Analysis of the Mechanism of Assembly of Cleaved Barnase from Two Peptide Fragments and Its Relevance to the Folding Pathway of Uncleaved Barnase

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ABSTRACT: A peptide corresponding to residues 1−22 of barnase that contains its major α-helix (residues 6−18) binds rapidly to the complementary peptide (residues 23−110) to form a catalytically active complex with near-native structure. Peptide 1−22 is approximately 3% helical in aqueous solution at 25 °C. A set of mutations in the helical regions of (1−22) cause the helix to be destabilized. We have investigated the mechanism of assembly of the peptides by analyzing the kinetics and equilibria of association of those mutants of (1−22) with native (23−110). The association reaction follows second-order kinetics. Virtually all the change in stability of the complex on mutation is reflected in changes in the association rate constant with the dissociation rate constant being very little affected. Both Brønsted and Φ-value analyses show that the helix is essentially fully formed in the transition state for the association at all the positions probed (residues 13−18). Peptide (23−110) contains all the residues necessary for catalysis. The complexes between all mutants peptides (1−22) with (23−110) are all only 10% active, however. The noncovalent complex is destabilized less by mutations in the helix than is the intact protein. Double-mutant cycle and other analyses show, however, that the intrahelical interactions are as strong in the noncovalent complex as in the intact protein and so the interactions between the helix and the rest of the protein must be weakened on cleavage of the 22−23 bond. This could well lead to effects on catalysis. Further, the energetics of the helix in the major transition state for the folding/unfolding of barnase are very similar to those in the looser noncovalent complex of (1−22)·(23−110). It is suggested, therefore, that the helix in the transition state for the folding/unfolding of intact barnase is similar to that in the noncovalent complex. This is consistent with earlier proposals that part of the rate-determining step in the folding of barnase is the docking of the preformed major helix onto the rest of the protein and the consolidation of the interactions between the two. The finding that fragments of barnase and the CI-2 inhibitor that are cleaved between regular elements of secondary associate by mechanisms similar to those of the folding of the intact proteins eliminates mechanisms of folding in which structure formation is propagated along the entire polypeptide chain. This also has implications for the folding of circularly permuted proteins.

Little is known about the early stages of protein folding because it is difficult to study experimentally. There are many open questions about the initiation of folding (Ptitsyn 1973; 1991; Kim & Baldwin, 1982; Dill, 1990; Shortle et al., 1990; Wodak & Rooman, 1993). For example, do local elements of native secondary structure in unfolded states act as foci for folding? One approach to the problem is to study fragments of proteins for they might give clues about the formation of secondary structure in the absence of tertiary interactions (Wright et al., 1988; Shortle & Meeker, 1989; Kim & Baldwin, 1990; Dyson et al., 1992; Sancho & Fersht, 1992; Sancho et al., 1992). Further, the structure of fragments may be analyzed by spectroscopy under conditions where their parent, full-length proteins adopt their native folds.

The folding pathway of barnase, a 110-residue extracellular ribonuclease from *Bacillus amyloquefaciens*, is being analyzed in depth (Matouschek et al., 1992; Serrano et al., 1992; Fersht, 1993), and so analysis of its fragments can be related to events that occur on the folding pathway of the intact enzyme. Two peptide fragments of barnase have been found to be particularly informative. The fragment consisting of residues 1−22, termed (1−22), contains the sequence corresponding to the major α-helix of barnase (residues 6−18). The fragment (23−110) contains the residues corresponding to the second α-helix and β-sheet structural elements of barnase and possesses all the catalytic residues. The 22−23 bond is not in an element of regular secondary structure. These peptides bind rapidly to produce a complex with native-like structure and some 10% of the catalytic activity of wild-type barnase (Kippen et al., 1994a). The circular dichroism spectrum of fragment (23−110) has some weak characteristics of that of the parent protein. These are enhanced on the addition of a substrate analog, and the fragment does have distinct catalytic activity (Kippen et al., 1994a). The fragment (1−22) is largely disordered in water but the addition of 2,2,2-trifluoroethanol (TFE) induces the formation of the native helix (Kippen et al., 1994b). Quantitative analysis by TFE-titration (Jasanoff & Fersht, 1994) shows that the equilibrium constant between the helix and coil (k_{helix} = [helix]/[coil]) is 0.03 (Kippen et al., 1994b). Further, the formation of helical structure in peptide (1−22) is destabilized by the mutation of specific residues that destabilize the native helix. However, these mutations also act by causing the helix to be truncated (Kippen et al.,

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1994b). The residues concerned, Tyr-13, Thr-16, Tyr-17, and His-18, are in the opposite face of the helix to that which packs against the β-sheet in the native protein (Mauguen et al., 1982; Bycroft et al., 1990). Tyr-13 makes extensive interactions with Pro-21 (Serrano et al., 1990, 1992), and His-18 interacts with Trp-94 in the native protein (Loewenthal., 1991, 1992).

The effects of mutation of Tyr-13, Thr-16, Tyr-17, and His-18 on the folding of the native protein have been extensively analyzed (Matouschek et al., 1992; Serrano et al., 1992; J. Matthews and A.R.F., manuscript in preparation). Here, we examine the equilibria and kinetics of the binding of the mutants of (1−22) to (23−110).

EXPERIMENTAL PROCEDURES

Materials. Cyanogen bromide was obtained from Fluka. GpUp was from Sigma. All other reagents were obtained from Sigma, Fluka, or Amersham.

Preparation and Purification of Peptides. The barnase peptides (1−22), (1−22 H18G), (1−22 T16S), (1−22 Y17A), (1−22 Y13A) and (1−22 Y13&17A) were prepared using a peptide synthesizer (Applied Biosystems 432A). Each peptide is amide protected at the C-terminal. The peptides were purified by gel filtration and preparative reverse-phase HPLC (Kippen et al., 1994b). The peptide (23−110) was prepared from chemical cleavage of the barnase mutant D22M with CNBr and purified by preparative reverse-phase HPLC as described for previous studies on this peptide (Kippen et al., 1994a). The identity and purity of each peptide was determined by electrospray mass spectrometry, reverse-phase HPLC, and $NaDodSO_4$ gel electrophoresis. The concentration of tyrosine-containing peptides was determined from their absorbance at 280 nm using a known molar extinction coefficient from model compounds (Gill & von Hippel, 1989). The concentration of peptide (1−22 Y13&17A) was determined from the dry weight of the peptide and by protein assay using the method of Bradford (1976).

Fluorescence and Circular Dichroism Spectra. Fluorescence spectra of peptides (1−22) mixed 1:1 with (23−110) were recorded in 50 mM MES buffer, pH 6.3, at 25 °C using a Perkin Elmer LS 50 B fluorimeter. CD spectra (far-UV) were recorded in 5 mM MES buffer, pH 6.3, at 25 °C using a Jasco J-720 spectropolarimeter. The concentration of each peptide (1−22) and (23−110) was 3 μM (wild-type), 10 μM (T16S and H18G), and 20 μM (Y13A, Y17A, and Y13&17A).

Catalytic Activity. Hydrolysis of the oligonucleotide GpUp by the complexes of peptides (1−22) with (23−110) was measured in 100 mM sodium acetate buffer, pH 5.8, at 25 °C using a Perkin-Elmer lambda 5 spectrometer, as described by Day et al. (1992) and Kippen et al. (1994a). The concentrations of peptide (23−110) and GpUp were 0.8 and 150 μM, respectively. The concentration of each peptide (1−22) was 20 μM (wild-type), 50 μM (T16S and H18G), and 100 μM (Y13A, Y17A, and Y13&17A).

Determination of Dissociation Constants of Peptides (1−22) from (23−110). Aliquots of each peptide (1−22) were added to a 0.5 μM solution of peptide (23−110) in 50 mM MES buffer, pH 6.3. The tryptophan fluorescence at 365 nm was recorded with excitation at 280 nm using a Perkin Elmer LS 50 B fluorimeter with sample holder equilibrated to 25 °C [for an analysis of the fluorescence spectrum of barnase, see Loewenthal et al. (1991)]. Changes in volume and contributions to the readings from the fluorescence of increasing amounts of peptide (1−22) were corrected for Kippen et al. (1994a).

Determination of Kinetics for Association of Peptides (1−22) with (23−110). Rate constants for association were measured under pseudo-first-order conditions (Fersht, 1985) in 50 mM MES buffer, pH 6.3, at 25 °C. A solution of peptide (1−22) was rapidly mixed with an equal volume of peptide (23−110) at $^1/_5$ the concentration in a stopped-flow fluorescence spectrometer (Applied Photophysics; Kippen et al., 1994a). The time dependence of the change in fluorescence at wavelengths above 315 nm on excitation at 280 nm was recorded.

RESULTS

Nature of the Complexes (1−22)•(23−110). The characterization of the complex between wild-type fragments has been described in detail (Kippen et al., 1994a). Similar results were found with all mutant complexes. Peptides (1−22) have a relatively low fluorescence since they do not contain any tryptophan residues. The fluorescence spectrum of peptide (23−110), which exhibits a maximum at 349 nm (Kippen et al., 1994a), is blue-shifted upon association of each peptide (1−22) to exhibit a maximum at 336 nm which is similar to that of native barnase (data not shown). The CD spectrum of each dissociated peptide exhibits a single minimum at 198 nm, which is characteristic of random coil conformation (Kippen et al., 1994a,b). Upon peptide association, a distinctive minimum at 231 nm develops, which has been assigned to Trp-94 in native barnase (Vuilleumier et al., 1993; Kippen et al., 1994a) (data not shown).

The large peptide (23−110) has residual activity with a turnover number of 0.8 s^{-1} with GpUp (Kippen et al., 1994a). Upon addition of excess peptide (1−22) in the presence of excess substrate, the maximum rate of hydrolysis is observed, allowing the turnover number to be estimated according to Michaelis−Menten kinetics (Fersht, 1985; Kippen et al., 1994a) (see Table 1). In all cases, the turnover number increases to 4.4−5.4 s^{-1}, compared with 53 s^{-1} for the turnover number of intact protein (Day et al., 1992).

Kinetics and Equilibria of Formation of the Complexes (1−22)•(23−110). The determination of the dissociation constant of the complex between wild-type (1−22) and (23−110), K_{diss}, by equilibrium titration is complicated because the concentrations of (1−22) employed were not sufficiently greater than that of [(23−110)]. The fluorescence data were fitted, therefore, to eq 1, which is the standard binding expression for 1:1 stoichiometry where the concentration of reagents are similar,

$$F = F_o - \alpha([N]_o + [C]_o + K_{diss}) - \sqrt{([N]_o + [C]_o + K_{diss})^2 - 4[N]_o[C]_o} \quad (1)$$

where F_o = fluorescence offset, $[N]_o$ = initial concentration of peptide (1−22), $[C]_o$ = initial concentration of peptide (23−110), and α is a constant containing the fluorescence intensities. The mutant peptides (1−22) exhibit weaker binding to (23−110) so that [(1−22)] ≫ [(23−110)] during titration and the standard simpler expression, eq 2, could be used (see Table 1).

$$F = F_o - F_\infty[N]_o/(K_{diss} + [N]_o) \quad (2)$$

The kinetic time courses were fitted to a double-exponential first-order rate expression. The phase with the larger amplitude and higher rate constant, k_{obs}, is that related to peptide association phase. The slower phase is the proline

Table 1: Turnover numbers and dissociation constants of complexes (1−22)•(23−110)

peptide (1−22)	k_{cat}[a] (s^{-1})	K_{diss}[b] (μM)
wild-type	5.4 (± 0.3)	2.7 (± 0.6)
H18G	5.2 (± 0.4)	7.7 (± 0.9)
T16S	5.3 (± 0.4)	14 (± 2)
Y17A	4.9 (± 0.4)	25 (± 3)
Y13A	4.7 (± 0.4)	30 (± 3)
Y13&17A	4.4 (± 0.4)	49 (± 4)

[a] Catalytic turnover number of the complex of peptides (1−22) and (23−110), in 100 mM sodium acetate buffer, pH 5.8, at 25 °C with GpUp. [b] Dissociation constant of peptides (1−22) from (23−110), measured from studies at equilibrium in 50 mM MES buffer, pH 6.3, at 25 °C. Errors quoted are standard errors of the mean.

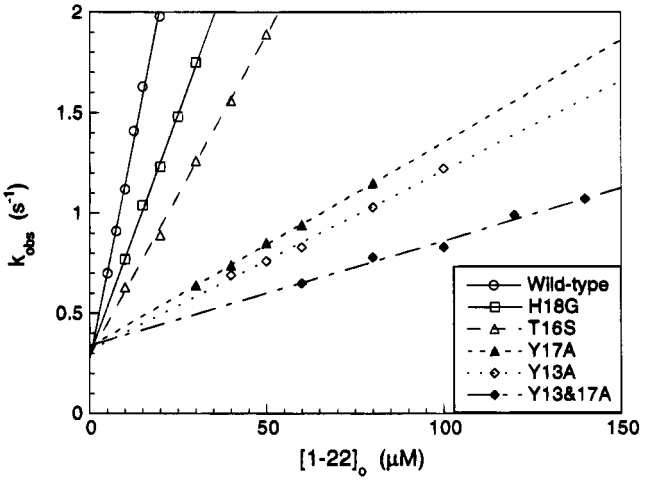


FIGURE 1: Determination of the rate constant for association of peptides (1−22) with peptide (23−110) measured under pseudo-first-order conditions at pH 6.3 and 25 °C, using eq 3.

Table 2: Rate Constants for the Association of Peptides (1−22) and (23−110) [a]

peptide (1−22)	k_1[b] (× 10^{-4}) ($s^{-1}M^{-1}$)	k_{-1}[c] (s^{-1})	K_{diss}[d] (μM)	Φ_{ass}[e]	Φ_{bar}[f]
wild-type	8.8 (± 0.3)	0.27 (± 0.04)	3.1 (± 0.5)	0	0
H18G	4.8 (± 0.1)	0.29 (± 0.02)	6.0 (± 0.4)	0.92	0.60
T16S	3.2 (± 0.1)	0.29 (± 0.03)	9.1 (± 0.9)	0.94	0.84
Y17A	1.02 (± 0.02)	0.33 (± 0.01)	32 (± 1)	0.92	0.50
Y13A	0.89 (± 0.04)	0.31 (± 0.03)	35 (± 4)	0.95	0.41
Y13&17A	0.53 (± 0.04)	0.34 (± 0.04)	64 (± 9)	0.93	0.32

[a] In 50 mM MES buffer, pH 6.3, at 25 °C. Errors quoted are standard errors of the mean. [b] Second-order rate constant for association. [c] First-order rate constant for dissociation. [d] Dissociation constant of peptides measured from k_{-1}/k_1. [e] Φ-value of peptide association. [f] Φ-value of barnase folding [taken from Matouschek et al. (1992)].

isomerisation-related phase (resulting from residues 47 and 64 in the large peptide) with a rate constant, k_{isom}, of 0.05 s^{-1} (Matouschek et al., 1992; Kippen et al., 1994a). k_{obs} follows the characteristic rate law for bimolecular association (Fersht, 1985):

$$k_{obs} = k_{-1} + k_1[(1-22)] \quad (3)$$

(see Figure 1 and Table 2) where k_1 is the second-order association rate constant and k_{-1} is the first-order dissociation rate constant, for the bimolecular association reaction

$$(23-110) \underset{k_{-1}}{\overset{k_1[1-22]}{\rightleftharpoons}} (1-22)\bullet(23-110) \quad (4)$$

The rate constant for the proline related phase was independent of the concentration of (1−22) at higher concentrations and independent of the nature of (1−22).

Table 3: Change in Free Energies of Association or Folding on Mutation

peptide (1−22)	$\Delta\Delta G_{ass}$[a] (kinetics) (kcal mol^{-1})	$\Delta\Delta G_{ass}$[b] (equilibrium) (kcal mol^{-1})	$\Delta\Delta G_{F\text{-}U}$[c] (barnase folding) (kcal mol^{-1})
H18G	0.39 (± 0.09)	0.62 (± 0.15)	1.0
T16S	0.64 (± 0.10)	0.97 (± 0.16)	1.7
Y17A	1.38 (± 0.09)	1.32 (± 0.15)	2.0
Y13A	1.44 (± 0.11)	1.43 (± 0.14)	3.3
Y13&17A	1.79 (± 0.11)	1.72 (± 0.14)	4.2

[a] Change in the free energy of association with (23−110) of peptides on mutation of (1−22), measured from kinetic studies. [b] Change in the free energy of association of peptides on mutation in (1−22), measured from studies at equilibrium. [c] Change in the free energy of folding of native barnase on equivalent mutations [determined by reversible urea denaturation studies by Serrano et al. (1992)]. Errors quoted are standard errors of the mean.

The equilibrium constant for the dissociation of each complex can also be calculated from the ratios of the rate constants for its formation and dissociation (see Table 2). The two sets of data for K_{diss} agree within experimental error (see Tables 1 and 2). The data set from the kinetics is the more reliable since the experiments are easier to perform accurately.

DISCUSSION

Equilibrium Association of Peptides (1−22) with (23−110). Residues 1−22 do not appear from examination of the crystal structure of barnase to be involved in catalysis (Mauguen et al., 1982). However, the reconstituted complex of (1−22) and (23−110) has only 10% of the catalytic activity of uncleaved enzyme. Interestingly, the stability of the complex is less sensitive to mutation than is the intact protein, most noticeably on the mutation of Tyr-13 and then Tyr-17 (see Table 3). [The change in free energy of association, $\Delta\Delta G_{ass}$, is calculated from the dissociation constants of wild-type and mutant proteins on mutation from $\Delta\Delta G_{ass} = RT \ln(K_{diss}^{mut}/K_{diss}^{wt})$.] Changes in energy of unfolding and dissociation should be related since the dissociation reaction represents a native-like complex being converted into fragments of which (1−22) was shown earlier to be largely unfolded (Kippen et al., 1994a). The following arguments suggest that cleavage of the bond between residues 22 and 23 causes a change in the interactions of the helix with the rest of the protein, and this could well affect catalysis.

The side chains of Tyr-13 and Tyr-17 are in contact according to a classical "aromatic−aromatic interaction" (Bycroft et al., 1990; Serrano et al., 1990, 1992). The coupling energy, $\Delta\Delta G_{int}$, between Tyr-13 and Tyr-17 has been calculated from equilibrium data from the wild-type and mutants Y13A, Y17A, and Y13&17A using a double mutant cycle to be 1.2 kcal mol^{-1} in the intact protein (Serrano et al., 1990). Using the same method with the values of $\Delta\Delta G_{ass}$, we obtain a similar coupling energy of 1.0 kcal mol^{-1} in the fragments, suggesting it is the interactions between the tyrosine side chains and the rest of the protein that are mainly weakened on cleavage. Tyr-13 packs closely onto Pro-21, and so the mutation affects this. Conversely, the γ-methyl group of Thr-16 makes its interactions solely intrahelically with the side chain of Tyr-17, and mutation to Ser-16 alters the energies of unfolding of the intact protein and dissociation of fragments by similar amounts. His-18 interacts with Trp-94 in addition to the

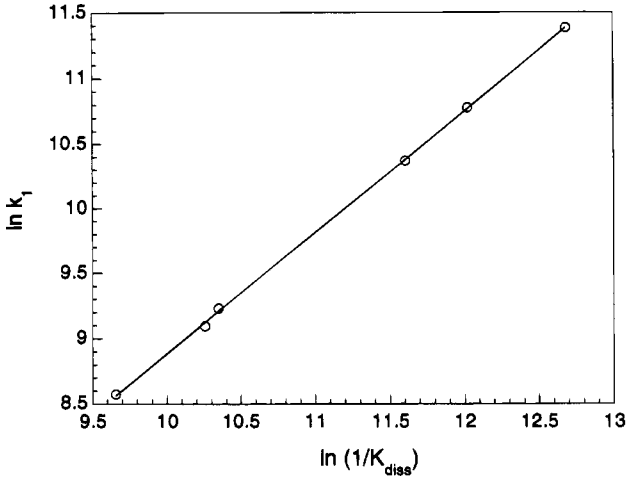


FIGURE 2: Brønsted plot of association rates and equilibrium constants of peptides (1−22) with peptide (23−110). The slope (β) is 0.93 ± 0.01.

helix, and its total energy appears less in the fragments. Thus cleavage by CNBr appears to weaken the interaction of the C-terminal portion of the helix with the rest of the protein.

Analysis of the Structure of the Transition State for Association. (i) The Helix Is Fully Intact in the Transition State for Association. The degree of structure formation in the transition state for protein folding can be analyzed by the Φ-value method: $\Phi = \Delta\Delta G_{‡\text{-U}}/\Delta\Delta G_{\text{F-U}}$, where $\Delta\Delta G_{‡\text{-U}}$ is the change in the free energy of the transition state on mutation and $\Delta\Delta G_{\text{F-U}}$ is the corresponding change in the free protein, both measured relative to the unfolded state. $\Phi = 0$ means that the protein is as unfolded as the unfolded state at the site of mutation whereas $\Phi = 1$ means that the site of mutation is most likely as folded as the folded state of the protein (Matouschek et al., 1989; Fersht et al., 1992). At the extreme, integral values of 0 and 1, Φ is identical to the Brønsted β-value, whereas, at fractional values, Φ and the extent of formation of structure are not necessarily linked in a linear manner.

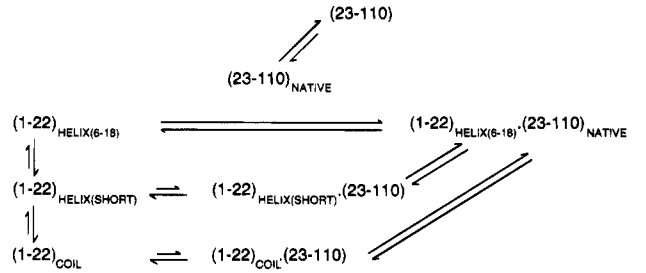
We can adapt this procedure to the formation of complexes (Prat Gay et al., 1994). The formation of interactions on the folding pathway of barnase have been analysed using Φ values (Fersht et al., 1992; Matouschek et al., 1992). The Φ values of peptide association were calculated using

$$\Phi_{ass} = \Delta\Delta G^{‡}_{ass}/\Delta\Delta G_{ass} \quad (5)$$

where $\Delta\Delta G^{‡}_{ass} = -RT \ln(k_1^{mut}/k_1^{wt})$ and $\Delta\Delta G_{ass}$ is calculated from the dissociation constant from kinetic data, as described above. The values of Φ_{ass} are all close to unity (see Table 2), showing that the helix is fully formed in the transition state for association. At the extreme value of 1, Φ and β should be identical. A plot of ln k_1 versus ln ($1/K_{diss}$) (see Figure 2), shows this, with $\beta = 0.93 \pm 0.01$. Virtually all of the destabilization energy of the complex is manifested in a decreased rate constant for association whereas the rate constant for dissociation is hardly affected.

(ii) Mechanisms for Peptide Association. The hydrophobic face of the amphipathic helix (6−18) packs against one side of the β-sheet in native barnase to form the major hydrophobic core. Leu-14 is a key residue in these interactions: its side chain interacts with those from the sheet so strongly that mutation to Ala-14 is one of the most destabilizing changes (Kellis et al., 1989; Serrano et al., 1992). Earlier NMR and CD data on the fragments show that wild-type

Scheme 1. Some Mechanisms for the Association of Barnase Peptides (1−22) and (23−110)[a]



[a] The top is the one-step reaction of a subpopulation of fully formed helix with a subpopulation of fully native 23−110. The middle is the preassociation of a truncated helix with the dominant conformation of 23−110, followed by a rate-determining rearrangement of the initial complex. The bottom is the preassociation of the dominant unfolded conformation of the helix with the dominant population of 23−110, followed by a rate-determining rearrangement of the initial complex. All pathways result in the conversion of the dominant unfolded conformation of the helix into a transition state which has helical structure.

(1−22) is 2.6% helical ([helix]/[coil] = 0.026) in aqueous solution at pH 6.3 and 25 °C, the conditions of the kinetic experiments in this study. Further, the helical region observed in solution spans the whole of the helical region found in the full length protein, residues 6−18 (Kippen et al., 1994b). On mutation of Tyr-17→Ala, the ratio of helix to coil changes only slightly, [helix]/[coil] = 0.018, but the helical region spans only residues 6−15 in the predominant species observable by NMR.

There are several mechanisms for association that can fit the observed data and the second-order kinetics (see Scheme 1). At one extreme, a small subpopulation of fragment (1−22) that is in its fully native conformation reacts in a simple bimolecular association reaction with a subpopulation of fragment (23−110) that is also fully native. The kinetics could also be explained by there being a pre-equilibrium association of nonnative fragments followed by a rearrangement. Provided the initial association step has a sufficiently high dissociation constant that its value is much higher than the concentration of either fragment, then saturation kinetics will not be observed for the association step- and pseudo-second-order kinetics will be observed. The mechanisms cannot be distinguished between using Φ- or β-values: the ground state of (1−22) for each mechanism is the predominantly random coil species, and the transition state for all three mechanisms contains the residues in their helical conformations, and Φ- or β measures the difference between transition and ground states. It seems likely, however, that there should be some preformed structure in the species that associate because some recognition must be required, and we have observed such partly structured species (Kippen et al., 1994b). The most likely mechanism is that the conformation of the helix observed previously by NMR in (1−22) (Kippen et al., 1994b) reacts with the partly structured conformation of (23−110) that has been characterized (Kippen et al., 1994a) in a weak association step, followed by some rearrangement.

Relationship to Folding of Intact Protein. At first sight, the effects of mutations in the major α-helix of barnase on its folding and unfolding appear quite different from the effects of the same mutations on the association and dissociation of the fragments (1−22) and (23−110): the Φ-values for association and folding differ considerably (see

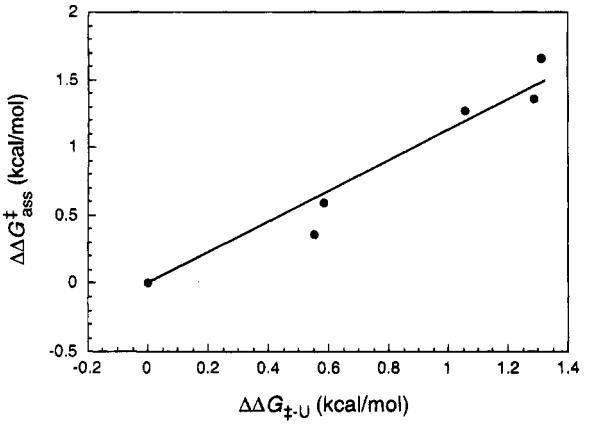


FIGURE 3: Plot of the change on mutation of the free energy of activation for the association of fragments (1−22) and (23−110) against the corresponding changes in the free energy of the major rate-determining transition state for the folding/unfolding of intact barnase. $\Delta\Delta G^{\ddagger}_{ass}$ is measured relative to the separate fragments, $\Delta\Delta G_{\ddagger\text{-}U}$ is measured relative to the unfolded state. The slope is 1.13 ± 0.07.

Table 2). However, the differences in Φ may result from the different effects the mutations have on the value of $\Delta\Delta G_{F\text{-}U}$ for the folding of the intact protein and on the value of $\Delta\Delta G_{ass}$ for the stability of the noncovalent complex between (1−22) and (23−110). Figure 3 is a plot of $\Delta\Delta G^{\ddagger}_{ass}$, the free energy changes on going from the mainly nonnative fragments (1−22) in solution to the transition state, against $\Delta\Delta G_{\ddagger\text{-}U}$, the free energy changes on going from the unfolded state to the transition state for folding of the same mutants of intact barnase. The two are linearly correlated with slope 1.13 ± 0.07 (standard error). A similar plot of $\Delta\Delta G_{ass}$ versus $\Delta\Delta G_{\ddagger\text{-}U}$ has a slope of 1.21 ± 0.07 (not shown). This shows that residues 6−18 in the major transition state for the unfolding of barnase are very similar energetically to the α-helix in the looser noncovalent complex of (1−22)•(23−110). The structure of the helix in the transition state for peptide association appears, therefore, to be very similar to that in the folding/unfolding of the native protein. This may be of direct relevance to studies on the folding pathway of barnase, where the helix is largely formed in the major folding intermediate and the rate-determining step involves the consolidation of its interactions with the rest of the protein (Matouschek et al., 1992; Serrano et al., 1992; Fersht, 1993). These studies indicate that the association of barnase fragments follows a similar pathway of folding. The early formation of this region of secondary structure may, in part, stabilize the formation of interactions later on the pathway of folding.

The finding that there is rapid association of fragments of barnase and of the barley CI-2 inhibitor (Prat Gay et al., 1994), via transition states that are similar to those of the folding of the intact parent proteins gives information on the mechanism of protein folding. In both cases, the proteins have been cleaved between regular elements of secondary structure. Clearly, their folding pathways do not depend on the order in which those elements of regular secondary structure are covalently attached. This eliminates mechanisms of folding in which structure formation is propagated along the entire polypeptide chain. Further, this implies a general principle: if the N- and C-termini of the native proteins are sufficiently close together to be linked covalently without too greatly destabilizing the protein, then the circularly permuted proteins that are thus linked but cleaved as in the fragments should readily fold. This is consistent with recent experimental findings on circularly permuted small proteins [T4-lysozyme (Zhang et al., 1993); ribonuclease T1 (Mullins et al., 1994)].

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