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Urea Denaturation of Barnase: pH Dependence and Characterization of the Unfolded State†

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ABSTRACT: To investigate the pH dependence of the conformational stability of barnase, urea denaturation curves were determined over the pH range 2-10. The maximum conformational stability of barnase is 9 kcal mol^{-1} and occurs between pH 5 and 6. The dependence of ΔG on urea concentration increases from 1850 cal mol^{-1} M^{-1} at high pH to about 3000 cal mol^{-1} M^{-1} near pH 3. This suggests that the unfolded conformations of barnase become more accessible to urea as the net charge on the molecule increases. Previous studies suggested that in 8 M urea barnase unfolds more completely than ribonuclease T1, even with the disulfide bonds broken [Pace, C. N., Laurents, D. V., & Thomson, J. A. (1990) *Biochemistry 29*, 2564-2572]. In support of this, solvent perturbation difference spectroscopy showed that in 8 M urea the Trp and Tyr residues in barnase are more accessible to perturbation by dimethyl sulfoxide than in ribonuclease T1 with the disulfide bonds broken.

In the 1960's, Tanford's group published a series of studies aimed at better characterizing the denatured states of proteins. The major conclusion was that proteins in 6 M GdnHCl with their disulfide bonds broken closely approach a randomly-coiled conformation (Tanford, 1968). The same seemed true in 8 M urea (Lapanje, 1969). Even with their disulfide bonds intact, proteins seemd to unfold as completely as possible given the restraints imposed by the disulfide bonds (Tanford, 1968). During the past five years, interest in the denatured states of proteins has been reawakened. Much of this was due to studies from Shortle's group suggesting that the denatured states of proteins are more complicated than previously thought (Dill & Shortle, 1991). Another contributing factor was the recognition that an intermediate folding state, now generally referred to as the "molten-globule" state, is formed by a number of different proteins under various conditions and has common characteristics: a secondary structure similar to the folded proteins and a tertiary structure similar to the unfolded protein (Kuwajima, 1989; Fink et al., 1991; Ptitsyn & Semisotnov, 1991; Dobson et al., 1991). Studies with NMR have been especially useful in providing information about the structure that may be present in unfolded proteins (Howard & Lian, 1984; Evans et al., 1991). For example, Evans et al. (1991) suggest "...a rather nonspecific clustering of residues in the thermally denatured form, rather than the persistence of well-defined elements of structure." In the summary of their recent review, Dill and Shortle (1991) state "There is now considerable evidence that even in strong denaturants such as 6 M GdnHCl and 9 M urea, some structure may remain in protein chains." The results presented here are consistent with both of these suggestions.

In a previous paper, we studied the pH dependence of the urea denaturation of ribonuclease A (RNase A) and ribonuclease T1 (RNase T1) (Pace et al., 1990). The pH dependence of both the conformational stability and the dependence of ΔG on urea concentration differed markedly for

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the two proteins. This was not surprising since these enzymes do not resemble one another in amino acid sequence or three-dimensional structure (Heinemann & Saenger, 1982). Barnase (RNase Ba) resembles RNase T1 in both amino acid sequence and three-dimensional structure, but, like RNase A, it is a basic protein with an isoelectric pH near 9 (Hill et al., 1983). This prompted our studies of the pH dependence of the urea denaturation of barnase presented here.

In our previous study, we concluded that proteins unfold to different extents in GdnHCl and urea even in the absence of disulfide bonds (Pace et al., 1990). In particular, RNase T1 with its disulfide bonds broken appeared to unfold ≈25% less completely than barnase in 8 M urea, and, out of the six proteins compared, barnase appeared to unfold most completely. In this paper, we use solvent perturbation difference spectroscopy (SPDS)[1] to show more directly that barnase unfolds more completely in 8 M urea than RNase T1 and reduced carboxamidomethylated RNase T1 (RCAM-RNase T1).[1]

Experimental Procedures

RNase T1 was prepared from a gene expressed in *Escherichia coli* (Shirley & Laurents, 1990). RCAM-RNase T1 was prepared as previously described (Pace et al., 1988). For most of the experiments, the barnase was prepared from a gene expressed in *E. coli* using a procedure developed by R. W. Hartley (personal communication). For four denaturation curves, the barnase used was kindly provided by S. Vuilleumier and A. Fersht (Kellis et al., 1989). All of the buffers and other reagents were the best grades available from Sigma (diglycine, MES, MOPS, HEPPSO) or Fisher (glycine, sodium formate, sodium acetate). "Ultrapure" urea was purchased from Schwarz/Mann Biotech, and stock solutions were prepared as described previously (Pace et al., 1989).

The urea denaturation curves were determined by measuring the intrinsic fluorescence (279-nm excitation and 320-nm emission) of solutions (≈0.5 μM in barnase) equilibrated for about 8 h at 25 ± 0.1 °C with either a Perkin Elmer MPF 44B or an SLM 8000 spectrofluorometer. For the four denaturation curves with the barnase supplied by Fersht's lab, the intrinsic fluorescence (290-nm excitation and 315-nm emission) was measured after ≈6 h of equilibration (pH 2.86 and 7.00) or ≈2 h of equilibration (pH 2.59 and 3.17). After the fluorescence measurements, the pH of several solutions near the midpoint of the transition was measured at room temperature after a double buffer adjustment on a Radiometer model 26 pH meter. The average of these pH measurements is the pH given in Table I and the figures. For some of the curves determined at pH values less than 3.5, the pH of all of the solutions in the transition region was measured and a correction was made for the small variation of the pH over the transition region as described under Results.

The solvent perturbation difference spectra were determined using double sector cells as described by Herskovits (Herskovits, 1967; Herskovits & Sorensen, 1968). The experiments with the folded proteins were done at pH 6 in 30 mM MES buffer. The experiments with the unfolded proteins were done in 8 M urea at pH 6 with 30 mM MES buffer or at pH 3 with 30 mM formate buffer. The perturbant was dimethyl sulfoxide (DMSO) for all of the experiments given in Table II except for barnase at pH 3 where the perturbant was ethylene glycol. For the experiments with RNase T1 and RCAM-RNase T1, the protein concentrations were in the range 30–40 μM based on a molar absorption coefficient of 18 500 M^{-1} cm^{-1} at 278 nm (Pace and Vajdos, unpublished observations). For the experiments with RNase Ba, the protein concentrations were in the range 10–20 μM based on a molar absorption coefficient of 25 900 M^{-1} cm^{-1} at 280 nm (Lees & Hartley, 1966). All of the difference spectra were determined at room temperature with a Cary 15 spectrophotometer.

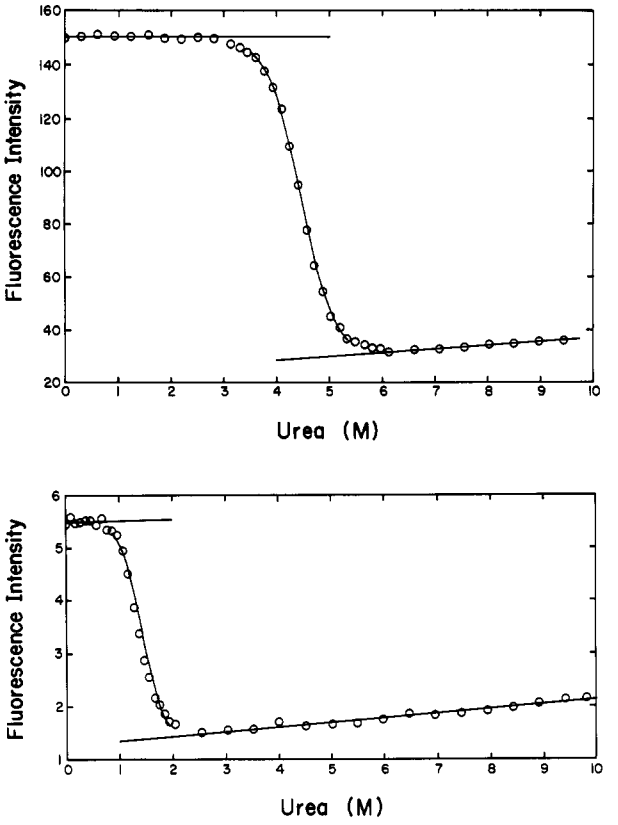

FIGURE 1: Urea denaturation curves for barnase at 25 °C in 30 mM MOPS buffer, pH 7 (A, top) or 30 mM diglycine, pH 2.96 (B, bottom). For panel A the fluorescence intensity was measured at 315 nm after excitation at 290 nm. For panel B, the fluorescence intensity was measured at 320 nm after excitation at 279 nm. The solid lines in the pre- and posttransition regions were used to determine F_F and F_U in the transition region for calculating ΔG with eq 1. The solid lines in the transition region were calculated using the parameters characterizing the transition region after the data were analyzed with eq 2. For all of the urea denaturation curves reported here, the data in panel A gave the best fit to eq 2, and the data in panel B gave the worst fit to eq 2.

Results

Urea denaturation curves for barnase were determined over the pH range 2–10. Typical curves at pH 7.00 and 2.96 are shown in Figure 1. To analyze the urea denaturation curves, a two-state folding mechanism was assumed (Hartley, 1968). This allows the calculation of ΔG as a function of urea concentration from the points in the transition region using

$$\Delta G = -RT \ln K = -RT \ln [(F_F - F)/(F - F_U)] \quad (1)$$

where K is the equilibrium constant, F is the observed fluorescence intensity, and F_F and F_U are the values of the fluorescence intensities characteristic of the folded and unfolded conformations of the protein (Pace et al., 1989). F_F and F_U were obtained by extrapolation of the pre- and posttransition baselines into the transition region, as illustrated in Figure 1. In all cases, ΔG was found to vary linearly with

[1] Abbereviations: SPDS, solvent perturbation difference spectroscopy; RCAM-RNase T1, reduced carboxamidomethylated ribonuclease T1; DMSO, dimethyl sulfoxide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPPSO, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-hydroxypropanesulfonic acid).

urea concentration, and the data were fit to

$$\Delta G = \Delta G(H_2O) - m[\text{urea}] \quad (2)$$

where $\Delta G(H_2O)$ is the value of ΔG in the absence of urea and m is a measure of the dependence of ΔG on urea concentration. $\Delta G(H_2O)$ and m were also determined by fitting the entire denaturation curve using nonlinear least-squares as described by Santoro and Bolen (1988). The values of $\Delta G(H_2O)$ and m obtained by the two procedures were always in good agreement.

For all of our denaturation curves, the intrinsic fluorescence of the unfolded protein increased with increasing urea concentration, i.e., the posttransition baselines were not flat. (The fluorescence intensities are corrected for the contributions to the fluorescence from the buffer and urea solutions used in preparing the solutions for the measurements.) For the folded protein, a similar but smaller and more variable dependence of the fluorescent intensity on urea concentration was generally observed. In contrast, Horovitz et al. (1990) state "The values of F_F and F_U are independent of the urea concentration within the range of 0 to 6.5 M urea...". Note in Figure 1A that this would be a resonable conclusion if only the data between 0 and 6.5 M urea were considered. However, it is clear that this is not the case when the data are extended to higher urea concentrations beyond those needed to complete unfolding (see Figure 1B). We faced this same situation in our studies with RNase T1. In the early stages, we were not making measurements over such a wide range of urea concentrations, and it was difficult to decide if the posttransition base line was flat or not. However, in our studies of the pH dependence of the denaturation, it became clear that the intrinsic fluorescence intensity of unfolded RNase T1 increases with both urea and GdnHCl concentration (Pace et al., 1990). This is the expected behavior since Schmid (1989) has shown that the fluorescence of both Tyr and Trp increase with increasing urea concentration. This question is important because the m values for barnase can be changed by as much as 10–15% depending on the baseline chosen. Using the Santoro and Bolen (1988) approach avoids this problem, but it then becomes important to have extensive data in the pre- and posttransition regions. If the nonlinear program has little data to use to determine a fit in these regions, it will sometimes assign an unreasonable value for the slope of a pre- or posttransition baseline.

A urea denaturation curve for barnase was first reported by Hartley (1968), and many have since been determined on barnase and mutants thereof by Fersht's group (Kellis et al., 1989; Serrano et al., 1990; Horovitz et al., 1990). Hartley (1968) reported $[\text{urea}]_{1/2}$ = 4.18 M for unfolding in 0.1 M Tris, pH 8, and we find $[\text{urea}]_{1/2}$ = 4.18 M in 30 mM HEPPSO, pH 8. Fersht's group reported $[\text{urea}]_{1/2}$ = 4.63, 4.57, and 4.58 M (Kellis et al., 1988, 1989; Serrano et al., 1990; Horovitz et al., 1990) in 50 mM MES, pH 6.3, and we find $[\text{urea}]_{1/2}$ = 4.61 M in 30 mM MES at pH 6.3. Thus, the agreement between the $[\text{urea}]_{1/2}$ values is excellent. The $[\text{urea}]_{1/2}$ values do not depend significantly on the pre- and posttransition baselines, but the m values do. Hartley (1968) reports a value of 15.8 for the slope of a plot of ln K vs ln [urea]. This corresponds to an m value of 2240 cal mol^{-1} M^{-1} at 4.18 M urea, the midpoint of the transition. Hartley (1968) followed unfolding by measuring absorbance changes at 286 and 291 nm with identical results. He reported that the absorbance was independent of urea concentration outside the transition region. [At least for the unfolded protein, a dependence on urea concentration would be expected on the basis of studies with model compounds (Schmid, 1989).] At the pH Hartley used, we find $m \approx 1850$ cal mol^{-1} M^{-1} (see Figure 2B). The m values reported by Fersht's laboratory at pH 6.3 in 50 mM MES buffer were as follows: m = 2270 cal mol^{-1} M^{-1} [difference spectroscopy at 270 and 286 nm (Kellis et al., 1988)]; m = 2060 [fluorescence (Kellis et al., 1989)]; m = 2020 [fluorescence (Horovitz et al., 1990)]; and m = 2170 ± 106 (average deviation) [this is the average of nine curves all determined using fluorescence (Serrano et al., 1990)]. The average of our m values near pH 6.3 is 1905 ± 50 cal mol^{-1} M^{-1}. If flat pre- and posttransition baselines are used for the analysis, larger m values are obtained. For example, at pH 6.04 the m value is increased from 1886 to 2060. In general, the m values are about 10 ± 5% greater when flat baselines are used.

Table I: Parameters Characterizing the Urea Denaturation of Barnase under Various Conditions at 25 °C[a]

buffer and pH	$[\text{urea}]_{1/2}$ (M)	m (cal mol^{-1} M^{-1})	$\Delta G(H_2O)$ (kcal mol^{-1})
30 mM MOPS, pH 7.00			
1	4.46	2008	8.95
2	4.52	1878	8.48
30 mM DiGly, pH 3.17			
1	1.68	3353	5.63
2	1.67	3390	5.65
3	1.67	2989	4.98
30 mM DiGly, pH 2.87			
1	1.17	3301	3.86
2	1.12	3386	3.80
3	1.12	2891	3.24
30 mM DiGly + 0.1 M NaCl, pH 2.82			
2	1.21	3133	3.78
3	1.21	2795	3.37
30 mM DiGly + 0.5 M NaCl, pH 2.76			
2	1.61	2782	4.47
3	1.60	2453	3.92
100 mM DiGly, pH 2.98			
2	1.38	3389	4.69
3	1.39	3160	4.40
100 mM DiGly, pH 2.50			
2	0.52	3425	1.78
3	0.51	3014	1.54

[a] A least-squares analysis of plots of ΔG vs urea molarity was used to determine the parameters of eq 2. The midpoint of the unfolding curves $[\text{urea}]_{1/2} = \Delta G(H_2O)/m$. The errors are estimated to be ±0.05 M in measuring $[\text{urea}]_{1/2}$ and ±7% in measuring m. For the first three entries, the results on line 1 were obtained with barnase supplied by the Fersht laboratory using wavelengths of 290 nm for excitation and 315 nm for emission, and the results on line 2 were obtained with barnase prepared in our laboratory using Hartley's procedure and using wavelengths of 279 nm for excitation and 320 nm for emission. For line 3, the results from line 2 have been corrected for the variation in pH observed across the transition region as explained in the text. (For the first entry, no correction was necessary since the urea denaturation curve was determined at pH 7.)

The Fersht lab kindly supplied us with some of their barnase so that we could compare results obtained with their protein and conditions with results obtained with our protein and conditions. The results are given in lines 1 and 2 for the first three entries in Table I. It can be seen that the agreement is good. Thus, the fact that we observe lower m values than the Fersht group results mainly from the difference in the posttransition baselines used in the analysis.

The urea denaturation curves determined at pH values below 3.5 required special attention. Below pH 3.5, the 30 mM buffers being used were not adequate to maintain a constant pH in the transition region, and the pH was found to decrease with increasing urea concentration for the solutions in the transition region. For example, the decrease was 0.06 pH units at pH 3 over the seven to nine solutions used to

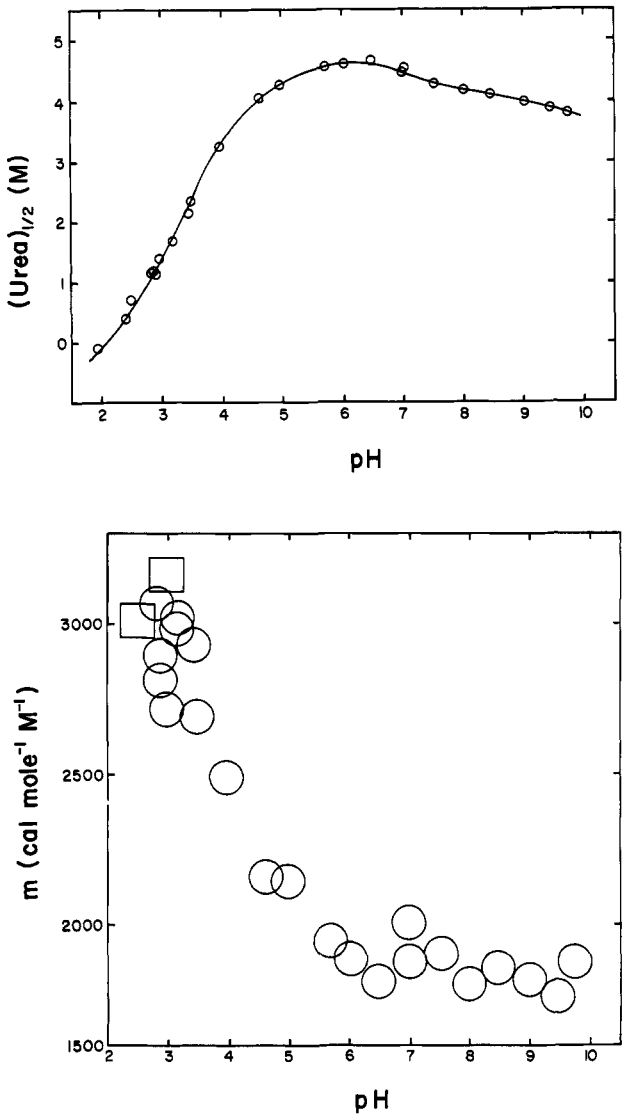

FIGURE 2: (A, top) $[urea]_{1/2}$ and (B, bottom) m as a function of pH for the urea unfolding of barnase at 25 °C. For the points designated with a circle, the curves were determined in the presence of 30 mM buffer. For the two points designated with squares, the curves were determined in the presence of 100 mM diglycine buffer. For panel B, all of the m values below pH 3.5 were corrected for the effect of the variation of pH in the transition region, as described in the text and illustrated in Table I.

determine the parameters in eq 2. The decrease becomes larger at lower pHs. This has no effect on the midpoint of the transitions, $[urea]_{1/2}$, but it does affect the steepness of the transitions, i.e., the m values. To correct for this, we measured the pH of all of the solutions in the transition region for several curves determined below pH 3.5. The slope of a plot of $[urea]_{1/2}$ vs pH is 1.7 M urea/pH unit near pH 3. Using this plus the initial m value allows the variation of ΔG with pH to be calculated. This value is used to correct the individual observed ΔG values to the pH measured at the midpoint so that a new m value can be calculated. After one reiteration with the new m value, a pH corrected m value is obtained. For each entry in Table I, line 2 gives the m value before the correction, and line 3 gives the pH corrected m value. In the presence of 30 mM buffer, the correction to the m value is ≈5% at pH 3.5, ≈10% at pH 3, and ≈15% at pH 2.5. All of the data in Figures 2A,B and 4 have been corrected for this effect. Note that the increase in m with decreasing pH shown in Figure 2B would be even greater if this correction were not made.

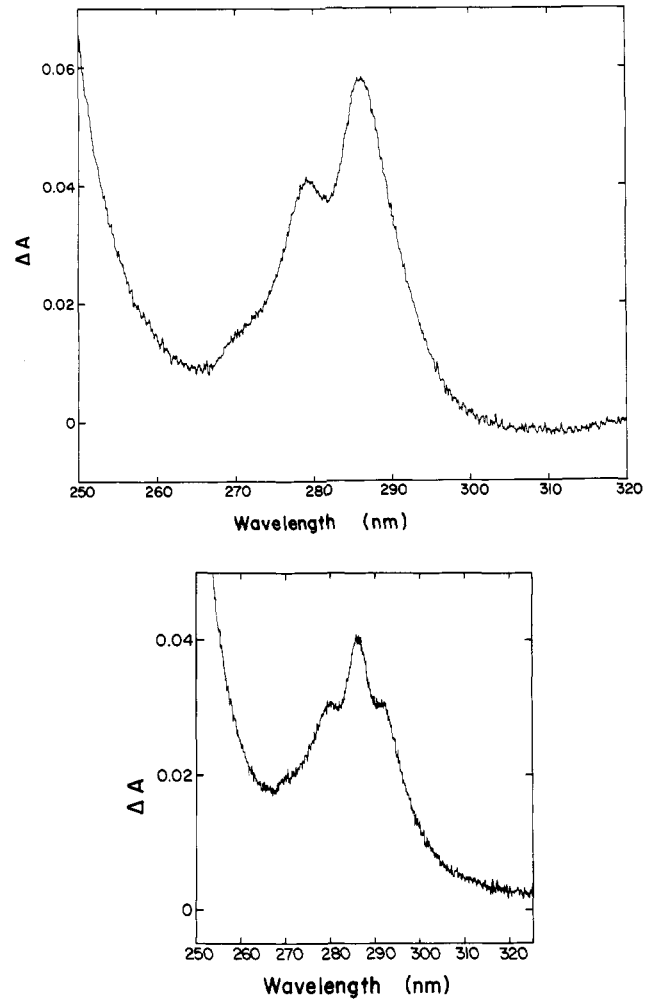

FIGURE 3: Solvent perturbation difference spectra of RNase T1 (A, top) and barnase (B, bottom) in the presence of 8 M urea and 30 mM MES buffer, pH 6. The perturbant was 20% dimethyl sulfoxide, and the protein concentrations were 30 μM for RNase T1 and 15 μM for barnase.

The $[urea]_{1/2}$ value is a measure of the midpoint, and the m value is a measure of the steepness of urea unfolding curves. The $[urea]_{1/2}$ and m values for the unfolding of barnase are shown as a function of pH in Figure 2. The maximum in $[urea]_{1/2}$ occurs near pH 6.3. This is much lower than the isoelectric pH, which is expected to be ≈9 on the basis of the amino acid composition (Hartley & Barker, 1972). The pH dependence of T_m between pH 1 and 7 observed by Hartley (1969) is similar to the pH dependence of $[urea]_{1/2}$ shown in Figure 2A. The pH dependence of m for barnase is similar to the pH dependence of m for RNase A but different from that for RNase T1 (Pace et al., 1990).

Herskovits (1965) used SPDS[1] to show that the percentage exposure of the Tyr and Trp residues in RNase A, lysozyme, and bovine serum albumin in 8 M urea increased by ≈20% when the disulfide bonds were broken (see Table III). This encouraged us to use SPDS to see if we could observe a difference in the accessibility of the Tyr and Trp residues in RNase T1, RCAM-RNase T1, and barnase in the presence of 8 M urea. Previous results indicated that barnase unfolds more completely than RNase T1, even with the disulfide bonds broken in RCAM-RNase T1 (Pace et al., 1990). Typical solvent perturbation difference spectra for RNase T1 and barnase in 8 M urea are shown in Figure 3. The perturbant used for most of the experiments was DMSO because it is a good perturbant (Herskovits & Sorenson, 1968) and is only

Table II: Tryptophan and Tyrosine Accessibility of Barnase, RCAM-RNase T1, and RNase T1 Determined by Solvent Perturbation Difference Spectroscopy[a]

	no. Trp exposed + no. Tyr exposed = total no. exposed		
	folded (pH 6)	unfolded (pH 6)	unfolded (pH 3)
RNase Ba	1.2 + 5.2 = 6.4 (7.0)	3.3 + 8.8 = 12.1 (14.1)	3.4 + 11.4 = 14.8 (nd)
RCAM T1		1.4 + 8.1 = 9.5 (9.8)	1.3 + 8.2 + 9.5 (9.8)
RNase T1	0.8 + 3.6 = 4.4 (4.2)	0.8 + 7.8 = 8.6 (9.1)	1.0 + 8.2 = 9.2 (9.7)

[a] The perturbant was 20% DMSO, and the data were analyzed using the model compound data and the methods described in Herskovits and Sorenson (1968). (For the experiment with unfolded barnase at pH 3, the perturbant was ethylene glycol.) For the folded proteins, the solvent was 30 mM MES. For the unfolded proteins, the solvent was 8 M urea containing either 30 mM MES (pH 6) or 30 mM formate (pH 3). The values in parentheses were obtained by comparing the $\Delta\epsilon$ values for the proteins with $\Delta\epsilon$ values obtained by perturbing a model compound mixture containing a 3:7 (for barnase) or a 1:9 (for RNase T1) mixture of *N*-acetyltryptophan ethyl ester and *N*-acetyltyrosine ethyl ester. For barnase, the average of the values obtained at 286 and 292 nm is given. For RNase T1, only the value from the data at 286 nm is given. The data at 292 nm cannot be used because the absorbance depends too sharply on wavelength near 292 nm (see Figure 3A).

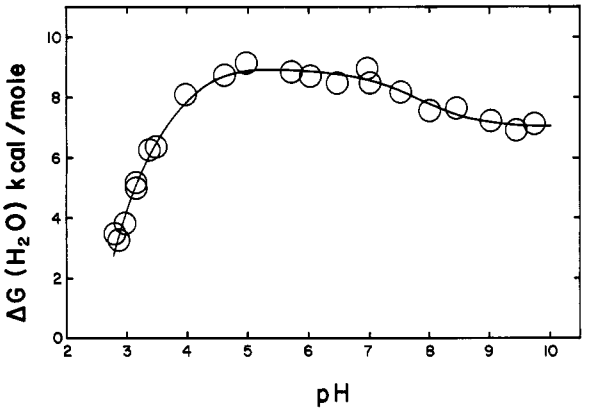

FIGURE 4: $\Delta G(H_2O)$ as a function of pH for the unfolding of barnase at 25 °C. The solid curve between pH 5 and 10 was calculated with eq 3 using $pK_F = 8.5$, $pK_U = 7.1$, and $\Delta G(H_2O)(pH < pK) = 8.87$ kcal mol^{-1}. The solid curve below pH 5 has no theoretical significance.

a very modest protein denaturant (Pace & Marshall, 1980). The shoulder near 292 nm, indicating the perturbation of Trp residues, is clearly visible with barnase, which contains three Trp and seven Tyr, but not with RNase T1, which contains one Trp and nine Tyr. This was also true of the SPDS that were determined with mixtures of *N*-Ac-Trp-OEt and *N*-Ac-Tyr-OEt present in the same ratios as in the proteins.

The SPDS were quantitated in two ways: first, by using the $\Delta\epsilon$ values for the perturbation of *N*-Ac-Trp-OEt and *N*-Ac-Tyr-OEt by 20% DMSO in both water and 8 M urea given in Table I by Herskovits and Sorenson (1968), and, second, by using the SPDS obtained by perturbing the model compound mixtures noted above. The results are listed in Table II.

Discussion

The $\Delta G(H_2O)$ value is one measure of the conformational stability of a protein. Figure 4 shows the variation of $\Delta G(H_2O)$ with pH for barnase. The maximum stability of barnase, ≈9 kcal mol^{-1}, occurs between pH 5 and 6. This is in reasonable agreement with estimates from the Fersht lab at pH 6.3: 9.5 kcal mol^{-1} from thermal denaturation results, 9.4 kcal mol^{-1} from urea denaturation results, and 8.9 kcal mol^{-1} from GdnHCl denaturation results (Kellis et al., 1989). For RNase T1 and RNase A, the maximum stabilities are also about 9 kcal mol^{-1} (Pace et al., 1990). However, for RNase T1 there is a relatively sharp maximum near pH 4.5, and for RNase A there is a broad maximum with very little variation in $\Delta G(H_2O)$ between pH 6 and 10. It is interesting that these proteins have similar conformational stabilities since barnase has no disulfide bonds, RNase T1 has two, and RNase A has four. It is estimated that the disulfide bonds contribute ≈19 kcal mol^{-1} to the stability of RNase A (Konishi et al., 1982) and ≈7 kcal mol^{-1} to the stability of RNase T1 (Pace et al., 1988). Since barnase and RNase T1 are related proteins (Hill et al., 1983), it was of interest to consider how barnase compensates for the stability that RNase T1 gains from its disulfide bonds. Analysis using the Lee and Richards program (Richards, 1977) showed that barnase buries more hydrophobic groups on folding than RNase T1 [based on the barnase crystal structure reported by Mauguen et al. (1982) and the RNase T1 crystal structure reported by Martinez-Oyanedel et al. (1991)]. Using *n*-octanol as a model for the interior of barnase suggests that these extra hydrophobic interactions would contribute an additional 25 kcal mol^{-1} to the stability of barnase. In addition, barnase forms 91 intramolecular hydrogen bonds on folding (K. Gajiwala, unpublished observations), and RNase T1 forms only 86 (Shirley et al., 1992), so hydrogen bonding should also make a greater contribution to the stability of barnase.

The pH dependence of the stability of barnase between pH 6 and 9 was previously studied by Sali et al. (1988). They showed that the change in stability in this pH range is due mainly to the fact that His 18 has a higher pK in the folded protein than in the unfolded protein. On the basis of these results, we used nonlinear least-squares to fit our data between pH 5 and 10 to

$$\Delta G = \Delta G(pH < pK) - RT \ln [(1 + K_U/H^+)/(1 + K_F/H^+)] \quad (3)$$

where K_F and K_U are the dissociation constants for His 18 in the folded and unfolded conformations, H^+ is the hydrogen ion activity, and $\Delta G(H_2O)(pH < pK)$ is $\Delta G(H_2O)$ at low pH, where His 18 is fully protonated (Tanford, 1970). The values we obtained were $\Delta G(pH < pK) = 8870 \pm 200$, $pK_F = 8.5 \pm 0.4$, and $pK_U = 7.1 \pm 0.5$. These are in reasonable agreement with the values of $pK_F = 8.2$ and $pK_U = 6.6$ in 5 M urea determined directly using NMR by Sali et al. (1988). As with barnase, the pH of maximum stability for T4 lysozyme is shifted far below the isoelectric pH, and the reason has been shown to be the same: a His residue in the folded protein with an unusually high pK of 9.1 (Anderson et al., 1990). Thus, these are two examples of proteins that are most stable at pHs far from their isoelectric points.

The dependence of ΔG on pH is greatest near pH 3, where a plot of log K vs pH suggests that ≈3–4 protons are bound on unfolding. This is in agreement with Hartley's (1969) estimates based on studies of the pH dependence of the thermal unfolding of barnase. At low pH where barnase will have a large positive charge, the pKs of the carboxyl groups in the folded protein are expected to be lower than in the unfolded protein (Nozaki & Tanford, 1967). Thus, unfolded barnase binds protons more tightly than folded barnase, and this is the

Table III: Percentage Trp + Tyr Accessibility in 8 M Urea from Solvent Perturbation Difference Spectroscopy and Changes in Accessibility (α Values) Calculated from the Measured m Values Using Tanford's Model

	α value[a]	% Tyr + Trp accessibility[b]	
		-S-S- intact	-S-S- broken
BSA		64	86
lysozyme	0.23	69	93
RNase A	0.35	68	88
RNase T1	0.33 (0.38)[c]	86	95
RNase Ba	0.48[d]		121

[a] The α values were calculated from the measured m values for urea as described by Pace et al. (1990). [b] The percent Trp + Tyr accessibility values in 8 M urea for BSA, lysozyme, and RNase A are from Herskovits (1965), and the values for RNase T1 and barnase are calculated from the data in Table II. [c] The α value in parentheses is for RNase T1 with the disulfide bonds broken. [d] This α value is lower than that given by Pace et al. (1990) because the m values reported here were used for the calculation.

main reason for the sharp decrease in stability observed as the pH is lowered. A similar pH dependence is observed with RNase T1 and RNase A, but the effect is smaller since only about two protons are bound on unfolding (Pace et al., 1990).

The fact that $\Delta G(H_2O)$ decreases by only $\approx$5 kcal mol^{-1} in going from the isoelectric pH to pH 3 where barnase has a large positive charge argues that electrostatic interactions among the charged groups on the surface do not make a large contribution to the conformational stability (Pace et al., 1990). A similar conclusion was reached in recent studies of mutants of T4 lysozyme (Dao-pin et al., 1991) and barnase (Sali et al., 1991) and in earlier studies (Tanford, 1970; Hollecker & Creighton, 1982; Matthew & Richards, 1982). Thus, it now seems fairly certain that electrostatic interactions among the charges on the surface of globular proteins make at most a small contribution to the conformational stability.

The measured m values depend mainly on the number and type of groups which are freshly exposed to solvent when the protein unfolds. Tanford (1964, 1970) developed a model that can be used to estimate the fraction of the peptide groups and uncharged side chains that must become exposed to solvent on unfolding to account for the measured m values. We will refer to this fraction as the α value. Some α values are listed in Table III. Note that the α value for barnase is considerably larger than that for lysozyme, RNase A, and RNase T1. This is not surprising because these proteins all contain disulfide bonds that are expected to reduce the accessibility of the unfolded conformations of these proteins to denaturant. Note also, however, that the α value for barnase is substantially higher than the α value for RNase T1 with its disulfide bonds broken. This is surprising. In fact, the α values for six proteins with no disulfide bonds ranged from 0.32 for dihydrofolate reductase to 0.48 for barnase, and this suggests that the unfolded states of these proteins in 8 M urea differ significantly in their ability to interact with denaturants (Pace et al., 1990).

On the basis of the crystal structures of the proteins and the Lee and Richards program (Richards, 1977), about 70% of the peptide groups and uncharged side chains are buried in folded RNase T1 and barnase. Thus, an α value of about 0.7 would be possible if the proteins unfolded completely. We have suggested that the increase observed in the m values at low pH results because the unfolded states expand due to electrostatic repulsion among the positive charges, allowing a more extensive interaction with urea. [This is supported by the decrease in the m value for barnase observed in the presence of 0.5 M NaCl (see Table I).] At low pH, the calculated α values increase to maximum values of 0.42 for RNase T1, 0.45 for RNase A, and 0.62 for barnase. Thus, the α value increase is greater for barnase, probably because of the absence of disulfide bonds, but is still less than the value possible if barnase unfolded completely.

The evidence is mounting that proteins do not unfold completely in 8 M urea and 6 M GdnHCl. On the basis of studies using NMR, the structure present in the unfolded states of proteins appears to be due mainly to hydrophobic clustering (Evans et al., 1991). Since the aromatic residues are among the most hydrophobic, they are likely to be important in hydrophobic clustering. SPDS[1] is a technique that provides information about the solvent accessibility of the Tyr and Trp side chains (Herskovits & Laskowski, 1962). Some typical data from Herskovits (1965) are presented in Table III. Note that there is a substantial increase in the solvent accessibility of the Tyr and Trp side chains in BSA, lysozyme, and RNase A in 8 M urea when the disulfide bonds are cleaved. These results encouraged us to see if we could observe differences in the accessibility of the Tyr and Trp residues in unfolded barnase and RNase T1 using SPDS.

It has been noted previously that SPDS overestimates the accessibility of the aromatic residues in folded proteins (Laskowski, 1966; Brandts & Kaplan, 1973; Kosen et al., 1980). We also observe this with barnase and RNase T1. Using the Lee and Richards program (Richards, 1977), the 10 Tyr and Trp side chains are estimated to be 16% exposed to solvent in barnase and 12% exposed to solvent in RNase T1. The estimates based on SPDS are $\approx$67% for RNase Ba and 43% for RNase T1 (Table II). When the proteins unfold in 8 M urea, the accessibility almost doubles (Table II). More importantly, we do indeed observe that the aromatic residues are substantially more accessible to the perturbant in barnase than they are in RNase T1. Thus, these results from SPDS are consistent with the conclusion that we reached from the α values: unfolded barnase is more accessible to solvent than unfolded RCAM-RNase T1.

Note that the results from SPDS indicate that the aromatic residues in barnase are more accessible to the perturbant than they are in the model compound mixtures. We do not have a good explanation for this observation. It is not likely to be a problem with the model compound data since similar results are obtained using the Herskovits and Sorenson (1968) data on the individual model compounds or our results with model compound mixtures. This plus the overestimation of the accessibilities of the aromatic residues in the folded proteins points out that SPDS is not reliable for giving an absolute measure of the accessibility of the aromatic residues. However, all of the *differences* in accessibility that we observe are reasonable. The accessibilities in 8 M urea are less for BSA, lysozyme, and RNase A, whose disulfide bond contents are $\approx$3% that for RNase T1, whose disulfide bond content is $\approx$2%; and these are less than for RCAM-RNase T1 and barnase, which contain no disulfide bonds. Also, the accessibilities increase when the disulfide bonds are broken, and the increase is less for RNase T1 than it is for BSA, lysozyme, and RNase A. For barnase, the accessibility is greater at pH 3 than it is at pH 6 as expected on the basis of the increase in the m value at low pH discussed above.

In summary, the results presented here show that the maximum conformational stability of barnase is $\approx$9 kcal mol^{-1} and occurs between pH 5 and 6. The large increase in the m value at low pH observed with barnase is similar to that observed previously with RNase A (Pace et al., 1990). This suggests that the unfolded states of barnase and RNase A interact more extensively with urea at low pH than at neutral

pH. This is probably due to an expansion of the unfolded states at low pH due to electrostatic repulsion among the positive charges. Finally, the interpretation of the *m* values in terms of accessibilities and the results from SPDS both suggest that barnase unfolds more completely than RNase T1 in 8 M urea. This suggests that the conformations assumed by unfolded proteins in 8 M urea depend to at least some extent on the amino acid sequence of the protein.

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