Nature and Consequences of GroEL−Protein Interactions

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ABSTRACT: The importance of chaperonin−protein interactions has been investigated by analyzing the refolding of the barley chymotrypsin inhibitor 2 in the presence of GroEL. The chaperonin retards the rate of refolding of wild type and 32 representative point mutants. The retardation of the rate drops to a finite level at saturating concentrations of GroEL, being lowered by a factor of 3−100, depending on the mutation. It is seen qualitatively that truncation of large hydrophobic side chains to smaller side chains weakens binding. Analysis of the magnitude of the rates of retardation shows further that hydrophobic and positively charged side chains tend to interact favorably with GroEL whereas negatively charged side chains tend to repel. There is an inverse correlation between the strength of hydrophobic interactions and the rate constant for refolding of the GroEL-complexed protein: the better the binding, the slower the folding. This shows directly that hydrophobic (and other favorable) interactions between the chaperonin and substrate are weakened during the refolding process and implies that unfolding can be catalyzed by the gain of such interactions.

The chaperonin GroEL of *Escherichia coli* is a member of the hsp60 class of molecular chaperones which recognize and bind unfolded or misfolded proteins in the cell (Goloubinoff et al., 1989; Badcoe et al., 1991; Buchner et al., 1991; Martin et al., 1991, 1993; Mendoza et al., 1991; van der Vies et al., 1992; Jackson et al., 1993a; Ellis, 1994; Fenton et al., 1994; Okazaki et al., 1994; Robinson et al., 1994; Zahn et al., 1994; Lin et al., 1995). How do proteins fold in the presence of the molecular chaperones such as GroEL? Does GroEL simply rescue misfolded proteins which are then released to fold in solution, or do proteins fold inside the central cavity of GroEL or when they are bound to its outer surface? If proteins do fold when bound to GroEL, is the pathway of folding similar to that in solution? The nature of recognition of unfolded proteins by GroEL is also unresolved. One approach to answering these problems is to study the influence of GroEL on the folding of proteins whose pathways are well-documented *in vitro*. The structures of barnase and the serine protease inhibitor chymotrypsin 2 (CI2) have been mapped at the level of individual residues by the protein-engineering method and NMR during their folding *in vitro* (Matouschek et al., 1989, 1992; Fersht et al., 1992; Fersht, 1993; Jackson et al., 1993c; Otzen et al., 1994). To provide answers to some of the questions about chaperonins, we have analyzed a series of mutants of barnase whose pathway of folding may be followed by changes of fluorescence. The denatured state of the 110-residue barnase, which refolds via a folding intermediate, binds to the molecular chaperone GroEL and refolds while still bound (Gray & Fersht, 1993; Corrales & Fersht, 1995). The 64-residue CI2, which consists of just a single structural domain, is distinguished by unfolding and folding via simple two-state kinetics in which the folded and denatured states are connected by just a single rate determining transition state (Jackson & Fersht, 1991a). As such, the folding of CI2 represents the folding of a basic unit of protein structure and perhaps a self-contained domain of a larger protein. Both barnase and CI2 consist of single polypeptide chains that do not have disulfide cross-links. This is an important characteristic for studying the earliest stages of protein folding since it allows the investigation of the interaction of the fully denatured protein with molecular chaperones, the formation of disulfide cross-links being a late stage in folding that is directed by the tertiary structure of the protein. Here, we examine the folding of CI2 to see if it also refolds while bound to GroEL, and, if it does, we take advantage of the simple kinetics to compare the influence of mutations on the folding kinetics in the presence and absence of GroEL.

MATERIALS AND METHODS

The CI2 mutants used in this study have been characterized previously (Jackson et al., 1993b,c; Otzen et al., 1994) or are unpublished (L. S. Itzhaki, D. E. Otzen, and A. R. Fersht). Mutants were prepared by inverse PCR and expressed and purified as described (Jackson et al., 1993b). GroEL was expressed and purified from *E. coli* by a modification of a described procedure (Gray & Fersht, 1991). The GroEL concentration (always expressed as that of the 14-mer) was determined using the Bio-Rad protein assay kit [based on the Bradford (Badcoe et al., 1976) assay] and by quantitative amino acid analysis; the two methods give identical results.

The refolding kinetics of CI2 was measured as described (Jackson & Fersht, 1991a) using a thermostated Applied Photophysics stopped-flow spectrophotometer model SF 17MV. A solution of 1 μM denatured CI2 in 20 mM HCl (pH 1.7) was rapidly mixed at 25 °C with an equal volume of 100 mM MES, pH 6.65 (containing 21.5 mM of the free acid and 78.5 mM of the sodium salt), to give a final pH of 6.25. The fluorescence of the single Trp residue in CI2 was followed by excitation at 290 nm; emission at wavelengths greater than 315 nm was collected via a cutoff filter. These conditions were found to be optimal for following the folding

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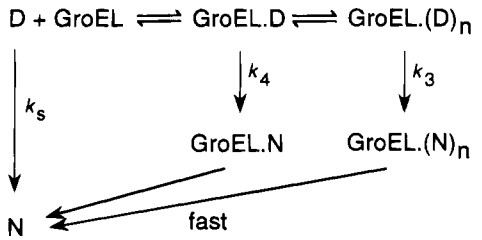

FIGURE 1: Mechanism for kinetics of folding of barnase bound to GroEL from Corrales and Fersht (1995).

of CI2 in the presence of GroEL, which does not have any tryptophan residues. GroEL, when required, was included in the MES buffer to give final concentrations between 0.0125 and 2 μM after mixing. A solution of 200 μM ATP, where used, was included in the solution containing denatured CI2 with the addition of an equimolar concentration of $MgCl_2$. In addition, 4 mM KCl was included in the GroEL−MES solution.

The change in the free energy of transfer of the hydrophobic side chains from *n*-octanol to water on mutation [$\Delta\Delta G_{\text{transfer}(n\text{-octanol}\rightarrow\text{water})}$] was calculated from the data of Leo and Hansch (1979) using the following equation:

$$\Delta G_{\text{transfer}(n\text{-octanol}\rightarrow\text{water})} = RT(\pi_{\text{wild type}} - \pi_{\text{mutant}}) \text{ (kcal/mol)} \quad (1)$$

where $\pi = \log P$ and where P is the partition coefficient of the mutated side chain between water and *n*-octanol.

RESULTS

Kinetics of Folding. The kinetics of folding is easier to understand in light of the analyzes of the kinetics of folding of barnase in the presence of GroEL (Figure 1; Corrales & Fersht, 1995) which are recapitulated now. On mixing denatured barnase with GroEL under conditions that favor folding, a series of refolding phases is observed. The first of these is the refolding of barnase in solution which competes with binding of denatured barnase to GroEL. The amplitude of this phase decreases with increasing [GroEL], and the rate constant remains constant at low [GroEL] at the value for folding in solution. At higher concentrations, the rate of binding to GroEL may be detected because there is a change in the fluorescence of barnase. At high ratios of [barnase]/[GroEL], the rate constant for the refolding of multiply-bound barnase is observed. while at low [barnase]/[GroEL] the much slower rate of singly-bound barnase is observed.

On mixing acid-denatured wild-type CI2 with MES buffer, there is a rapid decrease of fluorescence from the single tryptophan residue with a rate constant of 55 s^{-1}. This phase corresponds to the folding of the major form of denatured protein that has all of its peptidyl-prolyl bonds in the *trans* conformation (Jackson & Fersht, 1991a,b). There are additional slower decreases in fluorescence (~30%) as the forms with *cis*-peptidyl−prolyl bonds interconvert (Jackson & Fersht, 1991b). The rate of folding of CI2 in the presence of GroEL may be followed by the change in tryptophan fluorescence because GroEL lacks tryptophan residues. On increasing the concentration of GroEL in the MES buffer, the amplitude of the fast phase decreases and a slower phase appears. This is behavior identical to that previously found for the refolding of barnase in the presence of GroEL (Corrales & Fersht, 1995): there is competition between

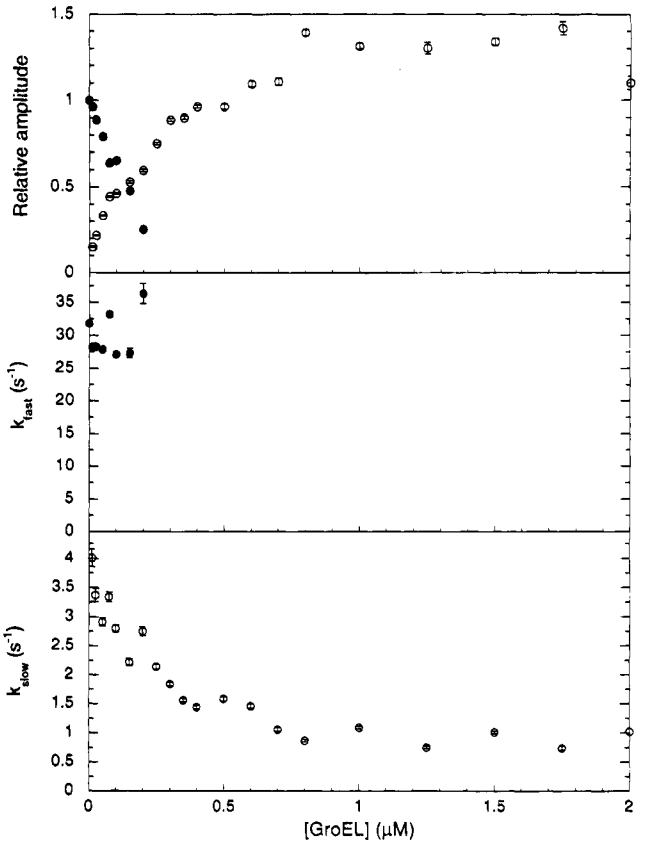

FIGURE 2: Folding of the CI2 mutant Asp→Ala52 at 0.5 μM in the presence of increasing concentrations of GroEL (0−2 μM). There is a fast phase, which represents folding in solution at 28 s^{-1} and is the major phase at [GroEL] < 0.1 μM. Its amplitude decreases as [GroEL] increases, and a second, slower phase appears of increasing amplitude: top panel, amplitudes of the fast (filled circles) and slow (open circles) phases; middle panel, rate constants for the fast phase; and bottom panel, rate constants for the slow phase. This mutant is a representative of the class where dissociation rate constants are lower than those for folding.

denatured protein folding in solution and being trapped by binding to GroEL where slower folding takes place. The sum of the amplitudes of the fast and slow phases approaches that of the amplitude of the fast phase in the absence of GroEL. The fast phase disappears by about 1 μM GroEL, and the rate constant of the slower phase levels off to a value which we denote k_g. Many of the mutants display behavior similar to that of wild type (for example Asp→Ala52, Figure 2), except that the concentrations of GroEL required for 50% inhibition of the fast phase vary (Table 1). There is a second category of mutations, however, for which we do not observe the appearance of a distinct phase on increasing the GroEL concentration. Instead, the amplitude of the folding phase observed in the absence of GroEL remains constant as increasing concentrations of GroEL are added, but the rate decreases (Figure 3). All the mutants in this category are characterized by the truncation of one or more large hydrophobic side chains. The binding step between denatured CI2 and GroEL is not observed since, unlike barnase, there is insufficient change in fluorescence on binding.

Analysis of Rate Law. The folding of CI2 in the presence of GroEL is a more general form of the kinetics of folding of barnase in the presence of GroEL (Figure 1) (Corrales & Fersht, 1995), of which the slowest phase has been unambiguously assigned to the regain of catalytically active protein when bound to GroEL in a 1:1 complex (Gray & Fersht, 1993; Corrales & Fersht, 1995). Since the dissociation rate

Table 1: Concentration of GroEL ([GroEL]$_{50\%}$) Required to Cause 50% Decrease of Fraction of CI2 Mutant (0.5 μM) Folding in Solution[a]

mutant	[GroEL]$_{50\%}$[b] (μM)
wild type	0.40 ± 0.01
LA8	0.28 ± 0.03
AG16	0.12 ± 0.02
IV20	0.22 ± 0.02
LG21	0.26 ± 0.01
LA32	0.22 ± 0.02
YG42	0.25 ± 0.01
LA49	0.44 ± 0.02
FL50	0.12 ± 0.01
VA51	0.21 ± 0.03
AG58	0.38 ± 0.10
VG60	0.25 ± 0.03
EA7	0.12 ± 0.01
EQ14	0.09 ± 0.02
ED14	0.28 ± 0.02
EN14	0.06 ± 0.002
QG22	0.42 ± 0.05
EA41	0.30 ± 0.03
DA45	0.10 ± 0.02
DA52	0.14 ± 0.01
KG17	0.13 ± 0.06
KG18	0.08 ± 0.03
KG24	0.30 ± 0.07
TV36	0.30 ± 0.05
VT60	0.28 ± 0.03
SA12	0.19 ± 0.02

[a] These mutants fold according to two phases: a fast phase for folding in solution that decreases with increasing [GroEL] and a slow phase of folding while bound to GroEL that increases with [GroEL]. A 0.5 μM solution of mutant was allowed to fold in the presence of increasing concentrations of GroEL (0−2 μM). A solution of 1 μM denatured CI2 in 20 mM HCl (pH 1.7) was rapidly mixed at 25 °C with an equal volume of 100 mM MES, pH 6.65 (containing 21.5 mM of the free acid and 78.5 mM of the sodium salt), to give a final pH of 6.25. GroEL was included in the MES buffer. [b] Visual estimate of concentration of GroEL at which the amplitude of the solution refolding phase had declined to half of the value in the absence of GroEL.

constant of barnase complexed to GroEL is much slower than the folding steps, distinct phases are always seen. For CI2, however, distinct fast and slow phases are seen for the wild type and the first category of mutants. The rate constant of the slower of the two folding phases (k_g) decreases with increasing concentration of GroEL and levels off at a constant value. For GroEL-bound barnase (Figure 1), there are two folding steps that are distinct: a higher rate constant for multiply-bound protein (k_3) and a lower one for singly-bound (k_4). The separation implies that the rate of interconversion of singly- and multiply-bound forms is slow in the case of barnase. If, however, the interconversion between the singly- and multiply-bound states is fast, as it could be for CI2 which is smaller and hence has fewer binding sites than does barnase, then there will be a single rate constant observed for folding while bound to GroEL that will be a combination of the two separate values:

$$k_g = \beta k_4 + (1 - \beta)k_3 \quad (2)$$

where β is the fraction of singly-bound CI2 and $(1 - \beta)$ is the fraction of multiply-bound CI2. (Note: In this analysis, k_3 is simplified to a single rate constant; it could be the sum of several individual rate constants, but the same qualitative behavior would be observed for k_g). At low concentrations of GroEL, CI2 folds via multiply-bound forms, while at high concentrations, the singly-bound state is predominant. The full analysis of the rate law is very complex. Here we just outline the basic principles and the behavior in extreme cases. The only rate constant we use quantitatively is k_4, and that is measured unambiguously.

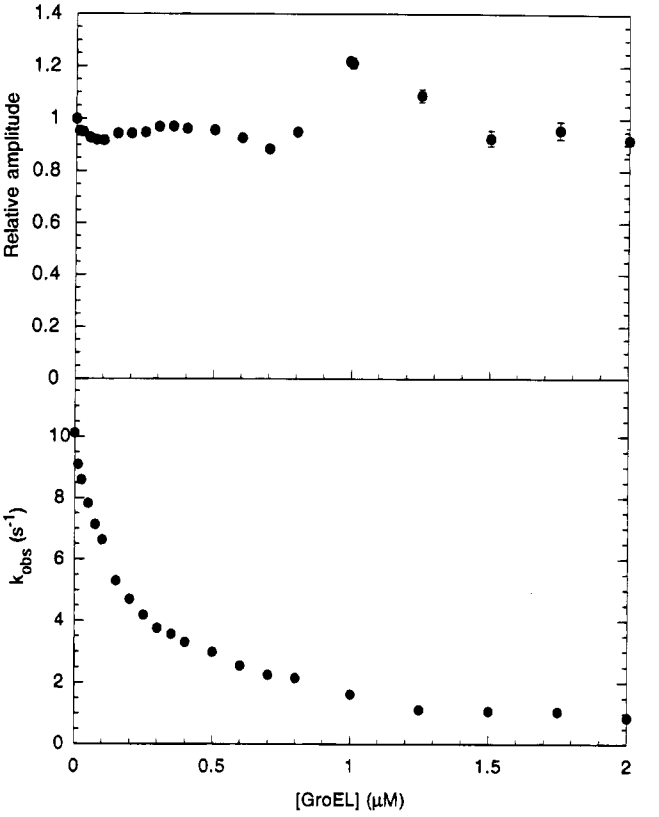

FIGURE 3: Folding of the CI2 mutant Phe→Ala50 at 0.5 μM in the presence of increasing concentrations of GroEL (0−2 μM). There is just a single phase of approximately constant amplitude (top panel) with a rate constant that decreases with increasing [GroEL] (bottom panel). This mutant is a representative of the class in which dissociation rate constants are fast compared with folding.

For the first category of mutants, we can measure the concentration of GroEL at which the amplitude of the first phase decreases to 50% of the initial value ([GroEL]$_{50\%}$) (Table 1), which is how much GroEL is required to compete with and bind 50% of the denatured state of CI2 before it can fold itself in solution. There is a significant correlation between [GroEL]$_{50\%}$ and k_s, the refolding rate in solution, which can be explained by competition between binding to GroEL and folding in solution: the faster the mutant protein folds, the more GroEL is required to have an effect.

The stoichiometry of binding of CI2 may be calculated for mutants in the first category using the decline in amplitude of the fast folding phase, as was done for barnase (Corrales & Fersht, 1995). The stoichiometry thus calculated is the number of moles of CI2 bound per mole of total GroEL and not per mole of complexed GroEL. This value of the stoichiometry is always less than or equal to the true stoichiometry of the number of moles bound per mole of complex. The true stoichiometry of the GroEL:CI2 complex tends toward 1:1 at high GroEL concentrations. At low concentrations of GroEL, the stoichiometry is significantly higher, particularly for mutants where negative charges have been deleted. For example, eight molecules of the mutant protein Glu→Gln33 are bound to each GroEL oligomer at 0.025 μM GroEL. Mutants involving the truncation of hydrophobic side chains bind at lower stoichiometry. A trend is present: the larger the truncation of hydrophobic side chains, the lower the stoichiometry. For example, at 0.025

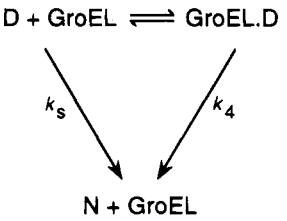

FIGURE 4: Simplified version of Figure 1 that applies to mutants of CI2 where association and dissociation steps are relatively rapid compared with folding, and [GroEL] > [CI2] so that the complex of stoichiometry 1 predominates.

Table 2: Dissociation Constants, K_D, of CI2 Mutants[a]

mutation	K_D[b] (μM)
IA29/IV57	0.012 ± 0.004
IA30	0.08 ± 0.05
LA32/FL50	0.05 ± 0.01
LA32/FA50	0.11 ± 0.02
VA38/FL50	0.05 ± 0.01
VA38/FA50	0.64 ± 0.16
FA50	0.05 ± 0.01

[a] These mutants fold in a single phase since there is a rapid equilibrium between free and bound CI2 compared with folding. [b] Calculated from eq 4.

μM GroEL, four molecules of Ala→Gly16, as opposed to only two of Phe→Leu50, bind to each GroEL oligomer. Some mutants (as well as wild type) apparently do not form multiply-bound states at all; these include Leu→Ala8, Gln→Gly22, Leu→Ala32, Thr→Val36, Leu→Ala49, and Val→Ala51.

For the second category of mutants of CI2, dissociation of the complex with GroEL must be relatively fast so that there is only a single observed folding phase which follows the rate law

$$k_{obs} = \alpha k_s + (1 - \alpha)k_g \quad (3)$$

where α is the fraction of CI2 free in solution and $(1 - \alpha)$ is that bound to GroEL. k_g is likely to be a composite of rate constants for singly- and multiply-bound CI2, but there is likely to be a smaller fraction of multiply-bound CI2. At high ratios of GroEL to CI2, the reaction scheme in Figure 1 approximates that shown in Figure 4, where CI2 is bound to GroEL in a 1:1 complex only. This simplification allows us to derive the following equation describing the relationship between the observed rate constant and the total concentration of GroEL:

$$k_{obs} = {}^1/_2\left\{\frac{-K_D - [G]_o(k_s - k_g)}{[D]_o} + k_s + k_g + \left[\left(\frac{K_D + [G]_o(k_s - k_g)}{[D]_o} + k_s + k_g\right)^2 - 4\left(-\frac{(K_D k_s + k_s[G]_o)(k_s - k_g)}{[D]_o} + k_s k_g\right)\right]^{1/2}\right\} \quad (4)$$

where $[G]_o$ is the total concentration of GroEL, $[D]_o$ is the total concentration of CI2 (0.5 μM), K_D is the dissociation constant for the 1:1 complex, and k_g is the rate of refolding of singly-bound CI2. The values of K_D obtained from fitting the observed rate constants for the second category of mutant proteins to the above equation are given in Table 2.

Nature of Chaperonin−Side Chain Interactions. The rate of folding of CI2 on GroEL does not appear to be as sensitive to mutation as is the rate of folding in solution. At high concentrations of GroEL, the majority of the refolding rate constants cluster between values of 1 and 2 s^{-1}, whereas in solution the refolding rates vary 5-fold between 10 and 50 s^{-1} (Table 3). There is no significant correlation between k_s and k_g; even if the structure of the transition state of CI2 folding on GroEL is identical to that of CI2 folding in solution, k_g may not appear to respond to mutation in a similar manner to k_s because of an additional affect of mutation on the binding affinity of GroEL which will be reflected in value of k_g. The change in binding affinity on mutation can be expressed in energy terms as

$$\Delta G_g = RT[\ln(k_s/k_g)_{mutant} - \ln(k_s/k_g)_{wild\ type}]\ (kcal/mol) \quad (5)$$

where k_s/k_g represents the retardation of the folding rate of each mutant on GroEL relative to that in solution. The relationship between the amount of retardation of the folding rate on complexation with GroEL and the types of structural changes on mutation become apparent when plotting the data in the bar chart in Figure 5. These data show clearly that the deletion of large hydrophobic side chains weakens the binding of the substrate to the chaperonin, as measured by the retardation of the folding rate (for example, Phe→Ala50 and Ile→Ala29/Ile→Val57). Mutation of the positively

Table 3: Rate Constant of Refolding of CI2 in Solution and on GroEL

mutant	k_s[a] (s^{-1})	k_g[b] (s^{-1})
wild type	56.8 ± 2.5	1.82 ± 0.01
Removal of hydrophobic side chains		
LA8	28.8 ± 1.1	1.66 ± 0.03
AG16	8.1 ± 0.2	0.37 ± 0.06
IV20	23.6 ± 0.6	1.85 ± 0.04
LG21	25.2 ± 0.7	1.25 ± 0.02
IA29/IV57	7.6 ± 0.3	1.45 ± 0.05
IA30	18.9 ± 0.9	4.50 ± 0.15
LA32	26.7 ± 3.0	3.33 ± 0.02
LA32/FL50	16.0 ± 0.1	1.49 ± 0.03
LA32/FA50	6.3 ± 0.6	1.90 ± 0.02
VA38/FL50	20.5 ± 0.1	1.80 ± 0.03
VA38/FA50	7.5 ± 0.3	2.00 ± 0.04
YG42	43.2 ± 1.2	3.66 ± 0.01
LA49	1.8 ± 0.1	0.18 ± 0.02
FL50	20.7 ± 0.8	1.14 ± 0.03
FA50	10.1 ± 0.01	1.04 ± 0.04
VA51	24.6 ± 0.9	2.00 ± 0.06
AG58	39.5 ± 0.9	1.97 ± 0.06
VG60	45.8 ± 1.2	1.87 ± 0.03
Removal of Negative Charges		
EA7	41.2 ± 2.3	1.00 ± 0.03
EQ14	30.8 ± 0.9	0.70 ± 0.01
ED14	47.6 ± 1.9	1.30 ± 0.01
EN14	23.1 ± 0.4	0.55 ± 0.02
QG22	50.2 ± 0.9	1.70 ± 0.04
EA41	35.1 ± 0.4	1.31 ± 0.03
DA45	33.6 ± 0.7	0.32 ± 0.01
DA52	28.3 ± 1.1	0.88 ± 0.02
Removal of Positive Charges		
KG17	12.9 ± 0.4	1.00 ± 0.02
KG18	17.9 ± 0.2	1.15 ± 0.02
KG24	33.7 ± 1.7	1.90 ± 0.02
Polar Switches		
TV36	44.3 ± 0.8	1.00 ± 0.02
VT60	40.8 ± 1.3	1.95 ± 0.06
SA12	29.8 ± 1.4	1.80 ± 0.05

[a] Rate constant for refolding of CI2 in the absence of GroEL. [b] Rate constant for refolding of CI2 at high ratios of GroEL to CI2 when CI2 is bound in a 1:1 complex.

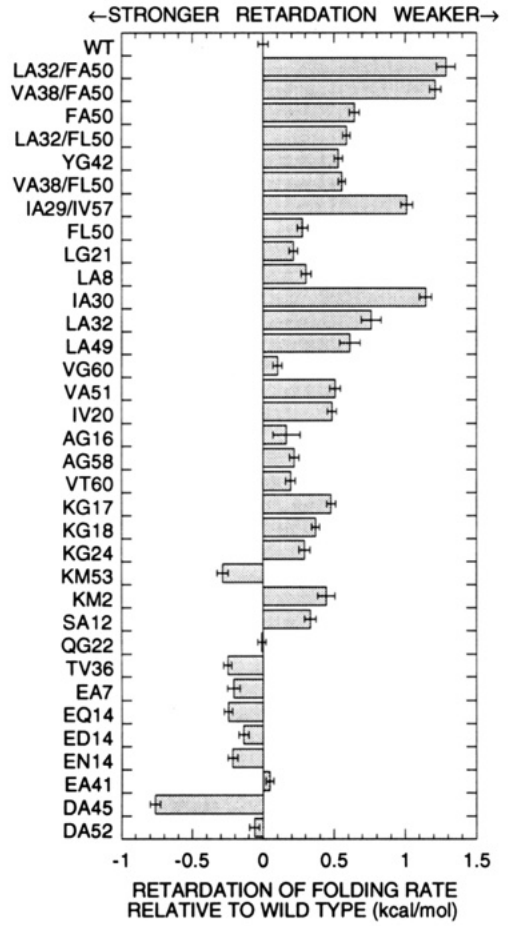

FIGURE 5: Relative retardation of folding on binding of different mutants of CI2 to GroEL. k_g was calculated by taking the average of the refolding rates measured at saturating concentrations of GroEL, at which concentrations the rates constants for folding had reached a plateau value (usually between 1.5 and 2 μM GroEL) and CI2 is bound in a 1:1 complex. $\Delta\Delta G_{retardation}$ for the mutation Lys→Met at position 53 was measured for the double mutant Lys→Met53/Met→Leu40 relative to the single mutant Met→Leu40. Mutant proteins are listed by type, i.e., mutations that delete hydrophobic groups (largest deletions first), mutations that remove positive charge, polar switch mutations, and mutations that remove negative charge.

charged side chain of Lys to a Gly (Lys→Gly17, Lys→Gly18, Lys→Gly24) also weakens binding. At first sight, this would suggest that positive charges are involved in binding. However, in addition to removing positive charge by such a mutation, a long hydrophobic chain is also deleted. The mutation Lys→Met, however, removes the positive charge while retaining the hydrophobic chain. The mutation Lys→Met2 weakens binding, indicating that the positive charge does favor binding here. Conversely, the mutation Lys→Met53 strengthens binding, showing that the effect is not uniform. Mutations that remove the negatively charged side chains of aspartate and glutamate cause the rate constant for folding to be retarded more. Thus, the more hydrophobic groups there are, the better the binding; positive charges on the substrate protein tend to enhance binding to GroEL, while negative ones are unfavorable.

All mutant proteins in the second category, i.e., those that do not show a distinct new folding phase in the presence of GroEL, have large values of $\Delta\Delta G_g$. Indeed, this can be seen clearly for two mutations at the same site; the mutants Phe→Ala50 and Phe→Leu50 belong to different categories, and $\Delta\Delta G_g$ is much larger for Phe→Ala50 than for Phe→Leu50. Thus the two different categories of CI2

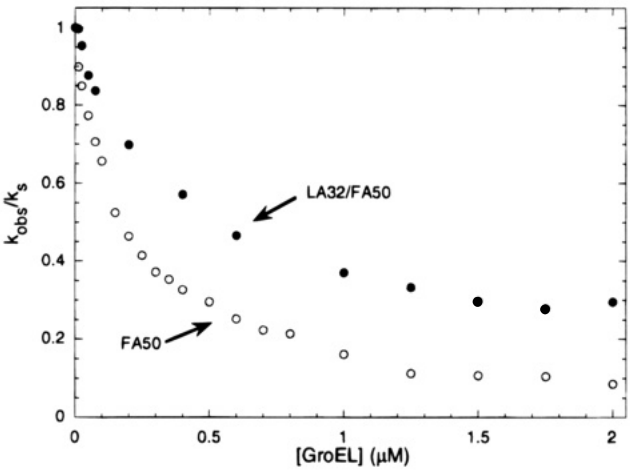

FIGURE 6: Folding of the CI2 mutant Phe→Ala50 (0.5 μM, open circles) and the double mutant Leu→Ala32/Phe→Ala50 (0.5 μM, filled circles) in the presence of GroEL. The rate constant for folding in the presence of GroEL (k_{obs}) is plotted as a fraction of the rate constant for folding in solution (k_s). Folding of the mutant with the greater truncation of hydrophobic side chains (Leu→Ala32/Phe→Ala50) is slowed down to a smaller extent on complexation with GroEL, and a greater concentration of GroEL is required to retard the folding rate by 50%.

mutants reflect the different binding affinities for GroEL and that this binding affinity is mediated in part by hydrophobic side chains. There appears to be a threshold value of $\Delta\Delta G_g$ of the order of 0.6 kcal mol^{-1}, above which a mutant protein folds according to the second category of behavior as a result of significant weakening of binding.

Changing the hydrophobic interactions affects both the strength of binding and the rate constant for folding of the bound CI2. First, as hydrophobic side chains are removed, the binding of the denatured state is weakened since a higher concentration of GroEL is required to bind 50% of the CI2 (Figure 6). Secondly, the retardation of the folding rate constant is also diminished: there is a nice correlation between the energy of retardation for the different mutants against the change on mutation in the free energy of transfer of the hydrophobic side chains from *n*-octanol to water [$\Delta\Delta G_{transfer(n\text{-octanol}\rightarrow water)}$] [calculated from the data of Leo and Hansch, 1979, using eq 1], with slope −0.3 (Figure 7). The small slope indicates that only a fraction of the hydrophobic energy is lost on going from the bound denatured state to the transition state. The negative sign means that the relative rates of refolding are inversely correlated with the strength of the binding interactions; the weaker the binding, the faster the rate of refolding. When the analysis is extended to include polar switch and charged mutations, the trend is observed to continue across the zero point of $\Delta\Delta G_{transfer(n\text{-octanol}\rightarrow water)}$ (slope = −0.17 for the linear fit of all the data).

Effect of ATP. Mg−ATP is required to release many proteins from their complexes with GroEL (Martin et al., 1991; Buchner et al., 1991). Both Mg−ATP and the cochaperonin GroES are required for other proteins (Goloubinoff et al., 1989; Martin et al., 1991). ATP enhances the rate of folding of CI2 on GroEL up to 4-fold. The magnitude of the enhancement varies with mutation, being inversely related to k_s (Table 4).

DISCUSSION

Folding of Small Proteins While Bound to GroEL. The folding of denatured wild-type CI2 and 32 representative

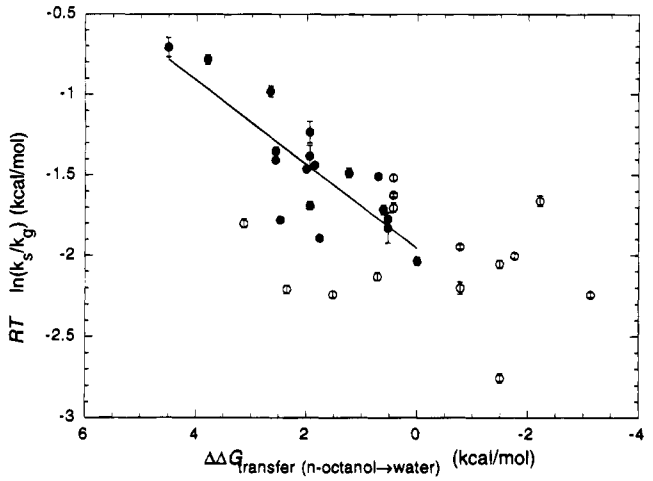

FIGURE 7: Plot of $RT \ln(k_s/k_g)$ against $\Delta\Delta G_{transfer(n\text{-octanol}\rightarrow water)}$, the difference in free energy of transfer of the wild-type and mutated side chain from *n*-octanol to water [calculated from the data of Leo and Hansch (1979); k_s is the rate constant for refolding in solution, and k_g is the rate constant for refolding at saturating concentrations of GroEL]. The contributions from multiple mutations were summed. Data for hydrophobic deletions are in filled circles and for polar switch/charged mutations in open circles. A linear fit of the data for the hydrophobic deletions only is shown, and gives a slope of -0.26 ± 0.04 and a correlation coefficient of 0.83 for 18 points. A linear fit for all mutations gives a slope of -0.17 ± 0.03 and a correlation coefficient of 0.67 for 32 points.

Table 4: Refolding Rates of CI2 Mutants in the Presence and Absence of ATP[a]

mutation	k_g (no ATP) (s^{-1})	k_g (100 μM ATP) (s^{-1})	k_g (no ATP)/ k_g(100 μM ATP)
EA7	1.00 ± 0.03	2.45 ± 0.06	2.45 ± 0.09
AG16	0.37 ± 0.01	1.56 ± 0.03	4.21 ± 0.14
LA32/FA50	1.90 ± 0.02	3.08 ± 0.02	1.62 ± 0.02

[a] Rate constant for refolding of CI2 at high ratios of GroEL to CI2 when CI2 is bound in a 1:1 complex.

mutants is retarded in the presence of GroEL. The rate laws for folding fall into two classes that are variations of that observed for the refolding of barnase (Figure 1). In the class that is similar to barnase, the rate constants for folding are fast compared with the dissociation steps, and so separate phases for folding in solution and folding while bound to GroEL are observed. In the second class, dissociation is rapid compared with folding, and so only a single folding phase is seen which has a rate constant which is the weighted average for folding in solution and when bound to GroEL (Figure 4).

It is clear that CI2 folds while bound to GroEL. The folding of both categories of mutants tends to a nonzero value of the rate constant at saturating concentrations of GroEL. Where two phases are observed, their amplitudes sum to the total change found for the folding in the absence of GroEL. Where only one phase is observed, its amplitude is the same as that for folding in the absence of GroEL. If folding occurs only when CI2 is free in solution, then by the law of mass action, the concentration of denatured CI2 in solution would fall to zero [cf. Gray and Fersht (1993) and Corrales and Fersht (1995)]. We find that on complexation with GroEL, the rate constants for folding of CI2 and its point mutations are slowed down by a factor of 3−100, dependent on the mutation. Further, the rate constant for folding is increased by ATP by a factor of up to 4. CI2 can fold while complexed to GroEL in the absence of ATP because of the weaker binding of this small protein compared with larger proteins. Barnase, which is almost twice as large as CI2, also folds when complexed with GroEL in a single step in the absence of ATP, but ATP has a larger effect on the refolding rate. It has now been found that native barnase binds weakly to GroEL with a dissociation constant in the mM region (R. Zahn, S. Perrett, G. Stenberg, and A. R. F., unpublished results). GroEL-bound denatured barnase can thus proceed directly to GroEL-bound native barnase without prior dissociation or folding coupled with dissociation.[1]

Although GroEL is not required for the folding of CI2 and barnase, their well-characterized folding pathways make them suitable for analyzing individual steps in chaperone-mediated folding. Barnase, which folds via multistep kinetics when free in solution, with the rate-determining step being the reaction of its folding intermediate, has the same rate-determining step in the presence of GroEL (Corrales & Fersht, 1995). CI2 folds by simple two-state kinetics without the accumulation of a folding intermediate. GroEL appears, therefore, to bind to the unfolded state of CI2. Its simpler kinetics allow an analysis of the chaperone−substrate interactions. The weak binding of CI2 to GroEL has the important advantage that it allows us detect the changes in affinity that occur on mutation of CI2, readily; conversely, it is difficult to study the effects of GroES on folding of GroEL-bound CI2 because it weakens binding further. Schmidt et al. (1994) have shown, however, that proteins that can fold spontaneously in solution, such as CI2, do not require GroES for GroEL-assisted folding.

Relationship to Mechanism of Action of GroEL. There are two different models for the role of GroEL, the first being a cage model in which the protein folds within the protected environment of the central cavity of the 14-mer (Creighton, 1991; Saibil et al., 1993) and the second postulating that proteins have to be released into solution for folding to occur (Lorimer et al., 1994; Weissman et al., 1994; Taguchi & Yoshida, 1995). Both require that the denatured state be bound, and so our results are relevant to both models.

Nature of Binding Interactions. The truncation of side chains of CI2 alters the affinity of the chaperone for its substrate. As seen in Figure 6, mutation of Leu→Ala32 in the mutant FA50 leads to a higher concentration of GroEL being required to give 50% inhibition. Further, the retardation of rate constant is also less for the weaker-binding mutant. Thus, the weaker the binding, the less the retardation of rate. It is difficult to analyze the binding data because of the complexity of the rate laws. The rates at saturating [GroEL] are more easily accessible. The relative rates of retardation (Figure 5) show that binding is mediated favorably by hydrophobic interactions and positively charged side chains, but negatively charged side chains mediate against binding. The significance of hydrophobic interactions for polypeptide binding to GroEL has been noted previously (Landry & Gierasch, 1991; Fenton et al., 1992; Landry et

[1] The only reaction schemes that could involve the folding of the protein free in solution and the rate constant not falling to zero with increasing [GroEL] are those that have the rate-determining release of a folding intermediate into solution, which could not rebind to GroEL. This is most unlikely since GroEL is expected to bind folding intermediates. Further, for CI2, there is no evidence for an intermediate on its folding pathway in the absence of GroEL. For barnase, the rate constants for the folding of a series of its mutants at saturating concentrations of GroEL parallel the folding rate constants in solution, showing that the process that is observed in the presence of GroEL specifically involves folding (Corrales & Fersht, 1995).

al., 1992; Dessauer & Bartlett, 1994; Lin et al., 1995; de Crouy-Chanel et al., 1995). Our studies show directly the importance of these interactions and that the interactions affect the rate of folding and not just the affinity of binding (see below).

The specific interactions between the chaperonin and protein were not seen previously for barnase. This may be because of the fewer mutants studied for barnase and the difference in its mechanism for folding. The rate-determining step for folding of GroEL-bound barnase is the conversion of a folding intermediate to the final folded state (Gray et al., 1993; Corrales & Fersht, 1995). The intermediate is known to be highly compact in solution (Fersht, 1993), whereas CI2 folds from a loose denatured state when free in solution. We surmise that GroEL-bound CI2 folds from an extended conformation in which most of its side chains are exposed to the chaperonin, whereas GroEL-bound barnase folds from a compact structure in which many residues are shielded from the chaperonin. Suitable mutants will have to be designed for identifying the interactions of barnase with GroEL.

Binding and Folding Are Correlated. The inverse correlation between strength of binding and rate is nicely illustrated for the hydrophobic side chains by plotting the change in energy of retardation against the change in hydrophobic transfer free energy on mutation of the side chain (Figure 7). The negative slope of the plot shows that the folding of the GroEL-complexed CI2 involves the breaking of hydrophobic interactions between the two proteins during the reaction. This shows directly that the chaperonin slows down the intrinsic act of folding because interactions between the chaperonin and the protein ligand have to be broken during the folding process itself: chaperonins are found to increase the yield rather than the overall rate of folding, and must do so by circumventing side reactions that inhibit folding. This inverse relationship suggests why the folding rate of the very small CI2 in its complex with GroEL is much faster than that of the larger barnase: fewer bonds have to be broken. In addition, it implies that the refolding rate constant for even larger proteins should be retarded more, and that is possibly why the folding of GroEL complexes of those proteins has a strong dependence on ATP, the binding of which is known to weaken the binding of protein substrates. Intriguingly, the direct link between rate constants and binding energy suggests the chaperonin can catalyze the unfolding rate constant of folded or misfolded states of proteins when they are bound.

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