

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 RuBisCO trapped in a misfolded condition (Shilterman *et al.*, 1999).

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

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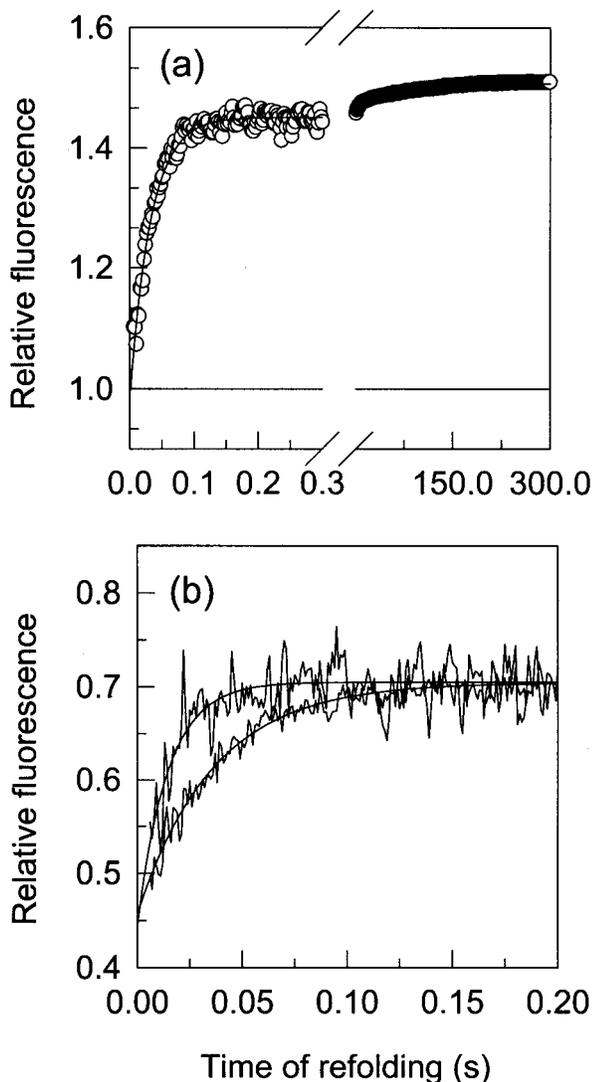


Figure 1. Refolding kinetics of barstar at 25°C, pH 7 in the absence and presence of GroEL. (a) Refolding in 0.6 M urea was initiated by 12-fold dilution of 12 μM barstar in 7.2 M urea into refolding buffer. The continuous horizontal line represents the fluorescence of 1 μM unfolded barstar. The continuous line through the data represents a non-linear least-squares fit to the data to the sum of two exponentials, which are characterized by apparent rate constants of $30(\pm 4) \text{ s}^{-1}$ and $0.018(\pm 0.004) \text{ s}^{-1}$. (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 $23,000 \text{ M}^{-1} \text{ cm}^{-1}$. Rapid 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.

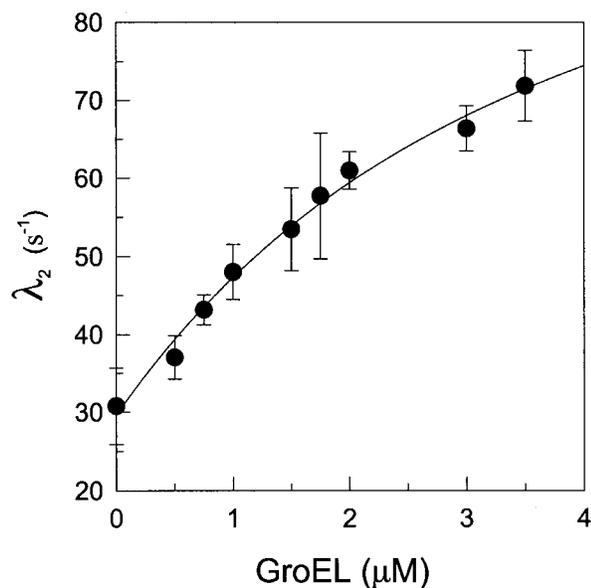


Figure 2. Dependence of the apparent fast folding rate, λ_2 , of barstar on GroEL concentration. The concentration of barstar is $0.5 \mu\text{M}$. The continuous line represents a non-linear, least-squares fit of the data to either equation (1) or (2). Values for the parameters obtained by the use of 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 GroEL-mediated 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

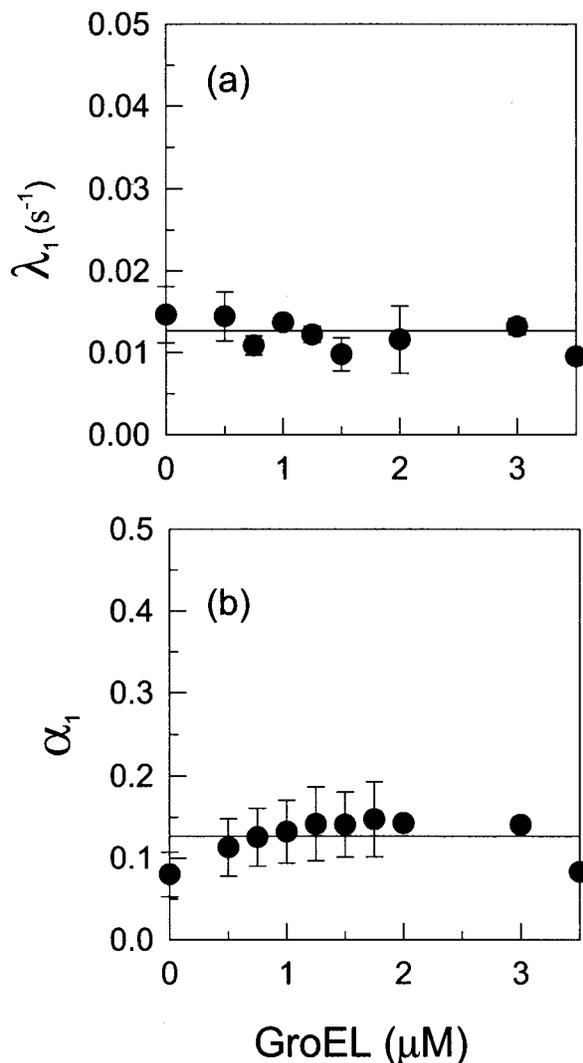
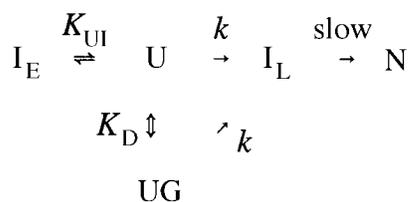


Figure 3. Effect of GroEL on the relative amplitudes, α_1 , and rates, λ_1 , of the slow phase of barstar refolding. The final concentration of barstar is $0.5 \mu\text{M}$. The continuous lines have been drawn by inspection only. Manual-mixing experiments were carried out using a SPEX fluorimeter (SPEX 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 $10^8 \text{ M}^{-1} \text{ s}^{-1}$ (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-

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 , and the following mechanism would account for the data:



Mechanism 5.

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

$$\lambda_2 = k \times \left[\frac{2P_T + K_{UI}(P_T + G_T + K_D(1 + 1/K_{UI}))}{2P_T(1 + K_{UI})} \pm \frac{K_{UI}\sqrt{((P_T + G_T + K_D(1 + K_{UI}))^2 - 4P_TG_T)}}{2P_T(1 + K_{UI})} \right] \quad (2)$$

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 $116(\pm 12) \text{ s}^{-1}$, $1(\pm 0.2) \text{ }\mu\text{M}$ and $3(\pm 0.6)$, 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 ($K_D = 1 \text{ }\mu\text{M}$) binding implies that the binding energy of GroEL ($\sim -0.5 \text{ kcal/mol}$) in the maximum concentration used here ($\sim 3.5 \text{ }\mu\text{M}$), 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 $I_E G$ 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 steady-state 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_{UI} in previous studies (Agashe *et al.*, 1995; Shastry & Udgaonkar, 1995). The thermodynamic coupling mechanisms 3 and 5, have now allowed values for K_{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 $U \rightarrow I_E$ 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_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 suf-

ferences. 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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