Thermodynamic Study of the Acid Denaturation of Barnase and Its Dependence on Ionic Strength: Evidence for Residual Electrostatic Interactions in the Acid/Thermally Denatured State

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ABSTRACT: We have investigated the acid denaturation of barnase and its dependence on ionic strength. From the pH dependence of the protein stability, we have obtained information about the titration properties of the native and denatured protein at temperatures ranging from 15 to 60 °C in the absence of chemical denaturant. It appears that both the native and the denatured state of barnase titrates at higher pH values in the presence of salt. The observation suggests that charge interactions are present, not only within the native fold but also within the denatured state, and that these interactions contribute to shift the pK_a values from those of isolated model compounds. Upon addition of salt these repulsive interactions are shielded, and the electrostatic free energy of the native state, as well as the denatured state, is reduced. Accordingly, we suggest that the thermally denatured state of barnase is not an extended random coil without residue–residue interactions but is sufficiently compact to contain intramolecular charge–charge repulsions. The results further reveal that the native state of barnase contains at least one residue with a highly anomalous pK_a value: At pH 0.3, the difference in degree of protonation between the native and the denatured state is still about 1 mol H^+/mol protein.

The pH dependence of protein stability is electrostatic in origin (Tanford, 1968). Upon titration with protons, the net charge as well as the charge distribution of the protein changes, and, so, the acid-unfolding transition is caused by repulsive forces between ionized residues. Since these interactions, in principle, can be treated by classical electrostatics, there is a fundamental interest in testing and developing theoretical models from existing experimental data [see, for example, Bashford and Karplus (1990) and Warshel and Aqvist (1991)]. The extent of interaction between a ionizable residue and the rest of the protein is typically reflected in its titration properties.

Although the titration behavior of native proteins has been studied in great detail, very little is known about the properties of the corresponding denatured conformations. One reason is that the denatured state titrates in a pH region where, normally, only the native state is present. Direct studies of the denatured state have been generally performed, therefore, in the presence of an additional denaturant, for example urea or guanidine hydrochloride (Nozaki & Tanford, 1967; Roxby & Tanford, 1971). The results from such experiments, however, could be misleading since the artificially induced denatured state may well be different from that of interest, the denatured state most stable in pure water. For instance, it may be deduced from classical electrostatics that any charge interactions within the denatured protein will be affected by the presence of guanide hydrochloride, since this charged denaturant contributes to the ionic strength of the solvent, (cf. Stigter & Dill, 1990). Consistent with this, it has been observed that stability calculations based on titration data from the guanidine hydrochloride-denatured states consistently differ from directly measured stabilities. For example, the calculated stabilities of ribonuclease A and hen egg-white lysosyme display an excessive pH dependence (Tanford & Roxby, 1972; Antosiewicz et al., 1993). The discrepancy, which also appears in similar studies of other proteins, can be accounted for if the pK_a values of the denatured protein are assumed to be slightly shifted from those determined in direct titration experiments (Antosiewicz et al., 1993). As a plausible explanation, it has been speculated that the thermodynamically relevant denatured state (under folding conditions) retains a significant amount of residual structure and electrostatic interactions, whereas the investigated proteins, denatured by urea or guanidine hydrochloride, are highly extended and solvent exposed with the residues effectively isolated from intramolecular interactions [for review of denatured states, see Dill and Shortle (1991)]. The presence of such residual structure may, thus, be manifested in titration properties shifted from those of residues isolated by solvent.

In the present work, we have investigated the titration behavior of the native and denatured states of barnase (EC 3.1.27.3), a small ribonuclease from *Bacillus amyloliquefaciens*. Barnase consists of 110 amino acid residues of which 12 are acidic and 16 basic (Mauguen et al., 1982). Structural constraints in the unfolded state are minimized since the protein does not contain any disulfide cross-links. The folding pathway of barnase has been extensively studied and demonstrated to proceed with the formation of a transient intermediate (Bycroft et al., 1990; Matouschek et al., 1990). Under equilibrium conditions, however, the occupancy of this intermediate is small and the unfolding transition displays a typical two-state behavior. In order to investigate the titration properties of barnase in the *absence* of chemical denaturants, the present study relies on the pH dependence of the thermal stability which, in turn, can be directly related to the difference in degree of protonation between the native and the denatured protein. Our results reveal that the pK values, not only of the native state but also of the denatured state of barnase, are shifted upward upon addition of salt. This provides new evidence for charge–charge interactions in denatured proteins in the absence of chemical denaturant and thus confirms earlier

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observations of residual structure in the thermally- and acid-unfolded states.

EXPERIMENTAL PROCEDURES

Materials. Barnase was overexpressed and purified from *Echerichia coli* (Serrano & Fersht, 1989). The buffers used were glycine/HCl (pH 1.5–3.0), sodium formate/formic acid (pH 2.7–4.2), sodium acetate/acetic acid (pH 3.7–5.3), and 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.3), all purchased from Sigma. HCl/KCl was used between pH 3.0 and 0.2. The ionic strength was controlled with KCl. The protein concentration was 1 μM, and the experiments were performed at 25 °C, unless otherwise stated.

Fluorescence-Monitored Acid Denaturation. The reversible acid denaturation was monitored by fluorescence at 25 °C using an Aminco Bowman spectrofluorimeter with excitation at 290 nm and emission at 320 nm. The analysis of the data is based on a two-state model where the equilibrium constant for the denatured and the native state of the protein is given by

$$K_{D/N} = \frac{I_N - I}{I - I_D} \quad (1)$$

where I_N is the fluorescence intensity of the native state, I_D is the fluorescence of the denatured state, and I is the measured fluorescence at a given pH value. If the pH dependencies of I_N and I_D are assumed to be linear

$$I_N = \alpha_N + \beta_N pH, \quad I_D = \alpha_D + \beta_D pH \quad (2)$$

and if $\log(K_{D/N})$ is approximated to be linearly dependent on pH in the transition region (since this occurs within a narrow pH range),

$$\log K_{D/N} = m_{eq}(MP_{ep}) - m_{eq}(pH) \quad (3)$$

eq 1 can be rewritten

$$I = \frac{I_N + I_D K_{D/N}}{1 + K_{D/N}} = \frac{(\alpha_N + \beta_N pH) + (\alpha_D + \beta_D pH)10^{-m_{eq}(pH - MP_{eq})}}{1 + 10^{-m_{eq}(pH - MP_{eq})}} \quad (4)$$

where α_N,β_N and α_D,β_D define I_N and I_D, respectively, MP_{eq} the pH at the transition midpoint and m_{eq} the number of protons taken up in the unfolding transition, i.e., the slope of $\log(K_{D/N})$ versus pH, eq 3 (cf. $\Delta Q_{D\text{-}N}$ in eq 11 below). The experimental data, i.e., plots of fluorescence intensity (I) vs pH (see Figure 2), were fitted with the data analysis program Kaleidagraph (Abelbeck Software) using eq 4.

CD-Monitored Temperature Denaturation. The reversible thermal denaturation of barnase was monitored by circular dicroism (CD) at 230 nm using a JASCO J-720 instrument fitted with a thermostated cell holder and interfaced with a Neslab RTE-110 water bath. Because of the low protein concentrations (1 μM), a cuvette with a 20-mm path length was used. The instrument was programmed to increase the temperature in the cuvette from 3 to 80 °C with a rate of 50 deg/h as that the CD signal was recorded every 0.2 °C. The results, i.e., the thermal transitions (cf. Figure 3a), were analyzed by using the following thermodynamic relations: At a given temperature, T, the enthalpy of unfolding is given by van't Hoff's equation

$$\Delta H_{D\text{-}N}(T) = -R\frac{\delta(\ln K_{D/N})}{\delta(1/T)} = RT^2\frac{\delta(\ln K_{D/N})}{\delta T} \quad (5)$$

The van't Hoff plots ($\ln K$ versus $1/T$), obtained from unfolding transitions of proteins, are found to be nonlinear. This is expected since $\Delta H_{D\text{-}N}$ is related to the difference in heat capacity between the native and denatured state by eq 6 and, therefore, temperature dependent if $\Delta C_p \neq 0$.

$$\Delta C_p = \frac{\delta(\Delta H_{D\text{-}N})}{\delta T} = T\frac{\delta(\Delta S_{D\text{-}N})}{\delta T} \quad (6)$$

where $\Delta S_{D\text{-}N}$ is the entropy of unfolding. Under the assumption that ΔC_p is independent of temperature and pH [i.e., the enthalpy of protonation of Asp/Glu is negligible and, hence, m_{eq} is constant with temperature (Privalov, 1979)], eq 6 was directly applied to the observed temperature dependence of $\Delta H_{D\text{-}N}$ (Figure 3b). The free energy of unfolding is related to the equilibrium constant, $\Delta H_{D\text{-}N}$ and $\Delta S_{D\text{-}N}$ by

$$\Delta G_{D\text{-}N}(T_m) = -RT\ln[K_{D/N}(T)] = \Delta H_{D\text{-}N}(T) - T\Delta S_{D\text{-}N}(T) \quad (7)$$

and, hence, at the temperature for the midpoint of the thermal transition (T_m) by

$$\Delta G_{D\text{-}N}(T_m) = 0 = \Delta H_{D\text{-}N}(T_m) - T_m\Delta S_{D\text{-}N}(T_m) \quad (8)$$

By first integrating the enthalpy and the entropy in eq 7 from T to T_m, then substituting the expressions for $\Delta H_{D\text{-}N}(T)$ and $\Delta S_{D\text{-}N}(T)$ into eq 7, and finally substituting $\Delta S_{D\text{-}N}(T_m)$ for $\Delta H_{D\text{-}N}(T_m)$ (eq 8), we obtain (Privalov, 1979)

$$\Delta G_{D\text{-}N}(T) = \Delta H_{D\text{-}N}(T_m)(1 - T/T_m) - \Delta C_p[(T_m - T) + T\ln(T/T_m)] \quad (9)$$

Equation 9, which relates the equilibrium constant with the thermodynamic parameters at the transition midpoint, i.e., $K_{D/N}(T) = e^{-\Delta G_{D\text{-}N}(T)/RT}$, was put into eq 1 to give an expression equivalent to eq 4,

$$\theta = \frac{(\alpha_N + \beta_N T) + (\alpha_D + \beta_D T)e^{-(\Delta G_{D\text{-}N}(T))/RT}}{1 + e^{-(\Delta G_{D\text{-}N}(T))/RT}} \quad (10)$$

where θ is the observed ellipticity, α_N β_N and α_D β_D define the temperature dependence of the ellipticity of the native state and the denatured state, respectively, and $\Delta G_{D\text{-}N}(T)$ represents eq 9. Analogously to the treatment of the fluorescence data, eq 10 was fitted to the thermal transitions, i.e., plots of ellipticity vs T (Figure 3a).

Thermal unfolding experiments were performed at different pH values and the parameters obtained at each pH value by eq 10 [$\Delta H_{D\text{-}N}(T_m)$ and T_m] are plotted in Figure 3b and fitted to a linear function. The linear function was then used to replace $\Delta H_{D\text{-}N}(T)$ in eq 9. Equation 9 is now suitable to calculate $\Delta G_{D\text{-}N}$ at 25 °C, knowing the midpoint for the thermal transition.

Finally, the pH dependence of the free energy was obtained by plotting the $\Delta G_{D\text{-}N}$ values, calculated at 25 °C by eq 9, versus pH (Figure 4). In order to calculate the derivative of these plots ($\partial\Delta G/\partial$pH), the free energy plots were first fitted to a predefined "smooth function" in the Kaleidagraph software (Figure 4), and then $\partial\Delta G/\partial$pH were derived from the smooth function (Figure 5). The smooth function were also used for the subtraction of the $\Delta G_{D\text{-}N}$ plots from one another (Figure 5).

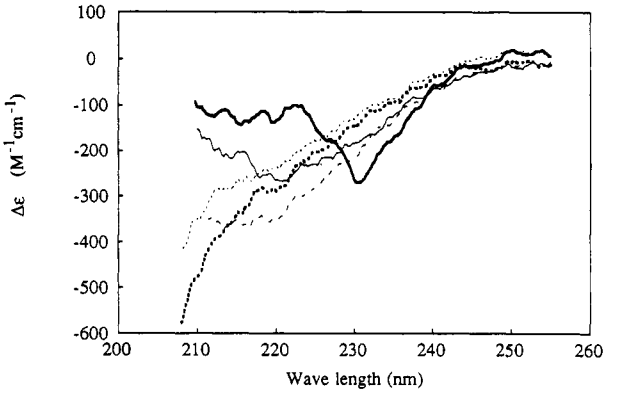

FIGURE 1: Far-UV CD spectra of native barnase and its denatured states. In order to minimize any effects of aggregation/association, the spectra were obtained at a low protein concentration (0.55 μM), hence the noisy recording. Native protein at 25 °C, pH 6.3, I = 50 mM (bold line), acid-denatured protein at 25 °C, pH 1.3, I = 50 mM (bold dotted line), thermally denatured protein at 70° C, pH 6.3, I = 50 mM (thin dotted line), acid-denatured protein at 25 °C, pH 0.8, I = 600 mM (thin line), acid-denatured protein at 70 °C, pH 0.8, I = 600 mM (thin dashed line).

The Relationship between the pH Dependence of the Protein Stability and the Difference in Degree of Ionization between the Native and the Denatured State. The pH dependence of protein stability is determined by the titration properties of the native protein relative to the titration properties of the denatured conformation. In general, the pK_a values of the denatured, solvent-exposed protein appear similar to those of model compounds, whereas the residues in the folded protein are found to titrate with anomalous pK_a values. The number of protons bound to a certain protein conformation at a given pH is easily calculated from its pK_a values. It has been demonstrated that the difference in number of bound protons between the native and the denatured state can be related to the pH dependence of the protein stability by (Tanford, 1968):

$$\frac{\partial(\Delta G_{D\text{-}N})}{\partial(\mathrm{pH})} = 2.3RT[Q_D(\mathrm{pH}) - Q_N(\mathrm{pH})] = 2.3RT\Delta Q_{D\text{-}N}(\mathrm{pH}) \quad (11)$$

where $\Delta G_{D\text{-}N}$ is the difference in free energy between the native and the denatured state. Q_D(pH) and Q_N(pH) are the number of protons bound to the denatured and the native state, respectively, and $\Delta Q_{D\text{-}N}$(pH) the change in the number of protons taken up on denaturation at this particular pH. If the observed pH dependence of the stability can be attributed entirely to protonation events (i.e., all other solvent effects are negligible), $\Delta Q_{D\text{-}N}$ at the pH for the transition midpoint at 25 °C equals m_{eq} in eq 3.

RESULTS

Far-UV CD Spectra of Acid- and Thermally-Denatured Barnase and Its Dependence on Ionic Strength. The far-UV CD spectrum of native barnase shows low overall spectral intensities with a distinct minimum around 230 nm (Figure 1). This minimum has previously been attributed to contributions mainly from aromatic residues (Vuilleumier et al., 1993). When denatured by acid at ionic strength I = 50 mM, barnase does not display the spectral features typical for a random coil, but a spectrum with significant negative contributions between 200 and 250 nm (Figure 1). Similar spectra has been observed for several other acid-denatured proteins (Goto & Fink, 1989) and are generally belived to be indicative of some content of secondary structure or compactness. Further, the absence of contributions in the near-UV region suggests that the acid-denatured state of barnase lacks any tertiary interactions (data not shown). The spectrum obtained from the thermally denatured protein at pH 6.3 and I = 50 mM is very similar to that of the acid-denatured state under conditions of low ionic strength (Figure 1) (cf. Privalov et al., 1989). At I = 600 mM, the spectrum of the acid-denatured state appears quite different and shows a minimum around 220 nm (Figure 1). It can be noticed, that similar spectra have been observed previously for so-called "molten-globule states", a collective name for compact denatured states with significant amount of secondary structure but no fixed tertiary interactions (Kuwajima, 1989; Ptitsyn et al., 1990; Ptitsyn, 1992). Another possibility is that the spectral change is due to aggregation or association processes taking place under conditions of high concentrations of salt. However, at the low protein concentrations used, no precipitate could be observed visually, nor could any material be spun down with a bench-top centrifuge. Further, the unfolding transition was found essentially reversible under all conditions. Although this may still be consistent with aggregation/association, it suggests that any aggregate or polymerized state is small and occurs in reversible equilibrium with a denatured monomer. At higher temperatures, the CD spectrum of the high-salt denatured state approaches that of the thermally unfolded state (Figure 1).

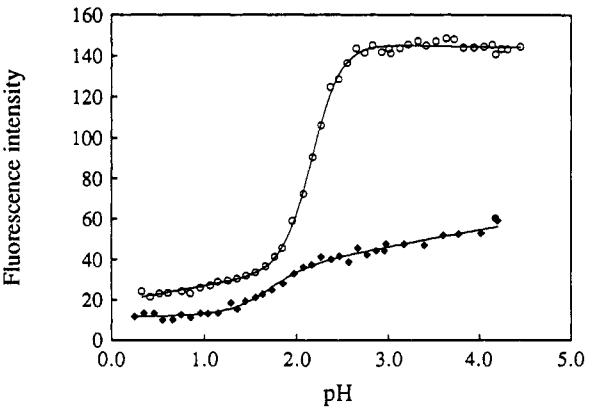

FIGURE 2: Titration of the fluorescence intensities of barnase (λ_{ex} = 280 nm; λ_{em} = 320 nm) with pH, at I = 50 mM (○) and I = 600 mM (◆). The solid curves represent theoretical two-state transitions fitted to the data (eq 4).

Fluorescence-Monitored Acid Denaturation. Figure 2 shows the fluorescence change of barnase upon titration with acid at ionic strengths I = 50 mM and I = 600 mM. The presence of salt is found to have a pronounced effect on the fluorescence properties of the protein, resulting in a less readily resolved transition under conditions of high concentrations of salt. It is clear, however, that in the presence of salt the protein unfolds at a lower pH and the number of protons taken up during the transition is decreased. The parameters obtained by eq 2 for 10, 50, 200, and 600 mM ionic strengths are listed in Table 1.

CD-Monitored Temperature Denaturation. The thermal denaturation curves show a single symmetric transition typical for a two-state process (Figure 3a). The midpoint of the transition, T_m, is lowered upon addition of acid, and the plot of T_m versus pH is consistent with earlier observations by Hartley (1969). Figure 3b shows the corresponding van't Hoff enthalpies plotted versus T_m, obtained from thermal transitions at pH 0.3–6 according to eq 4. The change in heat capacity, ΔC_p, obtained from the slope of this plot (eq 6) is 1.88 ± 0.7 kcal/mol deg. Figure 4 shows the free energy of

Table 1: Characteristics of the Acid Denaturation of Barnase at Varying Ionic Strength

ionic strength (mM)	MP_{eq}[a]	m_{eq}[b]	$pH_{\Delta G=0}$[c]	ΔQ at $pH_{\Delta G=0}$[d]
10	2.36 ± 0.02	4.0 ± 0.5		
50	2.19 ± 0.01	3.5 ± 0.1	2.12	3.3
200	2.05 ± 0.01	2.7 ± 0.15		
600	1.70 ± 0.1	2.0 ± 0.5	1.62	2.0

[a] pH at the midpoint for the acid transition at 25 °C, obtained from titration data using eq 4. [b] Number of protons taken up during the acid transition, obtained from titration data using eq 4. [c] pH where $\Delta G_{D\text{-}N}$ = 0, obtained from thermal denaturaion experiments (Figure 3). [d] The difference in degrees of protonation (mol of H^+/mol of protein) between the native and the denaturated states at $pH_{\Delta G=0}$, obtained from thermal denaturation data ($\Delta Q_{D\text{-}N}$, Figure 5).

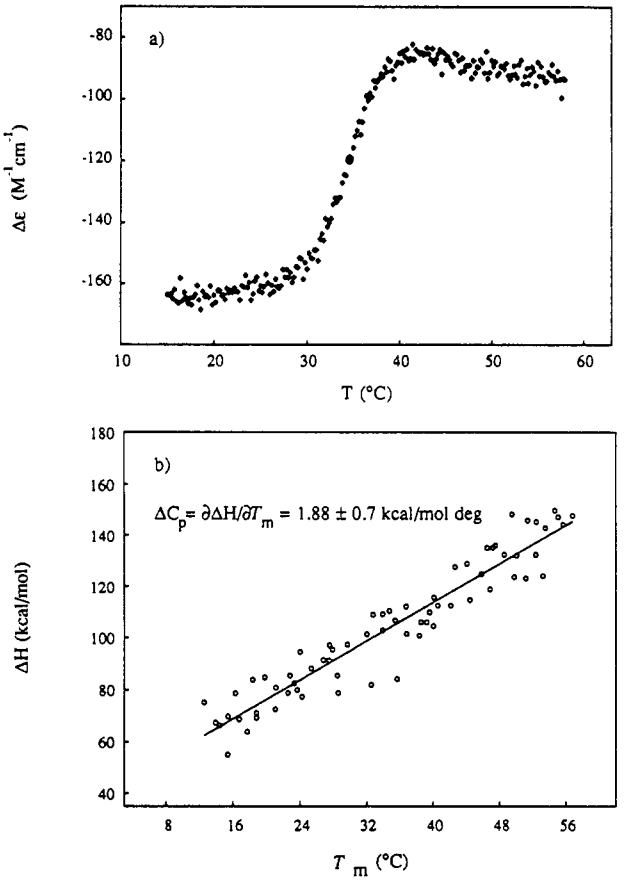

FIGURE 3: (a) Thermal denaturation of barnase monitored by CD at 230 nm, pH = 2.6. $\Delta H(T_m)$ and T_m are obtained from the thermal transition by nonlinear regression using eq 11. By this procedure, T_m is estimated with high precision whereas the values of $\Delta H(T_m)$ are subject to considerable scatter. (b) van't Hoffs enthalpies of the thermal transitions, obtained at different pH values and plotted versus T_m. To reduce scatter when extrapolating the free energy of unfolding from T_m to 25 °C (eq 7), a linear function was fitted to the plot and, in turn, this function was used to give the values of $\Delta H(T_m)$ and ΔC_p in eq 7.

unfolding at 25 °C, calculated by eq 4 and plotted versus pH under conditions of high and low ionic strength. The observed pH dependence of the free energy at I = 50 mM is in accord with recent observations by Pace et al. (1992), and the values obtained for $\Delta G_{D\text{-}N}$ (about 10 kcal/mol at pH 6) are in good agreement with data from scanning calorimetry (Martinez et al., 1994; C. M. Johnson, unpublished data from this lab), although they appear slightly higher than those determined by urea denaturation experiments, $\Delta G_{D\text{-}N}$= 8.8 ± 0.14 kcal/mol at pH 6.3 (Clarke & Fersht, 1993). Data obtained at I = 600 mM show that barnase is stabilized in the presence of

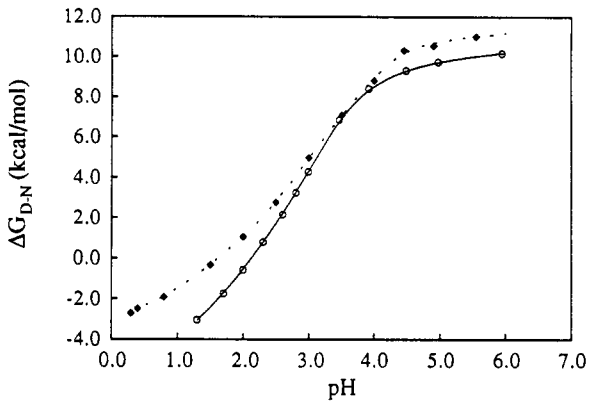

FIGURE 4: pH-dependent stability of barnase, $\Delta G_{D\text{-}N}$, and its dependence on ionic strength. I = 50 mM (○) and I = 600 mM (◆). The data set is obtained from thermal transitions according to eqs 9 and 10.

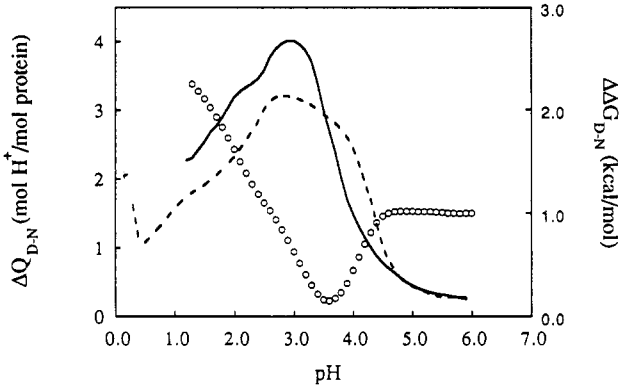

FIGURE 5: (Left y-axis) The lines represent the difference in bound protons, $\Delta Q_{D\text{-}N}$, between the native and the denatured state of barnase as a function of pH. I = 50 mM (—) and I = 600 mM (- - -). $\Delta Q_{D\text{-}N}$ is calculated from denaturation data (eq 11), using the fitted function in Figure 4. (Right y-axis) The circles (○) represent the corresponding difference in stability, $\Delta\Delta G_{D\text{-}N}$ = $\Delta G_{D\text{-}N}$ (I = 600 mM) − $\Delta G_{D\text{-}N}$ (I = 50 mM) (cf. Figure 4).

salt (Figure 4). In contrast, oxidized myoglobin (Friend & Gurd, 1979) and β-lactamase (Goto & Fink, 1989) have been observed to be destabilized by salt at acidic pH values. However, $\Delta G_{D\text{-}N}$ for barnase is determined to 11 kcal/mol at pH 6. This value is, again, somewhat higher than the stability determined by urea denaturation under corresponding conditions, $\Delta G_{D\text{-}N}$ = 9.9 kcal/mol at pH 6.3 and I = 550 mM (Horovitz et al., 1991). Below pH 5, where the protein begins to be destabilized by the acid, the loss of stability per pH unit is greater at I = 600 mM than at I = 50 mM. As a consequence, the stability appears independent of ionic strength around pH 3.5 where the two $\Delta G_{D\text{-}N}$ plots reaches a similar value (Figure 3). Below this apparent isoenergetic point, the plots diverge again as the protein remains stabilized by the presence of salt at lower pH values. The slope of the $\Delta G_{D\text{-}N}$ plots in Figure 4, i.e., $\partial\Delta G/\partial$pH, is related to the charge difference between the native and the denatured state of the protein according to eq 11. The charge difference, $\Delta Q_{D\text{-}N}$, which is equivalent to the number of protons taken up during the unfolding transition, is calculated at each pH and plotted in Figure 5. As expected, $\Delta Q_{D\text{-}N}$ increases sharply below pH 4.5 where the denatured state of the protein starts to become protonated and, hence, more positively charged (see Discussion). At high ionic strength, however, the increase is faster, resulting in a larger charge difference around pH 4. This difference, $\Delta\Delta Q_{D\text{-}N}$ = $\Delta Q_{D/N}$ (I = 600 mM) − $\Delta Q_{D/N}$ (I = 50 mM), corresponds to about 1 H^+ at pH 4. A maximum of $\Delta Q_{D/N}$

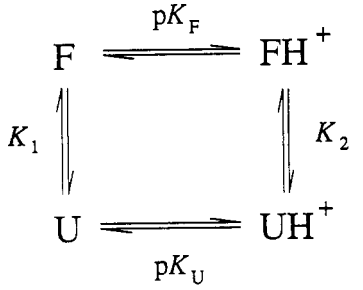

FIGURE 6: Thermodynamic cycle of a pH-dependent two-state transition of a protein containing only one protonable residue. F denotes the folded protein conformation and U the unfolded conformation (see the text for discussion).

is found around pH 3 at both high and low ionic strength (cf. Hartley, 1969). However, the total number of protons involved is larger at low ionic strength. Below pH 3, $\Delta Q_{D\text{-}N}$ is decreasing and at pH 0.3 is appears to be at least one residue that is still not protonated in the native state of the protein. It may be noticed that the lower number of $\Delta Q_{D\text{-}N}$ observed at I = 600 mM in this region, taken together with the increase around pH 4, can be interpreted in terms of an overall shift of $\Delta Q_{D\text{-}N}$ toward higher pH values. It is tempting to suggest that each step in the plot $\Delta Q_{D\text{-}N}$ versus pH corresponds to a residue titrating at the particular pH. At this stage, however, we are uncertain about the errors in the analysis, and a more detailed interpretation has to wait until full comparison with mutant protein is ready. Figure 5 also displays the difference between the two $\Delta G_{D\text{-}N}$ plots presented in Figure 4, i.e., $\Delta\Delta G_{D\text{-}N} = \Delta G_{D\text{-}N}$ (I = 600 mM) – $\Delta G_{D\text{-}N}$ (I = 50 mM). The difference plot is complementary to the $\Delta Q_{D\text{-}N}$ plots; between pH 4.5 and 3.5 the difference in $\Delta G_{D\text{-}N}$ is decreasing since the barnase is more rapidly destabilized at I = 600 mM than at I = 50 mM; at pH $\approx$ 3.5 $\Delta\Delta G_{D\text{-}N}$ = 0; and at lower pH values the protein becomes increasingly stabilized in the presence of salt.

DISCUSSION

To appreciate qualitatively the variation of the stability of proteins with pH, it is useful to consider a two-state transition of a hypothetical protein containing only one ionizable residue (Figure 6), for example an Asp, surrounded by permanent charges. In the unfolded state, where the peptide chain is highly open and solvent exposed, the spatial separation of the residues is maximized and residue–residue interactions are effectively screened by the intervening water. Therefore, the pK_a value of the Asp in the unfolded state (pK_U) is likely to be similar to that for the isolated peptide in water, about 4. In the folded conformation of the protein, on the other hand, where the Asp has been transferred into a specific site and interacts with other residues the pK_a value may well be shifted from pK_U. The factors that contribute to this shift are of electrostatic origin and may be rationalized as follows (Bashford & Karplus, 1990): (1) The interaction of the Asp with other ionized residues in the protein. Neighboring positive charges will tend to decrease its pK_a and while other negative charges will favor an increase. (2) The interaction with partial charges *other* than ionizable groups. These partial charges are the dipoles found in peptide bonds, polar residues, and bound water. It also includes dipole moment induced by larger structural elements, such as helices. (3) The desolvation effect, which arises from the energetically unfavorable process or transferring a charged group from a "good solvent", as water, to the hydrophobic interior of the protein. The desolvation penalty will favor burial of the neutral form of the residue and, hence, increase the pK_a of an Asp. If the residue is surface-exposed, it will be affected only marginally. Experimental observations reveal that the net pK_a shift for most residues is relatively small, often within one pH unit. In some cases, however, the pK_a values are found to be highly perturbed: For example, in native T4 lysozyme where Asp 70 appears to titrate below pH 1.5 (Anderson et al., 1990) and in hen egg-white lysosyme where the pK_a of Glu 35 is elevated 2 units to 6.1 (Kurimatsu & Hamaguchi, 1980).

If, accordingly, we assume that the pK_a value of the Asp in the folded state of our hypothetical protein is shifted down to, say, 1 (pK_F = 1), the equilibrium constants in the thermodynamic cycle in Figure 6 are related by

$$K_2 = K_1\left(\frac{10^{-pK_F}}{10^{-pK_U}}\right) = K_1 10^{-(pK_F - pK_U)} = K_1 10^{-3} \qquad (12)$$

It appears that the maximum pH-induced destabilization of the protein is determined by the shift of the pK_a value upon folding of the peptide chain, i.e., pK_F–pK_U. The corresponding pH dependence of the system falls into three regions, and its behavior is easily illustrated by studying these regions separately. At pH values higher than pK_U the residue is ionized in both the native and the denatured state (ΔQ = 0), the equilibrium is determined by K_1, and according to eq 11 $\partial\Delta G/\partial$pH = 0. At pH values lower than pK_U but higher than pK_F the Asp is protonated (neutral) in the denatured state but still ionized in the native protein, ΔQ = 1, and eq 11 gives $\partial\Delta G/\partial$pH = 1.36. Finally, at pH values below pK_F both states are neutral (ΔQ = 0), the equilibrium determined by K_2, and, again, $\partial\Delta G/\partial$pH = 0. If the thermodynamic cycle in Figure 6 is generalized to include n ionizable residues, it can be shown that the effect of each residue upon ΔG(pH) is additive. It must be realized, however, that the introduction/titration of a charged residue will perturb electrostatically the pK_a values of all other ionizable residues in the protein. As a consequence, these pK_a values are no longer constant but vary with pH [for a more extensive discussion of pH-dependent stability and coupled titrations, see Yang and Honig (1993)].

The pH-dependent stability of barnase (Figures 4 and 5) may now be interpreted analogously. At pH values above 4.5 (up to pH 6.5), $\Delta Q_{D\text{-}N}$ is small and, hence, the stability changes little with pH. Around pH 4, where the denatured state becomes more protonated than the native state, the protein starts to become destabilized. The charge difference, $\Delta Q_{D/N}$, reaches a maximum around pH 3 where the denatured state is likely to be fully protonated, whereas the native state still protects four ionized residues from protonation. At lower pH values, however, some of these protected residues become protonated as well and $\Delta Q_{D\text{-}N}$ decreases. It is interesting that at pH 0.3 there appears to be at least one residue which is still not protonated in the native conformation of the protein. The corresponding pK_a value for this group is thus lower than 0.3. At such extremes of pH, however, it is possible that also the peptide backbone starts to become protonated, which here may complicate the interpretation of our results.

Since the denatured protein seems to titrate around pH 4, it appears plausible that salt-induced changes in $\Delta Q_{D\text{-}N}$ which occur at much lower pH values can be assigned to the titration behavior of the native state. Accordingly, the salt-induced decrease of $\Delta Q_{D\text{-}N}$ observed below pH 3.5 (Figure 5) is explained by facilitated protonation of the native state. This interpretation is also consistent with earlier reports from direct titrations of the native states of ribonuclease and lysozyme (Tanford & Roxby, 1972). In general, the presence of anions is expected to screen electrostatic interactions between charges

within the protein. The effect could be caused by changes in the solvent dielectric, by Debye–Hückel screening, or by preferential ion binding to the protein (Goto et al., 1990). It is even possible that the titration properties are affected indirectly by chaotropic effects, i.e., salt-induced changes of the water structure. In the present study, we can conclude only summarily that the salt decreases the unfavorable intramolecular repulsion between positive charges and, hence, contributes to raise the pK_a values of the protein.

The salt-induced increase of $\Delta Q_{D\text{-}N}$ around pH 4, on the other hand, needs some further consideration. We start by examining the possible effects of aggregation/association of the denatured state. If such aggregation takes place in the transition region, where both the native and denatured state are present under equilibrium conditions, the resulting decrease in the concentration of denatured monomers would drive the equilibrium from the native state toward aggregates. Consequently, the transition midpoint would be shifted toward lower temperatures (or higher pH) and $\Delta G_{D\text{-}N}$ underestimated. If, for example, the aggregation is dependent only on ionic strength, and not on pH or temperature, the offset of $\Delta G_{D\text{-}N}$ would be constant and, hence, $\Delta Q_{D\text{-}N}$ unaffected throughout the pH range. To account for the increase in $\Delta Q_{D\text{-}N}$, the extent of aggregation can not be constant throughout the pH range (which would offset $\Delta G_{D\text{-}N}$ but not effect $\Delta Q_{D\text{-}N}$) but has to occur only below pH 4, the pH around which $\Delta G_{D\text{-}N}$ is observed to drop more rapidly in the presence of salt (cf. Figures 4 and 5). Such behavior, however, is unlikely. Conversely, aggregation is expected to be more pronounced above pH 4, where the net charge of the denatured state is comparatively low, rather than below pH 4, where the fully protonated denatured protein has a high positive charge and, hence, counteracts aggregation by intermolecular repulsion. This would, in fact, lead to an underestimate of $\Delta Q_{D\text{-}N}$, a phenomenon observed under similar conditions with CI2 (unpublished results). In several other studies, however, extremes of pH and salt have been found to give rise to partially structured denatured states populated under equilibrium conditions [for review, see Ptitsyn (1992)]. What would be the effect on the $\Delta G_{D\text{-}N}$ plot (Figure 4) of such a salt-induced change of the acid-denatured state? It was noted above that $\Delta G_{D\text{-}N}$ represents the difference in free energy between the native and the denatured state, and, consequently, the pH dependence of $\Delta G_{D/N}$ represents the pH dependence of this difference. The way $\Delta G_{D\text{-}N}$ is measured, however, can under some circumstances give misleading results. For example, under conditions where the protein is stable, the thermal transition takes place at high temperatures, here around 60 °C at pH 6. At this temperature, any partially structured species that constitutes the denatured state at 25 °C could be substantially destabilized and unfolded. The observed high-temperature transition may, therefore, involve a *different*, more unfolded, denatured state, and, if so, the stability derived from this transition may well be higher than the actual difference in free energy between the native state and the partially structured species. At lower pH values, however, where the thermal transition takes place around 25 °C, the partially structured species may indeed constitute the denatured state, and, in this case, the observed stability will correspond to the stability between the native state and the partially structured species. Under such circumstances, the stability as measured by thermal denaturation will refer to different denatured states throughout the pH range, and, hence, the free energy difference between these denatured states will be embodied in the pH dependence of $\Delta G_{D\text{-}N}$. That is, the free energy of unfolding will display variations with pH that result from effects other than $\Delta Q_{D\text{-}N}$, and, consequently, eq 11 is no longer valid. Following this reasoning, it could be suggested (Figure 5) that a change of the denatured state of barnase takes place around pH 4 at I = 600 mM, causing an apparent destabilization of the native state of about 1 kcal/mol. The CD spectrum obtained for barnase at high ionic strength (Figure 1) might then represent such a compact denatured species, or complexes thereof. It should be noted, however, that our results reveal two-state transitions at all pH values, which is against the idea of a gradual change of the denatured state. In addition, a change of the denatured state might have been seen as a curvature of ΔC_p (Figure 3b), if not exerted by changes in $\Delta H_{D\text{-}N}$ so by T_m. Unfortunately, the data are too scattered to allow any detailed analysis of this kind. The nature of the acid/thermally denatured state is being further investigated in a kinetic study of the acid denaturation of barnase (Oliveberg and Fersht, unpublished results).

As implied above, a more general interpretation of our observations is that the pK_a values of, not only the native state, but also of the *denatured state* are increased in the presence of salt. If so, it follows that the denatured state is not an extended polypeptide chain, with residues isolated from one another by the solvent, but it involves some degree of intramolecular electrostatic interactions. These interactions may reflect some degree of compactness or the presence of residual structure. The salt then decreases the electrostatic free energy of the denatured state (which may well result in an increased compactness of this conformation) and, hence, contributes to an apparent destabilization of the native state. By classical electrostatics, this effect is predicted to become increasingly pronounced with increased net charge of the denatured state. At pH values around 6, where the acidic residues are still ionized, the net charge of barnase is +3, and when the denatured state titrates around pH 4, the net charge is rapidly increased to +16, consistent with the observed drop in $\Delta\Delta G_{D\text{-}N}$ in this pH region (Figure 5). The idea of charge repulsions within the denatured state is further supported by preliminary stability calculations based on titration data from the native state of barnase (unpublished results): The pH for the transition midpoint of barnase at 25 °C (cf. Figure 2) can be reproduced only if the average pK_a value for the acidic residues in the denatured state is assumed to be around 3.6. That is, significantly lower than the pK_a values determined for denatured state in the presence of 6 M guanidine hydrochloride (Nozaki & Tanford, 1967; Roxby & Tanford, 1971) or the pK_a values for the isolated amino acids ($pK_a^{Asp} \approx 3.9$ and $pK_a^{Glu} \approx 4.3$).

The discrepancy, however, is readily understood upon closer examination: As pointed out above, the denatured (hydrophobic) state in a fairly "good solvent" as 6 M guanidine hydrochloride is likely to have properties different from those in pure water (Dill & Shortle, 1991). By analyzing how a particular property of a protein varies under such extreme denaturing conditions, estimates of the property can be made by extrapolating back to physiological conditions. This is routinely done in stability measurements, where the denaturant dependence of the equilibrium constant is analyzed and extrapolated back, either by the use of m values in urea/guanidinium hydrochloride experiments (Pace, 1986) or, as in this study, by the use of ΔC_p and $\Delta H_{D\text{-}N}(T_m)$. In contrast, the pH titrations of the denatured state have (so far) been carried out at only *one* concentration of denaturant and, consequently *not* extrapolated back to physiological conditions.

In this study we are indeed subject to the same problem. The results of this study reflect, in practice, the titration behavior of the protein at temperatures ranging from 60 °C at pH 5 to about 15 °C at the lowest pH values. It may be argued, however, that the thermally denatured state more resembles the reference (acid) denatured state (Figure 1) than does that unfolded by 6 M guanidine hydrochloride.

In summary, our results imply the presence of repulsive charge interactions within the protonated thermally denatured state. Upon addition of salt, this state is stabilized relative to the native protein by at least 1 kcal/mol at pH values below 4.

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