. B. (1999). Observation of multistate kinetics during the slow folding and unfolding of barstar. *Biochemistry*, 38, 9158-9168.
- Braig, K., Otwinowski, Z., Hedge, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L. & Sigler, P. B. (1994). The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature*, **371**, 578-586.
- Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X. & Kiefhaber, T. (1991). GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry*, **30**, 1586-1591.
- Clark, A. C., Ramanathan, R. & Frieden, C. (1998). Purification of GroEL with low fluorescence background. *Methods Enzymol.* 290, 100-118.
- Corrales, F. & Fersht, A. (1995). The folding of GroELbound barnase as a model for chaperonin-mediated protein folding. *Proc. Natl Acad. Sci. USA*, 92, 5326-5330.
- Coyle, J. E., Texter, F. L., Ashcroft, A. E., Masselos, D., Robinson, C. V. & Radford, S. E. (1999). GroEL

accelerates the refolding of hen lysozyme without changing its folding mechanism. *Nature Struct. Biol.* **6**, 683-690.

- Ewalt, K. L., Hendrick, J. P., Houry, W. A. & Hartl, F. U. (1997). *In vivo* observation of polypeptide flux through the bacterial chaperonin system. *Cell*, **90**, 491-500.
- Fayet, O., Louarn, J. M. & Georgopoulos, C. (1986). Suppression of the *Escherichia coli dna*A46 mutation by amplification of the groES and groEL genes. *Mol. Gen. Genet.* 202, 435-445.
- Fenton, W. A. & Horwich, A. L. (1997). GroEL-mediated protein folding. Protein Sci. 6, 743-760.
- Fenton, W. A., Kashi, Y., Furtak, K. & Horwich, A. L. (1994). Residues in chaperonin GroEL required for polypeptide binding and release. *Nature*, **371**, 614-619.
- Goldberg, M. S., Zhang, J., Sondek, S., Matthews, C. R., Fox, R. O. & Horwich, A. L. (1997). Native-like structure of a protein-folding intermediate bound to the chaperonin GroEL. *Proc. Natl Acad. Sci. USA*, 94, 1080-1085.
- Gray, T. E. & Fersht, A. R. (1993). Refolding of barnase in the presence of GroE. J. Mol. Biol. 232, 1197-1207.
- Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L. & Deisenhofer, J. (1996). The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature*, 379, 37-45.
- Katsumata, K., Okazaki, A. & Kuwajima, K. (1996). Effect of GroEL on the re-folding kinetics of α-lactalbumin. J. Mol. Biol. 258, 827-838.
- Lissin, N. M. (1995). *In vitro* dissociation and self-assembly of three chaperonin 60s: the role of ATP. *FEBS Letters*, **361**, 55-60.
- Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L. & Hartl, F. U. (1991). Chaperonin-mediated protein folding at the surface of GroEL through a "molten globule"-like intermediate. *Nature*, **352**, 36-42.
- Nath, U. & Udgaonkar, J. B. (1997). Folding of tryptophan mutants of barstar: evidence for an initial hydrophobic collapse on the folding pathway. *Biochemistry*, 36, 8602-8610.
- Nolting, B., Golbik, R. & Fersht, A. R. (1995). Submillisecond events in protein folding. *Proc. Natl Acad. Sci. USA*, 92, 10668-10672.
- Nolting, B., Golbik, R., Soler-Gonzalez, A. S. & Fersht, A. R. (1997). Circular dichroism of denatured barstar suggests residual structure. *Biochemistry*, 36, 9899-9905.
- Perrett, S., Zahn, R., Stenberg, G. & Fersht, A. R. (1997). Importance of electrostatic interactions in the rapid binding of polypeptides to GroEL. J. Mol. Biol. 269, 892-901.
- Roseman, A. M., Chen, S., White, H., Braig, K. & Saibil, H. (1996). The chaperonin ATPase cycle: mechanism of allosteric switching and movements of substratebinding domains in GroEL. *Cell*, 87, 241-251.
- Schreiber, G. & Fersht, A. R. (1993). The refolding of cisand trans-peptidylpropyl isomers of barstar. *Biochemistry*, 32, 11195-11203.
- Shastry, M. & Udgaonkar, J. B. (1995). The folding mechanism of barstar: Evidence for multiple pathways and multiple intermediates. *J. Mol. Biol.* 247, 1013-1027.
- Shastry, M., Agashe, V. R. & Udgaonkar, J. B. (1994). Quantitative analysis of the kinetics of denaturation and renaturation of barstar in the folding transition zone. *Protein Sci.* 3, 1409-1417.

- Shilterman, M., Lorimer, G. H. & Englander, S. W. (1999). Chaperonin function: folding by forced unfolding. *Science*, 284, 822-825.
- Sparrer, H., Lilie, H. & Buchner, J. (1996). Dynamics of the GroEL-protein complex: effects of nucleotides and folding mutants. J. Mol. Biol. 258, 74-87.
- Staniforth, R. A., Burston, S. G., Atkinson, T. & Clarke, A. R. (1994). Affinity of chaperonin-60 for a protein substrate and its modulation by nucleotides and chaperonin-10. *Biochem. J.* **300**, 651-658.
- Todd, M. J., Viitanen, P. V. & Lorimer, G. H. (1996). Chaperonin-facilitated protein folding: optimization of rate and yield by an iterative annealing mechanism. *Proc. Natl Acad. Sci. USA*, **93**, 4030-4035.
- Tsurupa, G. P., Ikura, T., Makio, T. & Kuwajima, K. (1998). Refolding kinetics of staphylococcal nuclease and its mutants in the presence of the chaperonin GroEL. J. Mol. Biol. 277, 733-745.
- Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P. & Lorimer, G. H. (1990). Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (groEL) are K⁺ dependent. *Biochemistry*, **29**, 5665-5671.
- Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H. & Gatenby, A. A. (1991). Complex interactions between the chaperonin 60 molecular

chaperone and dihydrofolate reductase. *Biochemistry*, **30**, 9716-9723.

- Walter, S., Lorimer, G. H. & Schmid, F. X. (1996). A thermodynamic coupling mechanism for GroELmediated unfolding. *Proc. Natl Acad. Sci. USA*, 93, 9425-9430.
- Weissman, J. S., Kashi, Y., Fenton, W. A. & Horwich, A. L. (1994). GroEL-mediated protein folding proceeds by multiple rounds of binding and release. *Cell*, 78, 693-702.
- Zahn, R. & Pluckthun, A. (1994). Thermodynamic partitioning model for hydrophobic binding of polypeptides by GroEL. II. GroEL recognizes thermally unfolded mature beta-lactamase. *J. Mol. Biol.* **242**, 165-174.
- Zahn, R., Spitzfaden, C., Ottiger, M., Wuthrich, K. & Pluckthun, A. (1994). Destabilization of the complete protein secondary structure on binding to the chaperone GroEL. *Nature*, **368**, 261-265.
- Zahn, R., Perrett, S. & Fersht, A. R. (1996a). Conformational states bound by the molecular chaperones GroEL and SecB: a hidden unfolding (annealing) activity. J. Mol. Biol. 261, 43-61.
- Zahn, R., Perrett, S., Stenberg, G. & Fersht, A. R. (1996b). Catalysis of amide proton exchange by the molecular chaperones GroEL and SecB. *Science*, **271**, 642-645.

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