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The folding of GroEL-bound barnase as a model for chaperonin-mediated protein folding

(chaperone/protein engineering)

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ABSTRACT **We have analyzed the pathway of folding of barnase bound to GroEL to resolve the controversy of whether proteins can fold while bound to chaperonins (GroEL or Cpn60) or fold only after their release into solution. Four phases in the folding were detected by rapid-reaction kinetic measurements of the intrinsic fluorescence of both wild type and barnase mutants. The phases were assigned from their rate laws, sensitivity to mutations, and correspondence to regain of catalytic activity. At high ratios of denatured barnase to GroEL, 4 mol of barnase rapidly bind per 14-mer of GroEL. At high ratios of GroEL to barnase, 1 mol of barnase binds with a rate constant of 3.5×10^7 $s^{-1} \cdot M^{-1}$. This molecule then refolds with a low rate constant that changes on mutation in parallel with the rate constant for the folding in solution. This rate constant corresponds to the regain of the overall catalytic activity of barnase and increases 15-fold on the addition of ATP to a physiologically relevant value of ≈ 0.4 s^{-1}. The multiply bound molecules of barnase that are present at high ratios of GroEL to barnase fold with a rate constant that is also sensitive to mutation but is 10 times higher. If the 110-residue barnase can fold when bound to GroEL and many moles can bind simultaneously, then smaller parts of large proteins should be able to fold while bound.**

The folding of proteins *in vivo* can be mediated by molecular chaperones that bind to the various forms of the denatured states of proteins (1–3). Ideas on the function of molecular chaperones are in a state of flux (4–7). The early consensus was that the role of molecular chaperones is to prevent aggregation of the denatured states, perhaps by actively catalyzing the unfolding of misfolded states (8, 9), and it is still strongly argued that proteins fold while free in solution (6, 10). Others propose that proteins may fold in the large central cavity of GroEL, which constitutes a cage that allows folding to proceed in isolation (4, 11–14). The reason why there is uncertainty over the location of the folding event is that the evidence so far is indirect, since the folding is usually monitored by measuring the overall regain of activity. By analogy with conventional enzyme kinetics, what is required to resolve questions of mechanism in such cases is to examine directly the progress of folding of GroEL-bound protein (15). A suitable substrate for such a study is the 110-residue single-chain ribonuclease barnase, which lacks disulfide crosslinks and whose folding pathway in solution has been well characterized (16).

It was shown earlier that the folding of denatured barnase is slowed down by binding to GroEL, as monitored by the rate of regain of catalytic activity (17, 18). Further, compelling evidence was presented that the folding occurs while the barnase is still bound to GroEL; the rate constant for folding levels off at a finite rate at saturating concentrations of GroEL. If the unfolded protein has to be released from GroEL to fold in solution, then the rate of folding should fall to zero when all the barnase becomes bound at saturating concentrations of GroEL.

We now analyze the folding pathway in detail by stopped-flow monitoring the change of tryptophan fluorescence of barnase, GroEL not having tryptophan residues. Unfortunately, the fluorescence of denatured barnase when bound to GroEL is so close to that of fully folded barnase that it is difficult to monitor the folding by using conventional stopped-flow fluorimeters. We have now overcome these difficulties by replacing Trp-94 by tyrosine (mutant W94Y), which removes internal quenching of the fluorescence of Trp-35 (19), and by using stopped-flow fluorimeters that can collect emitted light at a single wavelength. We have chosen to analyze in depth W94Y and two of its mutants: W94Y(S91A), which folds more slowly in solution because the second mutation disrupts the β-sheet, and W94Y(D8A/D12A/R110A), because this mutant folds more rapidly in solution as the triple mutation D8A/D12A/R110A stabilizes the protein (20). Using these mutants, we have been able to monitor the binding of denatured barnase to GroEL, measure the rate and stoichiometry of binding, observe the redistribution of barnase molecules with the changing stoichiometry, and follow the rate of folding of barnase bound to GroEL.

MATERIALS AND METHODS

Barnase mutants used in this study are W94Y (19), I4A, D12A/Y17G (J. Matthews and A.R.F., unpublished data), D8A/D12A/R110A (21, 22), H18Q, I55A, S57A, S91A, I51A, E60Q, and R110A (23). The mutants W94Y(S91A) and W94Y(D8A/D12A/R110A) were obtained by inverse PCR, identified by direct sequencing of the complete barnase gene, and expressed and purified as described (24). The concentration of barnase was determined from its absorbance at 280 nm, using ε_{280} = 27,400, except for variants containing W94Y mutation, where ε_{280} = 23,200 (calculated according to ref. 25). GroEL was expressed in and purified from *Escherichia coli* cells by a modification of a described procedure (26). The GroEL concentration (always expressed as that of the 14-mer) was determined by quantitative amino acid analysis, which gave identical results to the Bio-Rad protein assay kit [based on the Bradford assay (27)].

The kinetics of folding of barnase was measured as described (28). 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) to give final concentrations of 100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.3/2 mM KCl/2 mM $MgCl_2$/1 μM barnase at 25°C. ATP, when used, was included in the barnase denaturing solution to avoid any degradation from the GroEL ATPase activity. The concentration of buffer used here is twice that used previously. The higher concentration avoids a very slow

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and reproducible change in fluorescence on mixing GroEL with 32 mM HCl in the absence of any barnase in the less-concentrated buffer. This change in the folding buffer did not affect the folding rates of any of the mutants analyzed in the absence of GroEL.

Three stopped-flow spectrofluorimeters were used for the rapid mixing experiments. Slower rates of folding of barnase and barnase mutants ($t_{1/2} > 30$ ms) were monitored initially by following the intrinsic barnase fluorescence at an excitation wavelength of 290 nm and bandpass of 1.5 nm, with emission at 315 nm and bandpass of 10 nm by using a Perkin–Elmer MPF 44B fluorescence spectrophotometer fitted with a rapid-mixing device (28). Data were collected on a Nicolet Pro90 oscilloscope. Faster rates of folding were measured initially by using an Applied Photophysics SF.17MF stopped-flow spectrofluorimeter with an excitation wavelength at 290 nm (10 nm bandpass), and emission was collected at wavelengths higher than 315 nm by using a glass cut-off filter. Finally, the folding of the mutants W94Y, W94Y(S91A), and W94Y(D8A/D12A/R110A) were also analyzed by using an Applied Photophysics SX-17MV stopped-flow spectrofluorimeter fitted with an emission monochromator such that emitted light could be collected at a fixed bandpass over the entire time range. The excitation wavelength was 290 nm (10 nm bandpass), and the emission wavelength was 315 nm (10 nm bandpass). Identical rate constants were obtained on all three machines where the time scales overlapped.

Changes in the free energy of unfolding on mutation were measured as described (23). Introducing the mutation W94Y into wild-type barnase, S91A, and D8A/D12A/R110A destabilizes them by 0.98, 0.24, and 0.96 kcal·mol^{-1}, respectively.

RESULTS

Kinetics of Barnase Folding. Four distinct phases are observed in the folding of barnase in the presence of GroEL. The sum of the amplitudes for each phase is equal to the fluorescence change for the folding in the absence of GroEL when recorded on a single stopped-flow spectrofluorimeter. For example, in the presence of 2.0 μM GroEL, the amplitudes of phases 1–4 for W94Y(D8A/D12A/R110A) are 0.0052, 0.0025, 0.0011, and 0.0195, respectively, compared with a total change of 0.03 in the absence of GroEL.

Phase 1: Partitioning Between Folding in Solution and Binding to GroEL. This phase may be followed for wild type and most mutants. Folded barnase has a considerably higher fluorescence than does the denatured state, and its rate of folding is readily followed by exciting it at 290 nm and collecting all the fluoresced light at wavelengths greater than 315 nm. Folding is initiated by mixing acid-denatured barnase with Mes buffer to give a final pH of 6.3 and 1 μM barnase. When increasing concentrations of GroEL are included in the Mes buffer, there is initially ([GroEL] $<<$ 1 μM) a decrease in the amplitude of the normal phase of folding (Fig. 1). There is initially little change in the folding rate constant [Fig. 1; 9.8 s^{-1} for W94Y, 2.27 s^{-1} for W94Y(S91A), and 13.2 s^{-1} for W94Y(D8A/D12A/R110A)]. The amplitude of the exponential phase levels off at about 20% of the initial value as the concentration of GroEL (14-mer) increases above 1 μM, but the first-order rate constant then increases linearly with increasing [GroEL]. The kinetics at high [GroEL] are those of a second-order reaction corresponding to: $k_1 = k_{2nd}$[GroEL] where $k_{2nd} = 3.5 \times 10^7$ s^{-1}·M^{-1}. k_{2nd} is the same for several mutants that refold at different rate constants. This must correspond to the binding of GroEL with denatured barnase with a change in fluorescence of about 20% of the folding amplitude of W94Y. The fast phase corresponds to the competition between folding of the denatured barnase with its binding to GroEL. The addition of saturating concentrations of ATP does not affect the rate constants or amplitudes of binding.

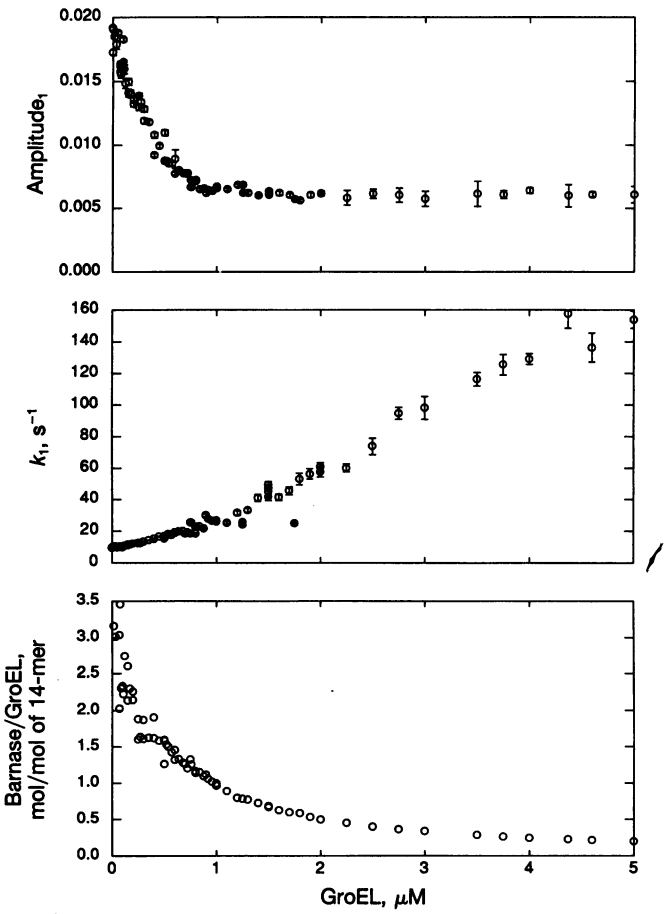

FIG. 1. Phase 1 of folding of the barnase mutant W94Y (1 μM) in the presence of GroEL. Stopped-flow fluorescence data were collected on excitation at 290 nm and emission at wavelengths greater than 315 nm. Error bars are shown. Filled circles are in the presence of 500 μM ATP. The stoichiometry in the bottom frame is the number of mol of barnase bound per mol of total GroEL and not per mol of complex. Since [GroEL] is greater than [barnase] at concentrations greater than 1 μM, the apparent stoichiometry must fall below 1. Phases 1 and 2 were examined by excitation at 290 nm and emission at 315 nm for the mutants W94Y(S91A) and W94Y(D8A/D12A/R110A).

The stoichiometry of binding of denatured barnase to GroEL can be calculated from the loss of amplitude of the kinetics of folding in solution (Fig. 1). If the amplitude of the folding phase of 1 μM barnase in the absence of GroEL is ΔF_o and that in the presence of α μM GroEL is ΔF_x, then the number of moles of barnase bound per 14-mer of GroEL is determined by the following: $(\Delta F_o - \Delta F_x)/\alpha(\Delta F_o - \Delta F_\infty)$, where ΔF_∞ is the limiting amplitude at saturating GroEL. (This assumes, as is general in such titrations, that the fluorescence of GroEL-bound barnase is the same for all moles of barnase that are bound if more than one mole is bound. Since the major change we monitor, however, is the decrease in amplitude of the refolding reaction in solution, any breakdown of the linearity assumption will cause little error, and certainly not affect the conclusions of this study.) At very low concentrations of GroEL—i.e., high ratios of barnase to GroEL—the stoichiometry tends toward 3.5 for W94Y and 4 for the other two mutants, which were monitored at 315 nm. At very high ratios of GroEL to barnase, the stoichiometry of the complex must tend toward 1:1. The second-order kinetics of binding observed at high values of [GroEL] are for 1 mol of barnase

binding per mol of GroEL 14-mer. There may well be an initial association that is faster than the overall rate constant of 3.5 $\times 10^7$ $s^{-1} \cdot M^{-1}$ that is observed, since association reactions can have fast preequilibria combined with a rearrangement step (15).

Phase 2: A Binding Event. Phase 2 is resolvable only for the W94Y series (Fig. 2). Its amplitude initially increases with increasing concentrations of GroEL but then decreases to a constant value under conditions where the stoichiometry of binding of barnase drops. Further, the rate constant for phase 2 varies little with mutations that change the rate of folding in solution [k_2 = 2.32 ± 0.06 s^{-1} for W94Y, 2.05 ± 0.06 s^{-1} for W94Y(S91A), and 1.8 ± 0.05 s^{-1} for W94Y(D8A/D12A/R110A)]. It is also unaffected by having the denatured barnase preincubated with ATP. Phase 2 must be associated, therefore, with an event involving the binding of ≥1 mol of barnase. It occurs after the initial binding since it does not follow second-order kinetics.

Phase 3: Folding of Multiply Bound Barnase. Phases 3 and 4 are measurable only when the cut-off filter for emission (>315 nm) is replaced by a monochromator and light is collected at 315 nm. The changes are clearly resolvable for wild-type barnase and most mutants, but there are large signals for the W94Y series. The rate constant for phase 3 reaches its maximum at 0.5–1 μM GroEL and then remains constant. The amplitude rapidly rises and then falls (Fig. 3). The initial increase in amplitude follows the increase in the amount of barnase that is bound to GroEL in a stoichiometry of greater than 1:1. The subsequent decay in amplitude parallels the lowering of the stoichiometry as the complex with singly bound barnase is formed. Phase 3 is sensitive to mutations in barnase. In the absence of GroEL, W94Y folds 4.3 times faster than the double mutant W94Y(S91A), whereas the quadruple mutant W94Y(D8A/D12A/R110A) folds 1.4 times faster than W94Y. These mutants behave in a similar way in phase 3 [k_3 for W94Y = 0.24 ± 0.01 s^{-1}, k_3 for W94Y(S91A) = 0.11 ± 0.007 s^{-1}, and k_3 for W94Y(D8A/D12A/R110A) = 0.32 ± 0.01 s^{-1}]. If phases 3 and 4 occur in a linear sequence—e.g., k_3 is the rate constant for the formation of a folding intermediate that precedes phase 4—then the amplitudes of phase 3 would rise to a maximum with increasing concentration of GroEL. As the amplitude initially increases and then decays with increasing concentrations of GroEL (Figs. 2 and 3), it must represent a competing or parallel pathway to phase 4. Since the amplitudes of phase 3 follow the formation of a complex with high stoichiometry, k_3 must be the rate constant for the folding of multiply bound molecules of barnase.

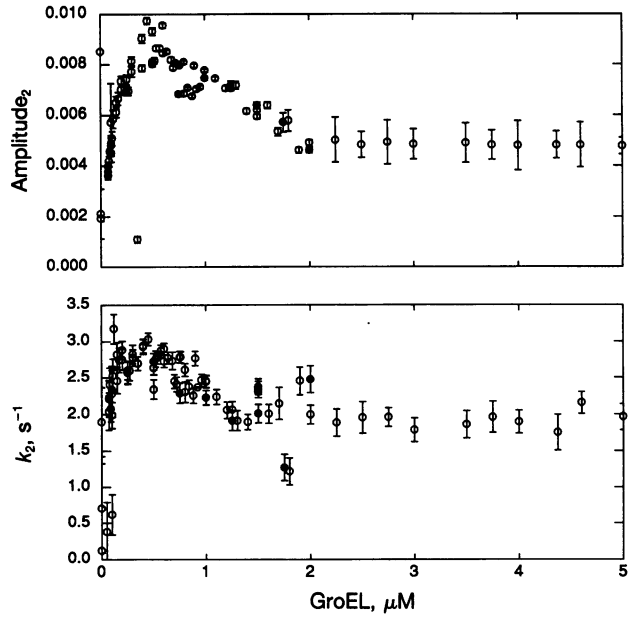

FIG. 2. Phase 2 of folding of barnase W94Y in the presence of GroEL. These are part of the same kinetic curves presented in Fig. 1 and so the amplitudes may be directly compared. Error bars are shown. Filled circles are in the presence of 500 μM ATP.

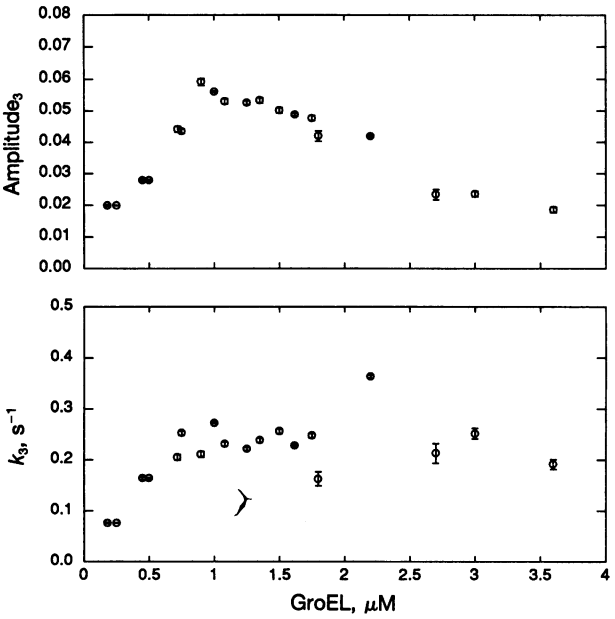

FIG. 3. Phase 3 of folding of barnase W94Y in the presence of GroEL. Stopped-flow fluorescence data were collected on excitation at 290 nm and emission at 315 nm. Error bars are shown.

Phase 4: Folding of Singly Bound Barnase. The changes are again clearly resolvable for wild-type barnase and most mutants, but there are larger signals for the W94Y series. The amplitude of phase 4 increases until the ratio of [GroEL] to [barnase] is sufficiently high that only one mol of barnase is bound per complex of 14-mer (Fig. 4). The rate constant for phase 4 is sensitive to mutation, and the values of k_4 parallel the rate constants for the folding of the mutants in solution (Fig. 5), as found previously for a limited data set in which the regain of catalytic activity was measured (18) ((k_4 for W94Y = 0.022 ± 0.0007 s^{-1}, k_4 for W94Y(S91A) = 0.0114 ± 0.0005 s^{-1}, and k_4 for W94Y(D8A/D12A/R110A) = 0.032 ± 0.0005 s^{-1}]. In all cases checked, the rate constant for the change in fluorescence is identical to that measured previously (18) for the regain of catalytic activity of barnase. Phase 4 is thus the rate constant for the conversion of singly bound, denatured barnase to the folded state.

ATP Enhances the Rate of Folding on GroEL. The rate constant for phase 4 is increased by [ATP] (Fig. 4), reaching 15-fold for each mutant. This is not due to release from the GroEL because k_4 has the same sensitivity to mutation in the presence of ATP as in its absence. There is a smaller effect on k_3 of about 3.5-fold for each mutant.

DISCUSSION

Kinetic Pathway of Folding of Barnase in the Presence of GroEL. Four kinetic phases are observed in the folding of GroEL-bound barnase monitored by its intrinsic tryptophan fluorescence. The changes in fluorescence during kinetics have been assigned to specific chemical or physical events by using data on the effects of mutations on barnase and on the rate of regain of catalytic activity. Phase 1 is the association of denatured barnase with GroEL: the reaction follows second-order kinetics and has a rate constant approaching the maximum value for protein–protein association. Phase 2 is an event

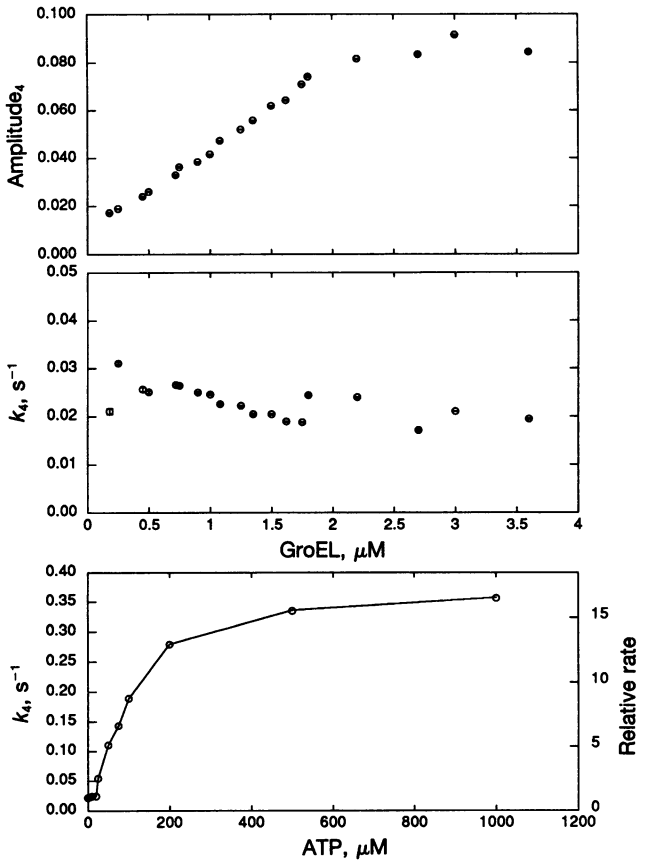

FIG. 4. Phase 4 of folding of barnase W94Y in the presence of GroEL. These are part of the same kinetic curves presented in Fig. 3 and so the amplitudes may be directly compared. The error bars are all within the circles of data points.

accompanying the binding of multiple numbers of barnase molecules to GroEL (up to 4 barnase molecules per 14-mer). Phase 3 is the folding of multiply bound molecules of barnase. Phase 4 is the formation of active barnase at high concentrations of GroEL, where there is just one mole of barnase bound per mole of complex of 14-mer. The rate constant for phase 4 is increased by the presence of ATP to a value ($\approx$0.4 s^{-1}) that is compatible with folding under physiological conditions.

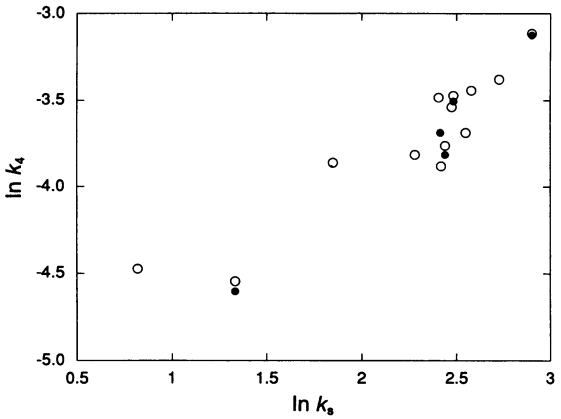

FIG. 5. Plot of the rate constant for folding of GroEL-bound mutants of barnase, k_4, against k_s, the rate constant for the folding of the same mutants in the absence of GroEL. Open circles are for the fluorescence traces; the filled circles were measured previously for the regain of catalytic activity of barnase (18). The linearity of the plot shows that the transition state for folding on GroEL is very similar to that for folding in solution.

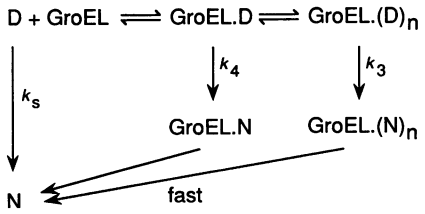

FIG. 6. Mechanism for the kinetics of folding of barnase bound to GroEL. At high ratios of barnase to GroEL, multiple numbers of barnase are bound ($n \geq 4$). At high ratios of GroEL to barnase, 1 mol is bound and folds more slowly in a step that is enhanced by ATP.

Perhaps k_3 is faster than k_4 because the multiply bound molecules of denatured barnase are bound more weakly and so have less binding energy to lose on folding.

The kinetic data eliminate mechanisms of folding in which unfolded barnase is released from GroEL to fold in solution. As argued previously (17), the folding cannot occur from a small amount of denatured barnase folding free in solution since, by the law of mass action, the fraction of free, denatured barnase would become vanishingly small at high concentrations of GroEL. Thus, a folded form of barnase has to be released from GroEL. It could be argued that a folding intermediate is released that does not bind to GroEL and that the final folding occurs in solution. However, this argument would still have to have a folding event occurring in the barnase–GroEL complex and would deny the role of GroEL being able to unfold partly folded molecules. The folding of barnase in the presence of GroEL is consistent with the mechanism shown in Fig. 6.

Implications for the Folding of Proteins *in Vivo*. The molecular chaperone pathway *in vivo* involves GroEL or the equivalent Cpn60 proteins in the final stages of folding (5, 29, 30). Generally, ATP is required for the release of the larger proteins from their complexes with GroEL. The cochaperonin GroES is also required in some but not all cases (7). Barnase, however, can fold from its complex with GroEL without the obligatory requirement of ATP, although the presence of ATP does speed up the reaction. The folding in the absence of ATP does not mean that the folding pathway of barnase is different from that of larger proteins but probably just results from its small size causing weaker binding to the chaperone and its being able to fold in a single step.

The evidence against proteins folding when bound to GroEL is that a GroEL mutant that binds but does not release denatured states of proteins can scavenge denatured proteins bound to wild-type GroEL (6). However, that mutant may be simply trapping partly folded proteins that are temporarily released during the folding and translocation processes on wild-type GroEL. Indeed, GroEL can cause the unfolding of folded proteins (26, 31), including barnase (R. Zahn, S. Perrett, and A.R.F., unpublished data). Since barnase can fold when bound to GroEL, smaller parts of larger proteins could refold when bound to GroEL. Further, the high stoichiometry of binding of 4 mol of barnase per mol of GroEL 14-mer means that a protein of some 4–500 residues could be bound to GroEL if the binding sites are not too far apart. It is likely, therefore, that large proteins refold when bound to GroEL, possibly folding in parts as the proteins undergo rounds of successive folding combined with either translocation or dissociation followed by reassociation.

1. Robinson, C. V., Gross, M., Eyles, S., Ewbank, J., Mayhew, M., Hartl, F. U., Dobson, C. M. & Radford, S. E. (1994) *Nature (London)* **372,** 647–651.
2. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L. & Hartl. F. U. (1991) *Nature (London)* **352,** 37–43.
3. Landry, S. J. & Gierasch, L. M. (1994) *Annu. Rev. Biophys. Biomol. Struct.* **23,** 645–669.

4. Ellis, R. J. (1994) *Curr. Opin. Struct. Biol.* **4,** 117–122.
5. Martin, J., Mayhew, M., Langer, T. & Hartl, F. U. (1993) *Nature (London)* **366,** 228–233.
6. Weissman, J. S., Kashi, Y., Fenton, W. A. & Horwich, A. L. (1994) *Cell* **78,** 693–702.
7. Schmidt, M., Buchner, J., Todd, M. J., Lorimer, G. H. & Viitanen, P. V. (1994) *J. Biol. Chem.* **269,** 10304–10311.
8. Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H. & Gatenby, A. A. (1991) *Biochemistry* **30,** 9716–9723.
9. Staniforth, R. A., Burston, S. G., Atkinson, T. & Clarke, A. R. (1994) *Biochem. J.* **300,** 651–658.
10. Todd, M. J., Viitanen, P. V. & Lorimer, G. H. (1994) *Science* **265,** 659–666.
11. Ellis, J. R. (1991) *Annu. Rev. Biochem.* **60,** 321–347.
12. Hendrick, J. P. & Hartl, F. U. (1993) *Annu. Rev. Biochem.* **62,** 349–384.
13. Hartl, F. U., Hlodan, R. & Langer, T. (1994) *Trends Biochem. Sci.* **19,** 20–25.
14. Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, S. G., Ranson, N. A., Clarke, A. R. & Saibil, H. R. (1994) *Nature (London)* **371,** 261–264.
15. Fersht, A. R. (1985) *Enzyme Structure and Mechanism* (Freeman, New York), 2nd Ed.
16. Fersht, A. R. (1993) *FEBS Lett.* **325,** 5–16.
17. Gray, T. E. & Fersht, A. R. (1993) *J. Mol. Biol.* **232,** 1197–1207.
18. Gray, T. E., Eder, J., Bycroft, M., Day, A. G. & Fersht, A. R. (1993) *EMBO J.* **12,** 4145–4150.
19. Loewenthal, R., Sancho, J. & Fersht, A. R. (1991) *Biochemistry* **30,** 6775–6779.
20. Horovitz, A., Serrano, L. & Fersht, A. R. (1991) *J. Mol. Biol.* **219,** 5–9.
21. Horovitz, A., Serrano, L., Avron, B., Bycroft, M. & Fersht, A. R. (1990) *J. Mol. Biol.* **216,** 1031–1044.
22. Horovitz, A. & Fersht, A. R. (1992) *J. Mol. Biol.* **224,** 733–740.
23. Serrano, L., Kellis, J. T., Cann, P., Matouschek, A. & Fersht, A. R. (1992) *J. Mol. Biol.* **224,** 783–804.
24. Serrano, L., Horovitz, A., Avron, B., Bycroft, M. & Fersht, A. R. (1990) *Biochemistry* **29,** 9343–9352.
25. Gill, S. C. & von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326.
26. Gray, T. E. & Fersht, A. R. (1991) *FEBS Lett.* **292,** 254–258.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
28. Matouschek, A., Kellis, J. T., Jr., Serrano, L. & Fersht, A. R. (1989) *Nature (London)* **342,** 122–126.
29. Frydman, J., Nimmesgern, E., Ohtsuka, K. & Hartl, F. U. (1994) *Nature (London)* **370,** 111–117.
30. Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N. & Furtak, K. (1993) *Cell* **74,** 909–917.
31. Zahn, R., Spitzfaden, C., Ottiger, M., Wuthrich, K. & Pluckthun, A. (1994) *Nature (London)* **368,** 261–265.