Folding of Barnase in Parts†

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ABSTRACT: Stretches of residual structure in the unfolded states of proteins could possibly constitute crucial regions that initiate protein folding. We are searching for such regions in barnase by dividing it into fragments. By this means, we can search for regions that just form within local sequences. We are also employing methods that can detect low levels of residual structure. In this study, we examine the fragment 1–22 and a large fragment (23–110) that contains all of the catalytic residues. Fragment 1–22 contains the first α-helix, and fragment 23–110 contains the second α-helix and β-sheet structure-forming residues of native barnase. These fragments bind together rapidly and tightly upon association to form a fully nativelike complex. Studies by circular dichroism and fluorescence spectroscopy indicate that each fragment is mainly disordered. However, we find by a procedure of titration with trifluoroethanol that about 3% of fragment 1–22 is helical in water at 25 °C. Importantly, we have detected residual catalytic activity in fragment 23–110 toward GpUp and RNA and the ability to bind the polypeptide inhibitor of barnase, barstar, suggesting that this fragment can form a nativelike conformation in water. The catalytic activity does not result from a small amount of contaminating impurity of parent enzyme or other ribonuclease, since the activity requires a 1:1 mole ratio of fragment to barstar for complete inhibition, and the activity is lost in much lower concentrations of urea than are required to denature the parent enzyme. There is a very weak signal in the near-UV CD spectrum of the large fragment. This is enhanced on the binding of CGAC, a tight-binding substrate analogue of barnase. This implies that there is small amount of preexisting structure in the fragment that is enhanced upon the binding of GpUp or barstar. Thus, evidence from this study on fragments, and from earlier studies on the intact enzyme, shows that barnase can fold by association of independently folded regions of structure.

Studies on protein folding have mainly concentrated on the later stages, involving the characterization of intermediate states and the interactions leading to the final stability of the folded protein (Creighton, 1978, 1985, 1990; Kim & Baldwin, 1982; Udgaonkar & Baldwin, 1988; Matouschek *et al.*, 1990; Matthews, 1991; Fersht, 1993). Little is known about the early events that precede the formation of extensive secondary structure and tertiary interactions. One speculation is that the formation of locally stabilized structures, sometimes referred to as nucleation sites, may be the first steps in the folding reaction (Ptitsyn, 1973, 1991; Karplus & Weaver, 1976; Argos, 1987; Matthews, 1987; Baldwin, 1989; Montelione & Scheraga, 1989; Dill, 1990; Kim & Baldwin, 1990; Shortle *et al.*, 1990; Wodak & Rooman, 1993). It is possible that the burial of local hydrophobic surface area drives the formation of nativelike secondary structure, which may act as a framework to direct subsequent folding events (Moult & Unger, 1991; Serrano *et al.*, 1992a,b; Dill *et al.*, 1993).

Regions of proteins that could initiate folding may be detected from residual structure in the unfolded state (Tanford, 1968, 1970; Karplus *et al.*, 1973; Shortle & Meeker, 1986; Dill & Shortle, 1991; Neri *et al.*, 1992) or from studies on protein fragments (Wright *et al.*, 1988; Shortle & Meeker, 1989; Kim & Baldwin, 1990; Dyson & Wright, 1991; Dyson *et al.*, 1992a,b). Fragments of proteins may lack the possibility of making the tertiary interactions formed in the full-length protein, and so may adopt the same conformation as those regions during the early stages of protein folding. With the advent of NMR methods, it is a relatively simple procedure to detect high degrees of formation of structure in, for example, helical peptides. It is of importance, however, to be able to detect very small amounts of structure that are masked by the overwhelming presence of random coil. To detect such persistence of structure, we are using a strategy of dividing barnase, a small extracellular ribonuclease from *Bacillus amyloliquefaciens* (Hartley, 1989), into fragments (Sancho & Fersht, 1992; Sancho *et al.*, 1992). It was found that the region of barnase containing its two major helices, fragment 1–36, binds rapidly and tightly to the region containing its β-sheet, fragment 37–110, to reconstitute an active enzyme. Examination of the structure of each fragment by CD showed that each was largely random coil. Each individual fragment should not have significant catalytic activity because important groups for catalysis are shared between the fragments. It was found that the α-helical region adopted helical structure in trifluoroethanol solutions and that the fraction of helix present in water could be estimated from titration with trifluoroethanol. It was postulated that one possible explanation for the rapid rate of association of the two fragments is that a small amount of the large fragment also adopted a nativelike structure. Moult (personal communication), using the procedure of Moult and Unger (1991), predicted that the helical region, residues 10–18 in barnase, could act as one initiation site and the β-hairpin, residues 85–100 in the center of the β-sheet, as another. Those regions are formed in the folding intermediate of barnase, and their docking appears to be part of the rate-determining step for folding (Matouschek *et al.*, 1992). In the present

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studies, we test this hypothesis by using a second set of fragments. Here, we examine the association of mainly fragment 1–22 and some experiments with 5–21, which mainly contains the major α-helix (residues 6–18), with the fragment (23–110) that contains all of the residues involved in catalysis and all strands of the β-sheet. The large fragment was prepared by chemical cleavage of the barnase mutant (Asp22→Met) with cyanogen bromide, and the smaller fragment was prepared by peptide synthesis. Spectroscopic studies are used to analyze residual structure in each fragment and the complex formed upon fragment association. Our results are compared to previous aspects of protein folding using barnase (Matouschek *et al.*, 1989, 1990; 1992; Serrano *et al.*, 1992a,b). There is strong evidence that barnase can fold by the association of independently folded parts.

EXPERIMENTAL PROCEDURES

Materials. GpUp, GpU, and RNA (from baker's yeast type XI) were from Sigma. Cyanogen bromide and 1,1,1-trifluoroethanol (TFE) were from Fluka. The barnase fragment (5–21) was obtained from Novabiochem (Nottingham, U.K.). All other reagents were of analytical grade and were purchased from either Sigma, Fluka, or Amersham.

Preparation and Purification of Fragments. Site-directed mutagenesis of the barnase gene (Paddon & Hartley, 1987; Serrano *et al.*, 1990) was performed by the method of Sayers *et al.* (1988) using the kit supplied by Amersham. Mutant plasmids were identified by direct sequencing. Purification of the mutant protein Asp22→Met was performed as described for the wild-type enzyme (Serrano *et al.*, 1990). The mutant protein (D22M) was chemically cleaved with cyanogen bromide, and the products were purified by FPLC and gel filtration, as described for previous studies on fragments of barnase (Sancho & Fersht, 1992). Subsequent purification of fragment 23–110 was carried out by preparative reverse-phase HPLC (Dynamax C8 300 Å, 10 × 250 mm column) with a linear gradient of acetonitrile (16–34% in 39 min) in 0.05% trifluoroacetic acid in water. Fragment 1–22 of barnase was prepared from a semisynthetic peptide synthesizer (Synergy, from Applied Biosystems) using the Fmoc strategy. The peptide was purified by gel filtration using a Sephadex G-15 column (2.1 × 29.8 cm) equilibrated in water at room temperature under a minimum flow by gravity (approximately 1 mL/min) and by preparative reverse-phase HPLC using the same conditions as above. The tetradeoxynucleotide, d(CGAC), was prepared from a DNA synthesizer (Applied Biosystems 380B) and purified by reverse-phase HPLC (Vydac C4 column) with a linear gradient of acetonitrile (20–50% in 30 min) in 0.1 M triethylammonium acetate (pH 7) in water.

The purity of each fragment was determined by $NaDodSO_4$–PAGE (Schägger & von Jagow, 1987), reverse-phase HPLC, and electrospray mass spectrometry (courtesy of Dr. J. Staunton, Department of Chemistry, Cambridge University). The concentrations of the fragments were determined from their absorbance at 280 nm. The extinction coefficient of each fragment was estimated from its number of tryptophan and tyrosine residues, using tabulated molar extinction coefficients of tryptophan and tyrosine at 280 nm in model compounds (Gill & von Hippel, 1989).

The buffers used were as follows: (i) 50 mM MES (pH 6.3), prepared from the dilution of a 1.00 M solution containing 387 mM acid form and 613 mM sodium salt; (ii) 2.5 mM sodium phosphate (pH 7), prepared from the dilution of a 0.1 M solution containing 39 mM monobasic and 61 mM dibasic sodium phosphate; (iii) 100 mM sodium acetate (pH 5.8), containing 92 mM sodium acetate and 8 mM acetic acid.

Fluorescence and CD Spectra of Dissociated and Associated Fragments. Fluorescence spectra were recorded in 50 mM MES (pH 6.3) buffer at 25 °C using an SLM Aminco fluorimeter. The fragment concentration was 1 μM. A fluorescence spectrum of fragment 23–110 was also recorded in the presence of 1 μM barstar, which binds 1:1 with native barnase (Hartley, 1989), in the same buffer at 25 °C. Circular dichroism spectra (far-UV and near-UV) were recorded in 2.5 mM sodium phosphate (pH 7) buffer at 25 °C using a Jasco J-720 spectropolarimeter. Fragment concentrations were 3 (far-UV spectra) and 20 μM (near-UV spectra). A CD spectrum (near-UV) of wild-type barnase was recorded under the same conditions as above at a concentration of 20 μM. A CD spectrum (near-UV) of fragment 23–110 was also recorded in the presence 50 μM tetradeoxynucleotide d(CGAC), a substrate analogue that binds to native barnase without digestion, in 100 mM sodium acetate (pH 5.8) buffer. The spectra of associated fragments were recorded by mixing the fragments in equal concentrations. The concentration of each fragment was varied individually to check for self-association.

Determination of Dissociation Constants of Fragments. Aliquots of fragment 1–22 (0–12 μM) were added to a 0.5 μM solution of fragment 23–110 in 50 mM MES (pH 6.3) buffer at 25 °C, and the fluorescence at 365 nm was recorded with excitation at 280 nm. Changes in volume and the contributions to the readings from the fluorescence of increasing amounts of fragment 1–22 were corrected for. The dissociation constant was calculated by fitting the correct fluorescence readings to a binding equation assuming 1:1 stoichiometry. The dissociation constant of fragments 5–21 and 23–110 was also determined from a similar procedure.

Determination of Rate Constants for Fragment Association. The association rate constant was measured by rapidly mixing the fragments in equal volumes with the concentration of fragment 5–21 in 5-fold excess of fragment 23–110. The concentration of unassociated fragment 5–21 is assumed to remain approximately constant throughout the reaction, and association was analyzed as a pseudo-first-order reaction (Fersht, 1985).

A solution of fragment 5–21 of 20–50 μM in 50 mM MES (pH 6.3) buffer was rapidly mixed in an Applied Photophysics stopped-flow fluorescence spectrometer at 25 °C, with an equal volume of fragment 23–110 at one-fifth the concentration. The time dependence of the change in emission fluorescence at wavelengths above 315 nm was recorded with excitation at 280 nm. Spectra at time bases of 10 and 100 s were recorded for each concentration. An average of seven runs at each time base was analyzed.

Trifluoroethanol Titration of Fragment 5–21. CD spectra (far-UV) of fragment 5–21 in a concentration of 6 μM were recorded in a series of TFE (0–37%) solutions in 5 mM MES (pH 6.3) buffer at 25 °C. Each solution was prepared separately and its pH measured. Data at a range of wavelengths were analyzed using a simple equation, as described for previous studies on fragments of barnase (Sancho & Fersht, 1992), to determine ΔG_{H_2O}, the standard free energy of the equilibrium between folded and unfolded states in water.

Catalytic Activity of Mutant Protein and Fragments. Catalytic activity measurements for the hydrolysis of the oligonucleotide GpUp by D22M protein and fragment 23–110 were performed essentially as described for wild-type barnase (Mossakowska *et al.*, 1989; Day *et al.*, 1992). Measurements were made in 100 mM sodium acetate (pH

5.8) buffer containing 100 μg/mL BSA at 25 °C using a Perkin-Elmer spectrometer. The concentrations of D22M protein and fragment 23–110 were 0.008 and 0.8 μM, respectively. Activity measurements in the presence of 100 μM GpUp were made from solutions containing a range of concentrations of fragment 23–110 (0–4 μM) in the same buffer at 25 °C. The activity of associated fragments in the presence of 100 μM GpUp was measured from solutions containing 0.8 μM fragment 23–110 and various concentrations of fragment 1–22 (0–20 μM) in the same buffer at 25 °C.

The hydrolysis of RNA (1 mg/mL) by D22M protein and the dissociated and associated fragments 1–22 and 23–110 was measured in 100 mM Tris-HCl (pH 8.6) buffer at 25 °C (Mossakowska *et al.*, 1989). The initial rate was recorded from the decrease in absorbance at 298.5 nm using a Perkin-Elmer lambda 5 spectrometer. The concentrations of D22M and each fragment were 0.1 and 8 μM, respectively. The concentrations of associated fragments 1–22 and 23–110 were 20 and 8 μM, respectively.

Inhibition of Catalytic Activity. The activities of solutions containing (i) 0.8 μM fragment 23–110, (ii) 0.8 μM fragment 23–110 and various concentrations of D22M protein (0, 0.008, and 0.016 μM), and (iii) 0.8 μM fragment 23–110 and 20 μM fragment 1–22 were measured in various concentrations of barstar (0–0.8 μM), the polypeptide inhibitor of barnase (Hartley, 1989; Schreiber & Fersht, 1993), in the presence of 100 μM GpUp under the same conditions as above.

The competitive inhibition by GpU of the hydrolysis of GpUp catalyzed by fragment 23–110 was measured from solutions containing 0.8 μM fragment 23–110, 60 μM GpUp, and various concentrations of GpU (0–200 μM) under the same conditions as above at 25 °C.

Urea Denaturation Assay. Urea has been shown to denature barnase reversibly and is employed as a measure of protein stability (Kellis *et al.*, 1989; Serrano *et al.*, 1992a). The catalytic activity of D22M protein and fragment 23–110 in the presence of 100 μM GpUp was measured in a series of urea (0–7.2 M) solutions. Measurements were made under the same conditions as above at 25 °C. The concentrations of D22M protein and fragment 23–110 were 0.008 and 0.8 μM, respectively.

RESULTS

Selection of Fragments. The mutation of aspartic acid to methionine at residue 22 in barnase was specifically chosen at a point between elements of secondary structure that is largely exposed to solvent and makes few contacts with other side chains. The chemical cleavage of the protein by cyanogen bromide, specifically at residue 22 since barnase contains no other methionine residues, yields two complementary fragments, 1–22 and 23–110. Since fragment 1–22 contains no tryptophan residues, it has a very low fluorescence intensity from 300 to 400 nm after excitation at 280 nm. This facilitates fluorescence experiments requiring an excess of this fragment. Also, since the N-terminal α-helix of barnase is the only element of secondary structure contained in fragment 1–22, only this particular element of secondary structure is studied. Fragment 1–22 could not be isolated from the cleavage of D22M and was instead prepared using a peptide synthesizer. Studies were also performed on fragment 5–21 since the first four residues of barnase have been shown to be disordered (Mauguen *et al.*, 1982; Bycroft *et al.*, 1990). The behavior of 1–22 and 5–21 is, in general, very similar. Fragment 23–110 contains the remaining elements of secondary structure

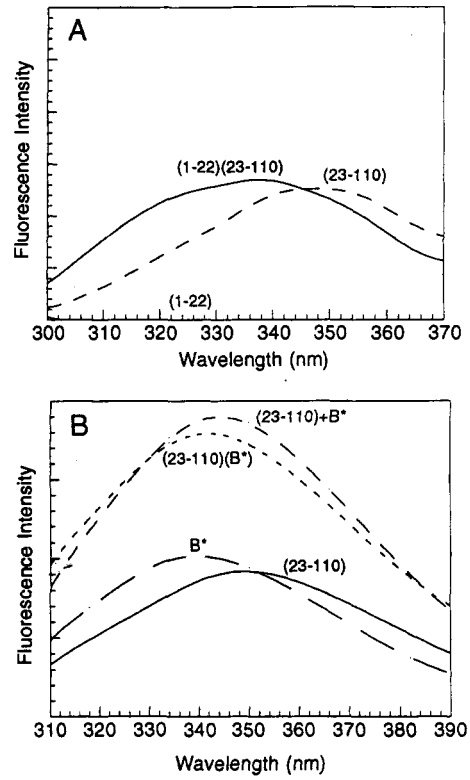

FIGURE 1: (A) Fluorescence spectra of the dissociated and associated (complex) fragments 1–22 and 23–110. (B) Fluorescence spectra of dissociated and associated fragment 23–110 and barstar. Spectra were recorded in 50 mM MES (pH 6.3) at 25 °C. Both fragment and barstar concentrations are 1 μM.

in barnase: the second α-helix and the five-stranded antiparallel β-sheet, containing all of the residues important for catalysis (27, 56–63, 73, 87, and 102; Mossakowska *et al.*, 1989).

Preparation of Fragments. The cleavage of D22M protein was monitored by reverse-phase HPLC over the 8-h cleavage period. The fragments 5–21, 1–22, and 23–110 were shown to be >99% pure by reverse-phase HPLC, mass spectrometry, and $NaDodSO_4$–PAGE. The yield of fragment 23–110 was approximately 10%, based on initial D22M protein.

Fluorescence and CD Spectra. The fluorescence spectra of dissociated and associated fragments are shown in Figure 1A. Fragment 1–22 does not contain any tryptophan residues and, hence, has a very low fluorescence intensity. The fluorescence spectrum of fragment 23–110 has an emission maximum (λ_{max}) at 349 nm. The fluorescence spectrum is shifted to the blue upon fragment association with a λ_{max} at 336 nm, which is similar to that of wild-type barnase (Loewenthal *et al.*, 1991). The fluorescence spectrum of fragment 23–110 with barstar is shown in Figure 1B. The spectrum is slightly shifted to the blue upon binding to give the (23–110)(B*) complex, as compared with an addition spectrum of the two individual spectra, 23–110 + B*.

The CD spectra of dissociated and associated fragments are shown in Figure 2A (far-UV) and 2B (near-UV, with wild-type barnase). The CD spectra indicate that fragments 1–22 and 23–110 are mainly disordered with a single peak in the far-UV at 198 nm, characteristic of random coil conformation. The spectrum of fragment 23–110 shows a less intense minimum at 198 nm than that of fragment 1–22 and in the near-UV displays low-intensity broad maxima from 270 to 290 nm and a minimum at 292 nm, which have been assigned to the interactions of tryptophans 35, 71, and 94 in wild-type barnase (Vuilleumier *et al.*, 1993). The CD spectra of

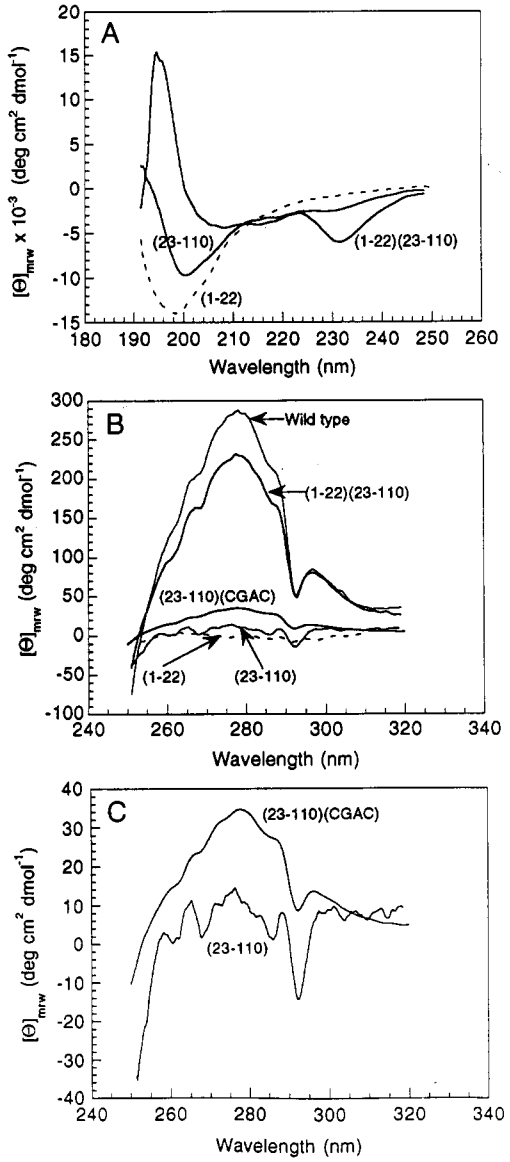

FIGURE 2: CD spectra of dissociated and associated fragments 1–22 and 23–110 in the (A) far-UV and (B) near-UV with wild-type barnase (recorded in 2.5 mM sodium phosphate (pH 7) at 25 °C) and (B and C) fragment 23–110 with the substrate d(CGAC) (recorded in 100 mM sodium acetate (pH 5.8) at 25 °C). Fragment concentrations are 3 (far-UV) and 20 μM (near-UV). Wild-type concentration is 20 μM and d(CGAC) concentration is 50 μM.

associated fragments exhibit a distinctive minimum at 231 nm in the far-UV, which has been assigned to tryptophan 94 in wild-type barnase (Vuillemier *et al.*, 1993), and distinctive spectral properties very similar to those of wild-type barnase in the near-UV. The CD spectrum of fragment 23–110 with d(CGAC) is shown in Figure 2B,C. The fragment spectrum becomes more distinctive upon binding the substrate, with a maximum of increased intensity from 270 to 290 nm. Data have the base line subtracted, and because of the high absorbance of the substrate, the spectra are smoothed.

Dissociation Constant. The dissociation constants of the complexes formed between association of fragments 1–22 and 23–110 (see Figure 3) and association of fragments 5–21 and 23–110 were determined as shown in Table 1 from fitting the data to a simple binding expression:

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

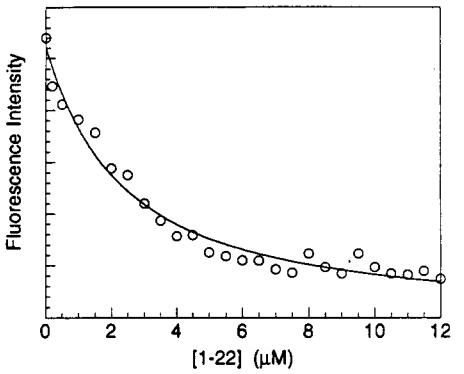

FIGURE 3: Determination of the dissociation constant between fragments 1–22 and 23–110 from the change in emission fluorescence at 365 nm after excitation at 280 nm. Data were recorded in 50 mM MES (pH 6.3) at 25 °C. The initial concentration of fragment 23–110 was 0.5 μM. Data are fit to a binding equation of 1:1 stoichiometry (see text).

Table 1: Rate and Equilibrium Data for the Association of Fragments[a]

small fragment	large fragment	K_{diss}[b] (μM)	k_1[c] (s^{-1} M^{-1})	k_{-1}[d] (s^{-1})
1–22	23–110	2.7 (±0.8)	e	e
5–21	23–110	3.1 (±0.1)	0.8 (±0.05) × 10^5	0.24 (±0.09)
1–36	37–110	2.4 (±0.5)	1.0 (±0.1) × 10^5	0.27 (±0.06)

[a] In 50 mM MES buffer (pH 6.3) at 25 °C. Errors quoted are standard errors of the mean. [b] Dissociation constant measured from studies at equilibrium. [c] Second-order rate constant for association. [d] First-order rate constant for dissociation. [e] Data not determined.

where F_o is the fluorescence offset, [N] is the concentration of the small fragment, [C] is the concentration of fragment 23–110, m is a constant containing the fluorescence intensities, and K_{diss} is the dissociation constant.

Rate Constant of Fragment Association. The change in fluorescence against time from association at each concentration of fragment was best fit to a double-exponential, first-order rate expression:

$$F = F_o + A_1 \exp(-k_{obs}t) + A_2 \exp(-k_{isom}t) \quad (2)$$

where F_o is the fluorescence offset and A is the amplitude of fluorescence change. The higher rate constant, k_{obs}, has a far larger amplitude than that associated with a second step, k_{isom}. A plot of the observed association rate constant (k_{obs}) against the concentration of fragment 5–21 follows the characteristic rate law,

$$k_{obs} = k_{-1} + k_1[5\text{–}21] \quad (3)$$

for the bimolecular association reaction (Fersht, 1985) (see Figure 4):

$$23\text{–}110 \underset{k_{-1}}{\overset{k_1[5\text{–}21]}{\rightleftharpoons}} (5\text{–}21)(23\text{–}110) \quad (4)$$

from which the second-order rate constant of association was determined as the gradient. The dissociation constant (K_{diss}), calculated from

$$K_{diss} = k_{-1}/k_1 \quad (5)$$

where k_{-1} is the rate constant for dissociation of the complex determined by extrapolation to 0 M fragment, agrees with that determined from equilibrium studies. Calculated rate constants are shown in Table 1.

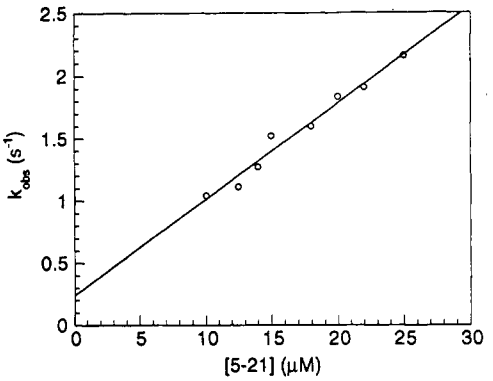

FIGURE 4: Determination of the rate constant for association of fragments 5–21 and 23–110 measured under pseudo-first-order conditions. The observed rate constant (k_{obs}) (see text) is plotted against the initial concentration of fragment 5–21, which is in excess over fragment 23–110, to give a linear relationship, from which the second-order rate constant for association was determined.

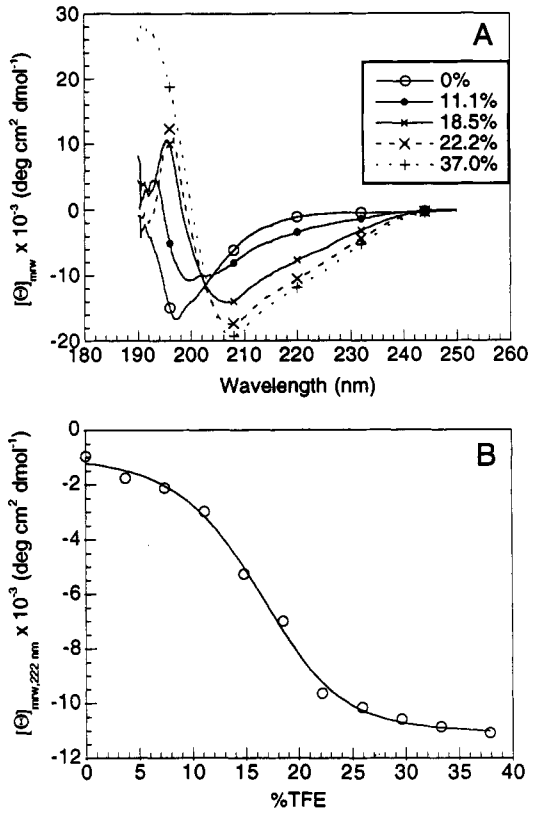

FIGURE 5: TFE titration of fragment 5–21. (A) CD spectra (far-UV) of fragment 5–21 in a concentration of 6 μM were recorded in a series of 11 TFE (0–37%) solutions in 5 mM MES (pH 6.3) at 25 °C. (B) The data at a range of wavelengths, including 222 nm, were analyzed using a two-state structural transition expression (see text).

Trifluoroethanol Titration. The CD spectrum of fragment 5–21 becomes more helical-like with increasing concentrations of TFE, with the development of a small shoulder at 222 nm and a minimum at 208 nm (see Figure 5A). The molar ellipticity at a series of wavelengths as a function of the concentration of TFE was fit to the simple eq 6 (see Figure

$$\Delta G_{TFE} = \Delta G_{H_2O} + m[TFE]/[H_2O] \quad (6)$$

5B) (Jasanoff & Fersht, 1994). This equation may be superior to the earlier one: $\Delta G_{TFE} = \Delta G_{H_2O} + m[TFE]$ (Sancho & Fersht, 1992). If the equilibrium between the folded and unfolded conformations of the peptide does approximate a

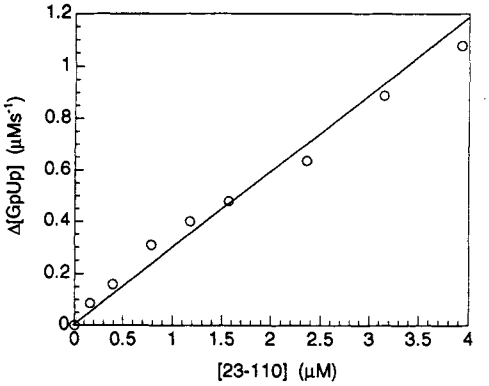

FIGURE 6: Specific catalytic activity of fragment 23–110 at various concentrations. Measurements were made with 100 μM GpUp in 100 mM acetate (pH 5.8) at 25 °C.

two-state model, the average free energy of unfolding of the peptide in water (ΔG_{H_2O}) is calculated to be −2.06 (±0.08 standard error) kcal mol^{-1} at pH 6.3. This indicates that 3.0% (±0.4%) of fragment 5–21 is folded in water at pH 6.3 and 25 °C.

From analysis of the mean residue ellipicity at 222 nm using standard theoretical methods to determine the mean residue ellipticity of a 100% helical peptide (Chen *et al.*, 1972; Gans *et al.*, 1991; see eq 7, where n is the number of residues

$$[\Theta]_{222,\ 100\%\ helical} = ((n - 4.6)/n)(-40\ 000) \quad (7)$$

in the peptide that are helical in native barnase), it is calculated that the fragment is approximately 43% helical in 35% TFE. The quantity 4.6 varies according to the individual studies, and a value of 2.5 is frequently used. However, Gans *et al.* (1991) find that the value is in the range 4.6–6.3. Further, Chakrabartty *et al.* (1993) have now shown that tyrosine residues in helices contribute a positive CD band in the 222-nm region that distorts and partly neutralizes the signal from the α-helix. There are two tyrosines in the barnase helix (residues 13 and 17). Vuilleumier *et al.* (1993) have also shown that they neutralize some of the helix signal. For this reason, the figure 43% is a severe underestimate of the helical content.

Catalytic Activity. The initial rate of GpUp hydrolysis by D22M protein *versus* the GpUp concentration was fit to Michaelis–Menten kinetics. Fragment 1–22 shows no catalytic activity. Fragment 23–110, however, does catalyze the hydrolysis of GpUp (see Figure 7A), with a turnover number (k_{cat}) 1.3% of that for the uncleaved enzyme. The rate of GpUp hydrolysis by fragment 23–110 was shown to be linearly proportional to the concentration of the fragment within the range of the experiment (see Figure 6), and so does not result from aggregation.

The initial rate of GpUp hydrolysis by associated fragments 1–22 and 23–110, as a function of the concentration of fragment 1–22, is fit to eq 8 (see Figure 7B), derived from

$$\begin{aligned} v &= [23\text{–}110]k_{cat}{}^{23\text{–}110} + [1\text{–}22][23\text{–}110]k_{cat}{}^{cpx} \\ &= (K_m k_{cat}{}^{23\text{–}110} + [1\text{–}22]k_{cat}{}^{cpx})[23\text{–}110]/(K_m + [1\text{–}22]) \end{aligned} \quad (8)$$

Michaelis–Menten kinetics in the presence of two active species, where $k_{cat}{}^{cpx}$ and $k_{cat}{}^{23\text{–}110}$ are the turnover numbers of the complex formed upon fragment association and the dissociated fragment (23–110), respectively. K_m, with respect to the concentration of fragment 1–22 = $(K_d K_s)/([GpUp] +$

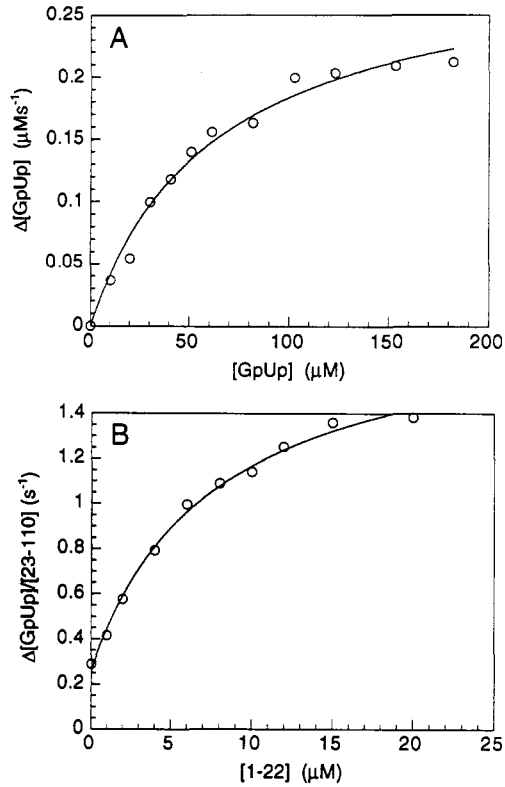

FIGURE 7: Activity assays of (A) fragment 23–110 and (B) association of fragments 1–22 and 23–110. The initial rates of GpUp hydrolysis *versus* the concentration of (A) GpUp and (B) fragment 1–22 were fit to Michaelis–Menten kinetics. Measurements were made in 100 mM acetate (pH 5.8) at 25 °C. The concentration of fragment 23–110 is 0.8 μM.

Table 2: Catalytic Activity of Proteins[a]

protein	K_m (μM)	k_{cat} (s^{-1})
wild type	20 (±2)[b]	53 (±2)[b]
D22M	26 (±3)	59 (±3)
23–110	64 (±9)	0.8 (±0.05)
(1–22)(23–110)	8 (±1)[c]	5.4 (±0.3)
37–110	0[d]	0[d]

[a] In 100 mM sodium acetate buffer (pH 5.8) at 25 °C with GpUp. Errors quoted are standard errors of the mean. [b] Data taken from Day *et al.* (1992). [c] Data recorded with respect to the concentration of fragment 1–22. [d] Data taken from Sancho and Fersht (1992).

K_s), where K_d is the the dissociation constant of fragments 1–22 and 23–110 and K_s is the dissociation constant of the complex and GpUp. There is a calculated 7-fold increase in rate upon fragment binding. All results are shown in Table 2.

Fragment 23–110 was shown to catalyze the hydrolysis of RNA 100 times slower than D22M protein under the same conditions. Association of fragment 23–110 with fragment 1–22 led to an approximate 7-fold increase in the initial rate of hydrolysis of RNA.

Inhibition of Catalytic Activity. The catalytic activity of fragment 23–110 is fully inhibited only by an approximately equal or greater concentration of barstar (see Figure 8). Barstar (Hartley, 1989) is the intracellular 89-residue polypeptide inhibitor of barnase that is found in *Bacillus amyloliquefaciens*. The inhibition of the catalytic activity of fragment 23–110 is not significantly affected when measured in the presence of D22M protein at a concentration where the rate of catalytic hydrolysis of GpUp by both species is approximately equal. The catalytic activity of fragment 23–110 in the presence of excess fragment 1–22 also is not fully inhibited

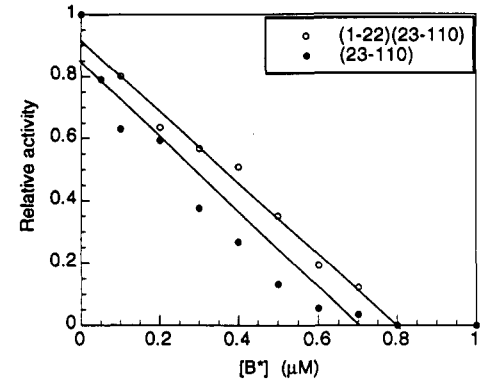

FIGURE 8: Inhibition of the activities of fragment 23–110 and associated fragments 1–22 and 23–110 by barstar. Measurements were made in the presence of 100 μM GpUp in 100 mM acetate (pH 5.8) at 25 °C. The concentrations of fragments 23–110 and 1–22 are 0.8 and 20 μM, respectively.

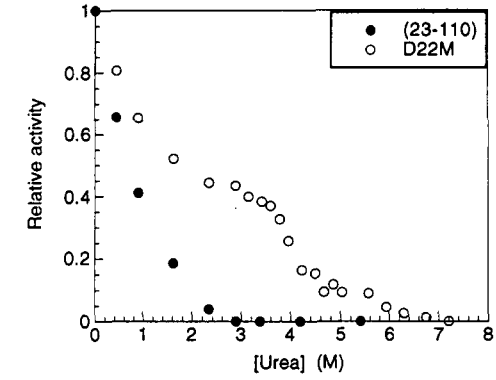

FIGURE 9: Urea denaturation assays of fragment 23–110 and D22M protein. Measurements were made in the presence of 100 μM GpUp in 100 mM acetate (pH 5.8) at 25 °C. The concentrations of D22M protein and fragment 23–110 are 0.008 and 0.8 μM, respectively.

until the concentration of barstar is equal to or greater than that of the enzyme (see Figure 8). Therefore, both fragment 23–110 and the associated fragment complex (1–22)(23–110) bind 1:1 with barstar, as seen with wild-type barnase (Hartley, 1989). The catalytic hydrolysis of GpUp by fragment 23–110 was not inhibited in the presence of GpU, suggesting that GpU does not bind to the fragment at the concentrations employed.

Urea Denaturation Assay. The catalytic activity of fragment 23–110 is exponentially reduced by an increasing urea concentration, showing negligible activity in urea solutions above 2.8 M (see Figure 9). The protein D22M shows an initial exponential decrease in catalytic activity in the presence of urea, which levels off from approximately 2 to 3.5 M urea, after which there is a transition with a midpoint at 4.3 M urea to fully inactive protein in solutions above 7 M urea (see Figure 9).

DISCUSSION

Spectroscopic Studies on the Structure of the Fragments. The fluorescence spectrum of barnase mainly depends on the environment of the tryptophan residues (Loewenthal *et al.*, 1991) and, hence, on the structure of the protein. The fluorescence spectrom of fragment 23–110 displays a λ_{max} for emission at 349 nm, which is in between those of folded (336 nm) and unfolded (354 nm) native barnase, suggesting ordered structure in this fragment. Although fragment 23–110 appears, from the CD spectrum in the far-UV (measure of secondary structure), to be mainly disordered, the spectrum

in the near-UV (measure of tertiary structure) suggests the formation of partial nativelike tertiary interactions.

Fragment 1–22 appears to adopt a random coil conformation from CD spectra. However, the lack of a weak positive signal at 222 nm in the far-UV, which is also characteristic of random coil peptides, may indicate partial formation of ordered structure in this fragment. Helical formation in peptides has been shown to be stabilized by TFE, which tends to promote α-helical conformation in regions with helical propensity (Lehrman *et al.*, 1990). The effect of TFE on the structure of fragment 5–21 is shown by CD spectra in the far-UV. The development of a shoulder at 222 nm and a minimum at 208 nm is indicative of an α-helical conformation. However, the CD spectra of helices have been recently shown to be distorted in the far-UV by the presence of tyrosine residues (Chakrabartty *et al.*, 1993; Vuilleumier *et al.*, 1993). These give a positive contribution that compensates for the negative ellipticity of the helix around 222 nm. An extensive study of mutant peptides derived from 1–22 that lack either Tyr13, Tyr17, or both shows more characteristic spectra (A. Kippen and A. R. Fersht, unpublished data). The titration data fit well to an expression (eq 6) similar to that derived for previous TFE studies on fragment 1–36 of barnase (Sancho *et al.*, 1992). The expression assumes that there is an equilibrium between the folded and unfolded states of the fragment, for which the change in free energy is proportional to the concentration of TFE. Equation 6 gives the value of ΔG_{H_2O}, which is the free energy change for the equilibrium between helical and random coil structures in water. Fragment 5–21 is thus estimated to be about 3.0% (±0.4%) helical in water at pH 6.3 and 25 °C, which is consistent with a low mean residue ellipticity at 222 nm in water.

These studies were confirmed by 2D ^{1}H NMR experiments performed on fragment 5–21, which indicate that the fragment forms partial helical structure in water and that in 35% TFE the fragment forms helical structure to a similar extent as in the corresponding region of native barnase (V. Arcus, A. Kippen, and A. R. Fersht, unpublished data). It is not known whether 3% of the population of the helix is fully helical or whether there are regions of fluctuating structure, as described by Wright *et al.* (1988) for "nascent" helices.

Spectroscopic Studies on the Association of Fragments. Association of fragments 1–22 and 23–110 leads to a shift in the fluorescence emission spectrum with a maximum at 336 nm, resembling that of wild-type barnase, indicating the formation of a complex with nativelike structure. Changes in the CD spectrum upon fragment association are also dramatic. The spectrum of the complex is very similar to that of the uncleaved enzyme, which is atypical for an $\alpha + \beta$ protein (Vuilleumier *et al.*, 1993). The observed minimum in the far-UV at 231 nm is associated with the presence of tryptophan 94 in native barnase (Vuilleumier *et al.*, 1993), which suggests that the secondary structural environment around residue 94 in the complex is similar to that in native barnase. The CD spectrum of the complex in the near-UV is also very similar to that of the uncleaved enzyme, displaying distinctive maxima from 270 to 290 nm, which have been associated with interactions mainly from tryptophans 35, 71, and 94 in native barnase, and a minimum at 292 nm that has been associated with tryptophan 71 in native barnase (Vuilleumier *et al.*, 1993). This strongly suggests that the tertiary structure of the complex is also very similar to that in native barnase.

The association of the fragments to form a nativelike complex is an extremely rapid process, comparable to the rate of protein folding from fully unfolded native protein (Matouschek *et al.*, 1992). The change in fluorescence with time is a direct measure of the folding of the fragments upon association, since fragment 5–21 exhibits very little fluorescence intensity. This fluorescence change with time exhibited two major phases, of which the faster rate constant (k_{obs}) corresponds to the observed fragment association rate. The slower rate constant (k_{isom}), calculated to be 0.05 s^{-1}, is the same as the rate constant of proline isomerization, seen from residues 47 and 64 in fragment 23–110. The second proline isomerization rate, seen from residue 21 in barnase folding experiments (Matouschek *et al.*, 1992; A. Kippen, E. Meiering, and A. R. Fersht, unpublished results), is not observed under these experimental conditions where the concentration of fragment 5–21 remains approximately constant throughout the reaction. The dissociation constant is very low, suggesting that the fragments become very tightly bound upon association.

The complex formed upon fragment association has a structure that is very similar to that of native barnase, although no covalent bonds are formed between the fragments as shown by NaDodSO$_4$–PAGE and reverse-phase HPLC (data not shown). Therefore, each fragment is strongly influenced by the presence of the other, possibly forming individual secondary structures before folding around each other to form tertiary interactions and their respective structures, as in the uncleaved protein.

Catalytic Activity of Dissociated and Associated Fragments. Fragment 1–22 displays no enzymatic activity, which is not surprising since none of the residues important for catalysis in barnase are present. Spectroscopic studies have indicated residual structure within fragment 23–110, and further evidence for this is given by the detection of residual catalytic activity with GpUp and RNA. The catalytic activity of fragment 23–110, which contains all of the residues important for catalysis, is 100 times lower than that of the native protein, suggesting that 1% or more of the large fragment is in a nativelike conformation in the presence of substrate. The catalytic rate is increased 7-fold upon fragment association to form the complex, which is approximately 10% of the activity of the uncleaved protein.

Fragment 23–110 was prepared many times, isolated from the D22M cleavage mixture and purified by many different techniques to ensure >99% purity, as shown by most techniques available. The specific activity of the fragment remained constant from each preparation, and k_{cat} for the fragment with GpUp remained invariable. This provides good evidence for an active fragment; however, impurities such as 1% of uncleaved D22M from the fragment preparation could not be ruled out at this stage.

Stronger evidence is demonstrated by the urea denaturation assay. The catalytic activity of fragment 23–110 is fully lost in solutions of urea above 2.8 M where D22M protein is still 40% active. The activity of uncleaved protein is not completely lost until urea solutions are above 7 M, suggesting that the protein does not completely unfold from nativelike structure until it is in urea solutions of concentration well in excess of those required by fragment 23–110. This is strong evidence that there is no uncleaved D22M protein still present in the fragment solutions; however, we still could not rule out the presence of a different nuclease. The midpoint of the transition seen in the urea denaturation assay of D22M agrees with that determined by fluorescence studies (data not shown), and the initial decrease of activity in low urea concentrations is thought to be from effects of urea on the active site before unfolding of the protein.

Conclusive evidence for the activity resulting from the fragment is provided by the titration with barstar. Fragment 23–110 is found to bind to barstar with a shift in the fluorescence emission maximum, indicating increased structure formation in the fragment. The catalytic activity of fragment 23–110 is inhibited by binding barstar in a ratio of 1:1 (see Figure 8). This is conclusive evidence that the activity results from the fragment and not from impurities in the fragment solutions. Further, fragment 23–110 can indeed form nativelike structure without the requirement of the remaining protein residues. The complex formed upon association of fragments 1–22 and 23–110 also binds to barstar in a ratio of 1:1, which strongly suggests that the catalytic activity of the complex is due to the formation of fully nativelike structure.

Native barnase is stabilized by a central hydrophobic core that is formed by the major α-helix packing against the β-sheet. Fragment 23–110 lacks the major α-helix, which exposes residues that normally form the hydrophobic core, thus destabilizing the fragment. The fragment does not appear to bind GpU; however, catalytic studies on native barnase have shown that the ratio k_{cat}/K_m is decreased 1000-fold when the second phosphate of the substrate GpUp is removed to form GpU. This is composed of a 100-fold decrease in k_{cat} and is, therefore, a result of decreased stability of the transition state relative to the ground state (Day *et al.*, 1992; Buckle & Fersht, 1994). The formation of structure in the fragment is increased in the presence of substrate, as indicated by the CD spectrum of the fragment in the presence of d(CGAC), a substrate which lacks the 2′-hydroxyl group that is necessary for enzyme cleavage. The inhibition constant, K_i, of binding d(CGAC) to fragment 23–110 in the presence of GpUp was determined to be 25 μM at pH 5.8 and 25 °C (data not shown), compared with 3 μM for preliminary studies on wild-type barnase (C. Johnson, personal communication). Upon substrate binding, the fragment CD spectrum exhibits stronger nativelike spectral properties in the near-UV, which suggests increased tertiary structure formation. Owing to the high absorbance of the substrate, we could not accurately analyze the binding spectrum in the far-UV.

We have subsequently prepared fragment 23–110 by expression of the barnase gene with the sequence relating to the first 22 residues deleted (A. Day, unpublished data). The fragment has been successfully purified by reverse-phase HPLC and identified by $NaDodSO_4$–PAGE and mass spectroscopy. Importantly, the specific activity of the fragment from this preparation is the same as that determined from the previous studies on the chemically prepared fragment. Thus, any possible impurity from the cleavage reaction, such as uncleaved D22M protein or the complementary fragment 1–22, is eliminated.

It is interesting to note from previous studies (Sancho & Fersht, 1992) that fragment 37–110 of barnase, which was prepared in a similar fashion from the cleavage of the mutant protein V36M, does not display any enzymatic activity with GpU, GpUp, or RNA. This fragment does not contain the second α-helix in barnase; it contains only the β-sheet structure-forming residues in native barnase and thus lacks one residue that is very important for catalysis in barnase, lysine 27. Studies on "modules" of barnase (Yanagawa *et al.*, 1993) have suggested that much smaller fragments of barnase exhibit enzymatic activity. The activity of these modules, lacking most of the active-site residues, is 4 orders of magnitude lower than the activity we have measured by very different techniques and may be due to just the net charge on the peptide, as shown by similar activity of non-barnase polypeptide chains of equal net charge.

Implications of Results for the Folding Pathway of Barnase. It has been speculated that the initial formation of regions of secondary structure could precede a global hydrophobic collapse early on the protein folding pathway (Kim & Baldwin, 1990; Moult & Unger, 1991; Serrano *et al.*, 1992a,b). Early formed nucleation sites are proposed to adopt a conformation that allows them to bury large portions of their surface area in the folded protein (Moult & Unger, 1991). Studies to determine initiation sites from residual structure in the unfolded states of proteins may allow insight into the very early stages of protein folding. Studies on barnase fragment 1–36 in this laboratory (Sancho *et al.*, 1992) are consistent with the prediction (J. Moult, personal communication) that there is an initiation site in the first α-helical region of barnase. Our studies show that a significant fraction of fragment 1–36 in water adopts a helical conformation in the region corresponding to the first α-helix in native barnase. It has also been shown by studies on the refolding of barnase (Matouschek *et al.*, 1989, 1990) that this region is formed in the barnase folding intermediate.

In the present studies on barnase fragments 1–22 and 23–110, we have shown further evidence that protein fragments can form nativelike structure in the absence of tertiary interactions. Fragment 1–22 appears to be 3% helical in water, and at least 1% of fragment 23–110 can form a nativelike conformation in water. The extremely rapid and tight association of fragments 1–22 and 23–110 to form a fully nativelike complex is further evidence that the regions of barnase analyzed by these fragments may adopt, from the early stages of folding, a conformation similar to that in the native protein. This is strong evidence that barnase can fold by the association of independent parts. Our results support the theory that localized regions of structure, which are formed early in the folding of a protein, could initiate the folding reaction. Further, these initiation points can be can be analyzed independently without the remaining protein residues, which may enable a more detailed kinetic study of the rapid processes in the early stages of protein folding. But, the very fact that the large fragment can fold in the absence of the small fragment shows that its α-helix is not a crucial nucleation site for the initiation of folding.

REFERENCES

Argos, P. (1987) *J. Mol. Biol. 197*, 331–348.

Baldwin, R. L. (1989) *Trends Biochem. Sci. 14*, 291–294.

Buckle, A., & Fersht, A. R. (1994) *Biochemistry* (in press).

Bycroft, M., Sheppard, R. N., Lau, F. T.-K., & Fersht, A. R. (1990) *Biochemistry 29*, 7425–7432.

Chakrabartty, A., Kortemme, T., Padmanabhan, S., & Baldwin, R. L. (1993) *Biochemistry 32*, 5560–5565.

Chen, Y. H., Yang, J. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120–4131.

Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231–297.

Creighton, T. E. (1985) *J. Phys. Chem. 89*, 2452–2459.

Creighton, T. E. (1990) *Biochem. J. 270*, 1–16.

Day, T., Parsonage, D., Ebel, S., Brown, T., & Fersht, A. R. (1992) *Biochemistry 31*, 6390–6395.

Dill, K. A. (1990) *Biochemistry 29*, 7133–7155.

Dill, K. A., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795–825.

Dill, K. A., Fiebig, K. M., & Chan, H. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1942–1946.

Dyson, H. J., & Wright, P. E. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 519–538.

Dyson, H. J., Merutka, G., Waltho, J. P., Lerner, R. A., & Wright, P. E. (1992a) *J. Mol. Biol. 226*, 795–817.
Dyson, H. J., Sayre, J. R., Merutka, G., Shin, H.-C., Lerner, R. A., & Wright, P. E. (1992b) *J. Mol. Biol. 226*, 819–835.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman & Co., New York.
Fersht, A. R. (1993) *FEBS Lett. 325* (1, 2), 1–160.
Gans, P. J., Lyu, P. C., Manning, M. C., Woody, R. W., & Kallenbach, N. R. (1991) *Biopolymers 31*, 1605–1614.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319–326.
Hartley, R. W. (1989) *Trends Biochem. Sci. 14*, 450–454.
Jasanoff, A., & Fersht, A. R. (1994) *Biochemistry* (in press).
Karplus, M., & Weaver, D. L. (1976) *Nature 260*, 404–406.
Karplus, M., Snyder, G. H., & Sykes, B. D. (1973) *Biochemistry 12*, 1323–1329.
Kellis, J. T. J., Nyberg, K., & Fersht, A. R. (1989) *Biochemistry 28*, 4914–4922.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.
Lehrman, S. R., Tuls, J. L., & Lund, M. (1990) *Biochemistry 29*, 5590–5596.
Loewenthal, R., Sancho, J., & Fersht, A. R. (1991) *Biochemistry 30*, 6775–6779.
Matouschek, A., Kellis, J. J., Serrano, L., & Fersht, A. R. (1989) *Nature 340*, 122–126.
Matouschek, A., Kellis, J. T., Jr., Serrano, L., Bycroft, M., & Fersht, A. R. (1990) *Nature 346*, 440–445.
Matouschek, A., Serrano, L., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 819–835.
Matthews, B. W. (1987) *Biochemistry 26*, 6885–6888.
Matthews, B. W. (1991) *Curr. Opin. Struct. Biol. 1*, 17–21.
Mauguen, Y., Hartley, R. W., Dodson, E. J., Dodson, G. G., Bricogne, G., Chothia, C., & Jack, A. (1982) *Nature 29*, 162–164.
Montelione, G. T., & Scheraga, H. A. (1989) *Acc. Chem. Res. 22*, 70–76.
Mossakowska, D. E., Nyberg, K., & Fersht, A. R. (1989) *Biochemistry 28*, 3843–3850.
Moult, J., & Unger, R. (1991) *Biochemistry 30*, 3816–3824.
Neri, D., Billeter, M., Wider, G., & Wüthrich, K. (1992) *Science 257*, 1559–1563.
Paddon, C. J., & Hartley, R. W. (1987) *Gene 53*, 11–19.
Ptitsyn, O. B. (1973) *Dokl. Acad. Nauk 210*, 1213–1215.
Ptitsyn, O. B. (1991) *FEBS Lett. 285*, 176–181.
Sancho, J., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 741–747.
Sancho, J., Neira, J. L., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 749–758.
Sayers, J. R., Schmidt, W., & Eckstein, F. (1988) *Nucleic Acids Res. 16*, 791–802.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Schreiber, G., & Fersht, A. R. (1993) *Biochemistry 32*, 5145–5150.
Serrano, L., Horovitz, A., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *Biochemistry 29*, 9343–9352.
Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A., & Fersht, A. R. (1992a) *J. Mol. Biol. 224*, 783–804.
Serrano, L., Matouschek, A., & Fersht, A. R. (1992b) *J. Mol. Biol. 224*, 847–859.
Shortle, D., & Meeker, A. K. (1986) *Proteins: Struct. Funct. Genet. 1*, 81–89.
Shortle, D., & Meeker, A. K. (1989) *Biochemistry 28*, 936–944.
Shortle, D., Stites, W. E., & Meeker, A. K. (1990) *Biochemistry 29*, 8033–8041.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Udgaonkar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 700–704.
Vuilleumier, S., Sancho, J., Loewenthal, R., & Fersht, A. R. (1993) *Biochemistry 32*, 10303–10313.
Wodak, S. J., & Rooman, M. J. (1993) *Curr. Opin. Struct. Biol. 3*, 247–259.
Wright, P. E., Dyson, H. J., & Lerner, R. A. (1988) *Biochemistry 27*, 7167–7175.
Yanagawa, H., Yoshida, K., Torigoe, C., Park, J.-S., Sato, K., Shirai, T., & Go, M. (1993) *J. Biol. Chem. 268* (8), 5861–5865.