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pK_A Values of Carboxyl Groups in the Native and Denatured States of Barnase: The pK_A Values of the Denatured State Are on Average 0.4 Units Lower Than Those of Model Compounds

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ABSTRACT: We have determined the pK_A values of the 12 carboxyl residues in the native and denatured state of barnase by a combination of thermodynamic measurements on mutants of charged residues and NMR titration data. The pK_A values of the 11 residues titrating under folding conditions (above pH 2.2) were determined by two-dimensional ^1H NMR. The pK_A value of the remaining residue, Asp 93 which forms a salt link with Arg 69 and titrates at much lower pH values, was determined by changes in the pH dependence of the stability of the protein upon mutation to Asn: pK_A^{Asp93} at low ionic strength (50 mM) and pK_A^{Asp93} at high ionic strength (600 mM). The overall titration of the native state is nonideal, and the protein retains fractionally ionized residues other than Asp 93 throughout the experimental pH range of 0.2−6.3. Protonation events taking place at pH values below 2 were further characterized by the pH dependence of the unfolding kinetics of wild-type and charge-mutant proteins. By comparing the observed pH dependence of the protein stability with that calculated from the pK_A values for the native protein, we demonstrate that the pK_A values of the denatured state are significantly lower than those reported for model compounds: the pK_A values of the denatured state appear on average 0.4 units lower than previous estimates in the presence of chemical denaturant. The results have direct implications for calculations of the energetics of proton equilibria and suggest that the acid/thermally denatured state is not an extended coil where the residues are isolated from one another by the intervening solvent but is compact and involves intramolecular charge repulsion.

Determination of the H^+-titration behavior of proteins and its coupling to protein stability forms a basic link in the understanding of electrostatic interactions in biomolecules. The transfer of an ionizable residue, from a loosely constrained solvent-accessible position in the denatured conformation, into a specific site in the folded protein is in most cases accompanied by a pK_A shift. The electrostatic factors that contribute to this shift have been the subject of intense theoretical interest (Tanford & Kirkwood, 1957; Stigter & Dill, 1989; Bashford & Karplus, 1990; Warshel & Åqvist, 1991; Antosiewicz et al., 1994) and may be rationalized as follows: (1) The interaction of the titrating group with other ionized residues in the protein—neighboring positive charges will tend to decrease its pK_A and negative charges will lead to 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 and include also 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 water to the hydrophobic interior of the protein. In practice, titration data from folded proteins show that a majority of the pK_A shifts are relatively small (Haruyama et al., 1989; Dyson et al., 1991; Forman-Kay et al., 1992; Inoue et al., 1992; Oda et al., 1994), but in some cases the pK_A values are highly perturbed. For example, in chymotrypsin, the pK_A value of the α-NH group of Ile 16 is shifted from 7.85 to 9.96 because of a salt link with Asp 194 (Fersht, 1972), in T4 lysozyme the salt-bridge partners Asp 70 and His 31 are found to have pK_A values which are shifted about 3 units to ~0.5 and 9.1, respectively (Andersson et al., 1990), and in ribonuclease HI, Asp 148 is still fully ionized at pH 2 (Oda et al., 1994).

It has been demonstrated that the pK_A values of residues in proteins which have been denatured with strong chemical denaturants like guanidine hydrochloride or urea are similar to those of isolated amino acid model compounds (Nozaki & Tanford, 1967; Tanford & Roxby, 1972). The results suggest that the denatured state under these conditions is significantly expanded and does not involve any intramolecular interactions (Nozaki & Tanford, 1967; Tanford & Roxby, 1972). In contrast, the acid/thermally unfolded state of barnase shows slightly anomalous titration properties, which reveal that, in the absence of chemical denaturants, the denatured conformation is more compact or involves elements of residual structure (Oliveberg et al., 1994). The presence of such interactions in the acid-denatured state of barnase is also supported by a recent structural analysis by NMR (Arcus et al., 1994). This apparent variation of the denatured state with denaturant is in accord with a wealth of other studies, which report marked structural/spectroscopic differences between denatured states induced by high or low temperature (Privalov et al., 1989; Griko et al., 1994), chemical denaturant (Kuwajima et al., 1976; Neri et al., 1992), or extremes of pH (Myer, 1968; Goto & Fink, 1989) [for a general review on denatured states, see Dill and Shortle (1991)]. In particular, combinations of low pH and high

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ionic strengths have been demonstrated to give rise to partially structured, compact denatured conformations, so-called "molten globule states" (Dolgikh et al., 1981; Kuwajima, 1989; Ptitsyn, 1992).

We have reported a salt effect on the titration properties of the acid/thermally denatured state of barnase, suggesting that its pK_A values are lowered relative to those of model compounds (Oliveberg et al., 1994). As similar results have been obtained also with another protein, chymotrypsin inhibitor 2 (Y. J. Tan, this laboratory, unpublished results), it is tempting to speculate that intramolecular charge interactions constitute a general feature for denatured proteins in pure water (cf. Stigter & Dill, 1990), especially since denatured conformations appear compact in the absence of chemical denaturants (Privalov et al., 1986), whose action is to facilitate the solvation of hydrophobic residues [see, for example, Dill and Shortle (1991) and references therein].

In the present work, we have extended our thermodynamic analysis on the titration behavior of barnase by determination of the titration curves of individual carboxyl groups of the native protein, using two-dimensional NMR and a thermodynamic/mutational approach. The titration data, taken together with the pH dependence of the energetics, enable us to quantify more precisely the previously observed pK_A shifts of the denatured state. These new pK_A values are essential for calculations of the energetics of ionization equilibria, which are based on theoretical estimates of electrostatic interactions in the native protein, since the values based on model compounds will result in an overestimate of the pH dependence of the protein stability. In addition, we report about nonideal titrations of the native protein, as well as on the properties and salt effects on the Asp 93–Arg 69 semiburied salt bridge, which becomes protonated at pH values only below 1.

Barnase (EC 3.1.27.3) is a small ribonuclease from *Bacillus amyloliquefaciens*, which consists of 110 amino acid residues, 12 of which 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 is demonstrated to proceed with the formation of a transient intermediate (Bycroft et al., 1990a; Matouschek et al., 1990; Fersht, 1993). Under equilibrium conditions, however, the occupancy of this intermediate is small and the unfolding transition displays a typical two-state behavior.

MATERIALS AND METHODS

Materials. Site-directed mutagenesis was carried out by standard procedures (Sayers *et al.*, 1988) using a kit from Amersham. Wild-type and mutant barnase were overexpressed and purified from *Escherichia coli* (Serrano & Fersht, 1989). The purified protein was dialyzed several times against distilled water, flash frozen, and stored in liquid nitrogen. Protein used for the preparation of NMR samples was lyophilized.

Thermal Unfolding Experiments. The buffers used in the thermal unfolding experiments were 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 reversible thermal denaturation of wild-type and mutant barnase was monitored by circular dichroism (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. The observed unfolding transition is independent of the wavelength used for analysis, with identical results being obtained from CD measurements at 210, 220, 230, and 280 nm, fluorescence spectroscopy with excitation at 280 nm, and differential scanning calorimetry (cf. Sanz & Fersht, 1993). CD measurements at 230 nm were chosen to allow the use of dilute samples and so minimize the possibility of any aggregation of the denatured protein. The low protein concentrations (1 μM) required the use of a cuvette with long path length (20 mm). The temperature in the cuvette was automatically increased from 3 to 80 °C with a rate of 50 degrees per hour, and the ellipticity was recorded every 0.2 °C. The experimental data, i.e., plots of ellipticity (θ) vs temperature (T), were fitted with the data analysis program Kaleidagraph (Abelbeck Software) using eq 1 (Oliveberg et al., 1994).

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

where θ is the observed ellipticity, $\alpha_N + \beta_N T$ is the ellipticity of the native state, $\alpha_D + \beta_D T$ is the ellipticity of the denatured state, and $\Delta G_{D\text{-}N}(T)$ is given by (Privalov, 1979):

$$\Delta G_{D-N}(T) = \Delta H_{D-N}(T_m)(1 - T/T_m) - \Delta C_p^{D-N}[(T_m - T) + T \ln(T/T_m)] \quad (2)$$

where $\Delta C_p^{D\text{-}N}$ is the difference in heat capacity between the native and the denatured state, and $\Delta H_{D\text{-}N}(T_m)$ is the change in enthalpy at the transition midpoint, T_m. For a more extensive discussion about the stability measurements see Oliveberg et al. (1994).

The parameters $\Delta H_{D\text{-}N}(T_m)$ and T_m were obtained by eq 1 at a set of pH values between 6.3 and 0.2 and are linearly correlated with a slope which is $\Delta C_p^{D\text{-}N}$, as is usually found (Privalov, 1979). In this kind of experiment, T_m can be determined with high accuracy (±0.2 °C) whereas the value of $\Delta H_{D\text{-}N}(T_m)$ is often subject of a considerable error (±10%). Therefore, to reduce data scatter, a linear function was fitted to the plot of $\Delta H_{D\text{-}N}(T_m)$ vs T_m, $\Delta H_{D\text{-}N}(T) = \Delta H^0_{D\text{-}N} + \Delta C_p^{D\text{-}N} T$, where $\Delta C_p^{D\text{-}N} = 1.88$ kcal/mol K (Oliveberg et al., 1994), and this function, which represents the "mean value" of $\Delta H_{D\text{-}N}(T_m)$ at each temperature, was subsequently used to replace the enthalpy term in eq 2. Knowing the midpoint for the thermal transition, eq 2 allows the calculation of $\Delta G_{D\text{-}N}$ at 25 °C. Finally, the pH dependence of the stability was obtained by plotting the $\Delta G_{D\text{-}N}$ values, calculated at 25 °C by eq 1, versus pH (Figure 1). The formate and acetate buffers used in the region where the carboxylate groups of the protein titrate have negligible enthalpies of ionization and so their pK_A values do not change with temperature, as is also expected for the Asp and Glu residues of the protein.

Unfolding Kinetics Studied by Stopped-Flow pH Jump. The unfolding reaction of barnase was induced by rapid mixing into acid conditions using an Applied Photophysics DX-17 MV stopped-flow spectrofluorimeter. The instrument was connected to a thermostated water bath, which maintained the temperature of the reservoir syringes and the

observation cell to within ±0.1 °C. The instrument was set up to mix the protein solution with HCl/KCl solution in a volumetric ratio of 1:1, yielding a final protein concentration of 8.0 μM, a final pH between 0.2 and 2, and an ionic strength of either 50 or 600 mM. The kinetics was monitored by fluorescence changes. Excitation was at 280 nm, and the emission was collected at wavelengths greater than 315 nm using a cut-off filter. The first-order rate constants were obtained by fitting a series of exponential functions to the experimental time courses, using the software Kaleidagraph, and the logarithm of these rate constants are plotted versus pH for further analysis (Figure 4).

Relationship between the pH Dependence of the Protein Stability and the Difference in Degree of Ionization between the Native and the Denatured State. It has been demonstrated from the law of mass action 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)

$$\frac{\partial(\Delta G_{D-N})}{\partial(\mathrm{pH})} = 2.3RT[Q_D(\mathrm{pH}) - Q_N(\mathrm{pH})] = 2.3RT\Delta Q_{D-N}(\mathrm{pH}) \quad (3)$$

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. In order to calculate the derivative of free energy ($\partial\Delta G/\partial$pH), the stability plots were first fitted to a predefined "smooth-function" in the Kaleidagraph software (Figure 1), and then $\partial\Delta G/\partial$pH was derived analytically from this smooth-function. The smooth-function was also used for the subtraction of the $\Delta G_{D\text{-}N}$ plots from one another (Figure 2).

Knowing the pK_A values for the denatured and native protein, respectively, the ionization state (Q) for these conformations, and thereby $\Delta Q_{D\text{-}N}$, can be directly calculated at any pH according to

$$\Delta Q_{D-N}(\mathrm{pH}) = Q_D(\mathrm{pH}) - Q_N(\mathrm{pH}) = \sum_{i=1}^{n}\frac{10^{(\mathrm{p}K^i_D-\mathrm{pH})}}{(1+10^{(\mathrm{p}K^i_D-\mathrm{pH})})} - \sum_{i=1}^{n}\frac{10^{(\mathrm{p}K^i_N-\mathrm{pH})}}{(1+10^{(\mathrm{p}K^i_N-\mathrm{pH})})} \quad (4)$$

Equations 3 and 4 were used to compare the pH dependence of the experimentally observed stability with that calculated from the observed pK_A values (Figure 6).

Relationship between the pH Dependence of the Unfolding Kinetics and the Difference in Degree of Ionization between the Native and the Major Transition State. Equation 3 is general and applies not only to overall protein stability but to any free-energy difference in the folding pathway. The first-order rate constant for the unfolding process [k_u(pH)] may then be related to the free-energy difference between the native state and the major transition state ($\Delta G_{‡\text{-}N}$) according to transition state theory

$$\log k_u(\mathrm{pH}) = \log\frac{k_B T}{h} + \frac{\Delta G^{‡}(\mathrm{pH})}{2.3RT} \quad (5)$$

where the pH dependence of log k_u(pH) is directly proportional to the pH dependence of $\Delta G_{‡\text{-}N}$(pH),

$$-\frac{\partial \log k_u}{\partial\,\mathrm{pH}} = \frac{1}{2.3RT}\frac{\partial\Delta G^{‡}}{\partial pH} = \Delta Q_{‡\text{-}N}(\mathrm{pH}) \quad (6)$$

which, in turn, can be directly related to the difference in number of bound protons between the native state and the transition state $\Delta Q_{‡\text{-}N}$(pH) according to eq 3. It must be noted that the validity of eq 6 relies on the assumption that protons can be exchanged during the conformational events taking place between the native state and the transition state and, hence, that the native state and the transition state can interconvert without having the same degree of ionization. The exchange need not happen in the transition state itself but could occur in any intermediate conformation between the ground state and the transition state.

NMR Sample Preparation. NMR samples were prepared by dissolving lyophilized protein in 0.5 mL of 90% H_2O/10% D_2O or 90% 1 mM HCl in H_2O/10% D_2O to a final concentration of 2−4 mM. The pH was adjusted using 1−5 μL aliquots of a 250 mM HCl solution. The pH value for each sample was measured before and after NMR acquisition and, with exception of that at pH 5.9, none showed pH changes larger than ±0.05 units. All pH values refer to uncorrected meter readings. NMR spectra were acquired at a set of pH values between 5.9 and 2.2, with the lower limit dictated by the disappearance of signals from the folded protein. The chemical shifts were referenced to 0.07 ppm against the internal standard 3-(trimethylsilyl)-d_4-propanoic acid (TSP).

NMR Spectroscopy. NMR spectra were acquired at 30 °C with a Bruker AMX500 spectrometer equipped with a X32 computer. For each sample, a total correlation spectrum (TOCSY) (Bax & Davies, 1985) was recorded in the phase-sensitive mode using time-proportional phase incrementation (Redfield & Kuntz, 1975; Marion & Wüthrich, 1983) and a mixing time of 55 ms. A total of 2048 data points were recorded in t_2 with 400−512 t_1 increments and spectral widths of 8000 Hz in both dimensions. Either 8 or 16 scans per increment were collected. Data processing was carried out using the Bruker UXNMR software package. Time domain data were zero filled, and phase-shifted sine bell window functions were applied prior to Fourier transformation; the final matrix contained 2048 × 1024 real data points in F_2 and F_1, respectively.

NMR Resonance Assignment and Data Treatment. Proton resonances were assigned in the TOCSY spectra at pH 5.1 and 4.2, by reference to the existing complete ^{1}H assignment for barnase at pH 4.5 (Bycroft et al., 1990b). pH-dependent chemical shifts [δ^j(pH)] were then followed incrementally to pH 2.2. The experimental data were fitted to an ideal titration curve which describes the titration of a residue (i) in a constant electrostatic environment with one proton (eq 7) (cf. Shrager et al., 1972).

$$\delta^j(\mathrm{pH}) = \frac{\delta^{i,j}_{base} + \delta^{i,j}_{acid}10^{(\mathrm{p}K^i_N-\mathrm{pH})}}{(1+10^{(\mathrm{p}K^i_N-\mathrm{pH})})} \quad (7)$$

where $\delta^{i,j}_{base}$ is the chemical shift of the jth resonance associated with the unprotonated residue i and $\delta^{i,j}_{acid}$ the corresponding shift associated with the protonated residue

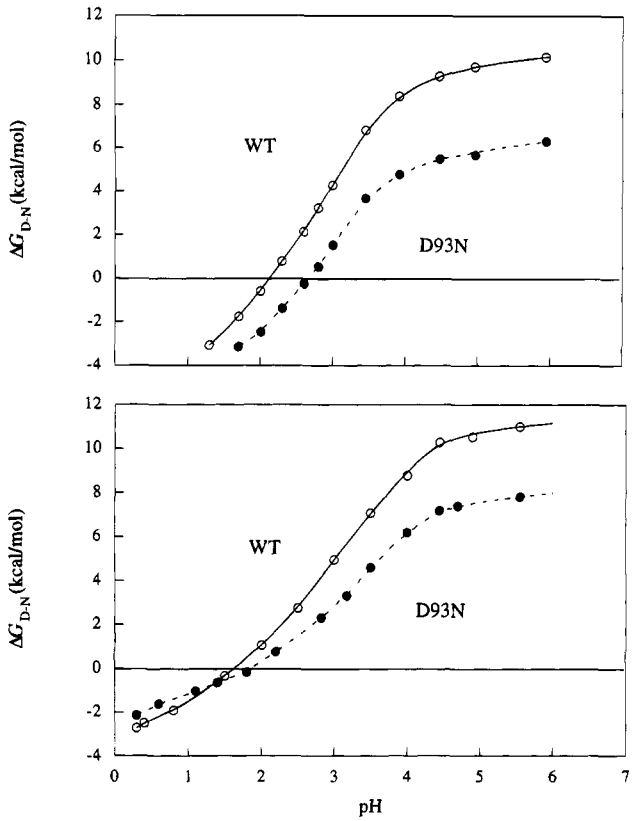

FIGURE 1: pH dependence of the stability of wild-type barnase (○) and the Asp→Asn 93 mutant (●), determined by thermal denaturation experiments. (Upper panel) μ = 50 mM. (Lower panel) μ = 600 mM. The lines show the fitted smooth functions which were used to represent ΔG_{D-N}(pH) when calculating ΔQ_{D-N}(pH) according to eq 3 (Figure 3), as well as the free-energy differences in Figure 2.

i. pK_N^i is the pK_A value of the *i*th residue in the native protein. For pH dependent resonances which show complex titration behavior, there are insufficient data points to fit confidently multiple titration curves, and these data are interpreted in a qualitative manner. Data were also fitted to a Hill equation that assumes there are n_H protons simultaneously titrating at each position. The mean value of n_H for all positions is 1.0, with the individual values being spread generally between 0.8 and 1.2. The pK_A values thus obtained are very similar in each case to those obtained from eq 7.

RESULTS

CD-Monitored Temperature Denaturation of WT Barnase and Its Mutant Asp→Asn 93. The pH dependence of the free energy of unfolding for WT barnase, ΔG_{D-N}^{WT}(pH), which has been described in detail in a previous report (Oliveberg *et al.*, 1994), is shown in Figure 1 [see also Hartley (1969) and Pace et al. (1992)]. In summary, ΔQ_{D-N}^{WT}(pH) derived from the stability data by eq 3 displays a rapid increase around pH 4 as the denatured state becomes protonated. The difference in ionization reaches a maximum value of about 4 (mol of H^+/mol of protein) at pH 3 and finally decreases at lower pH values as also the native state becomes protonated (Figure 3). The protonation of the native state, however, appears to be extended down to very low pH values so that at pH 0.2 (the lowest pH in the experimental range, μ = 600 mM) the value of ΔQ_{D-N}^{WT}(pH) is still about 1 (Figure 3). Upon addition of

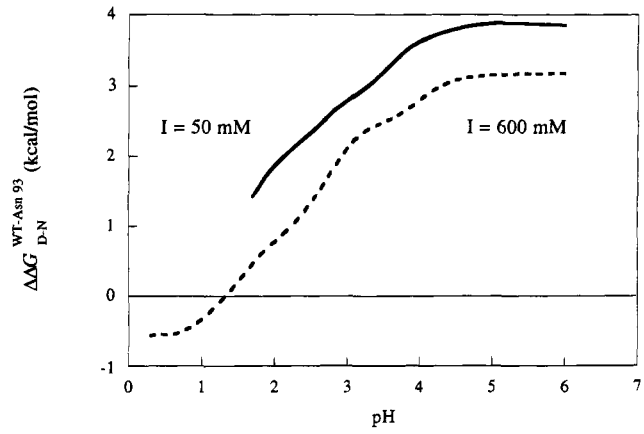

FIGURE 2: pH dependence of the free-energy difference between wild-type barnase and the Asp→Asn 93 mutant, derived from the data in Figure 1. (Solid line) μ = 50 mM; (broken line) μ = 600 mM.

salt, the titration of the denatured state as well as of the native state is shifted to higher pH values. The salt shows a net stabilizing effect on barnase at pH values above 4 (Figure 1), presumably exerted by charge screening within the positive clusters around the active site (Meiering et al., 1992).

There is no evidence of aggregation of denatured barnase at low ionic strengths. There is aggregation at high concentrations and high ionic strength. The melting temperature (T_m) in 600 mM KCl is independent of protein concentration below 20 μM, however, showing that the stability and titration properties of the protein are related to a monomeric form of the denatured state. Experiments were thus performed at 1 μM protein. The absence of aggregation under these conditions was corroborated by extensive kinetic studies of unfolding and refolding (unpublished results).

The thermal denaturation curve of Asp→Asn 93 shows a single symmetric transition typical for a two-state process, as was observed for wild-type protein. The value obtained for ΔC_p^{D-N} coincides precisely with that observed for the wild-type protein although the unfolding enthalpy of this mutant is somewhat lower than that observed for the wild-type protein (data not shown). The mutant protein is substantially destabilized at pH 6 but gains stability relative to the wild-type at lower pH values and becomes finally more stable than the wild-type at pH values below 0.7 (Figures 1 and 2). The salt effect on the stability of the mutant is less than that for wild-type, so that at μ = 600 mM the difference in free energy between the proteins is reduced (Figure 2).

It is evident by comparison of the titration behavior that a deviation in ΔQ_{D-N} starts to occur below pH 4.5, where the value for the wild-type protein becomes larger than that for the Asp→Asn 93 mutant (Figure 3). The difference reflects the titration of Asp 93 in the denatured state, which contributes to an extra ΔQ_{D-N} unit for the wild-type protein, but not for the mutant in which the "ionization state" of Asn 93 remains unchanged. Thus, the titration curve and pK_A value for Asp 93 in the denatured protein is given by $\Delta\Delta Q_{D-N}^{WT-Asn93}$(pH) = ΔQ_{D-N}^{WT}(pH) − ΔQ_{D-N}^{Asn93}(pH). It is seen in Figure 3 that $\Delta\Delta Q_{D-N}^{WT-Asn93}$(pH) appears to describe a nonideal titration curve, in particular at high ionic strength where its value never reaches one. This behavior which could well be due to the experimental error or inaccuracy in the derivation procedure makes assignment of a pK_A value by curve fitting unsatisfactory. By visual inspection, how-

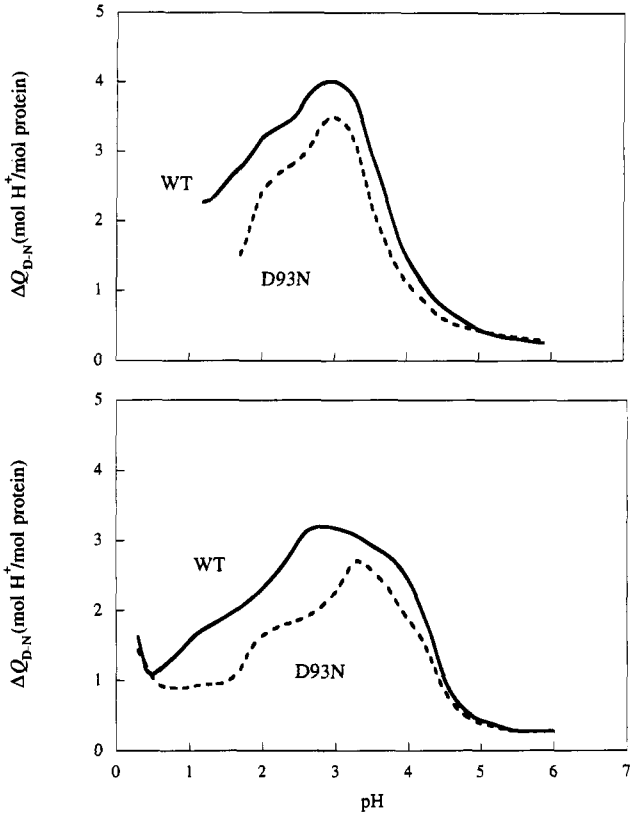

FIGURE 3: Difference in number of protons bound to the native and the denatured state, respectively, calculated from the stability data in Figure 1 according to eq 3. (Solid line) wild-type barnase; (broken line) the Asp→Asn 93 mutant. (Upper panel) μ = 50 mM. (Lower panel) μ = 600 mM. Deletion of Asp 93 decreases the pH dependence of $\Delta G_{D\text{-}N}$ between pH 5 and 0.5.

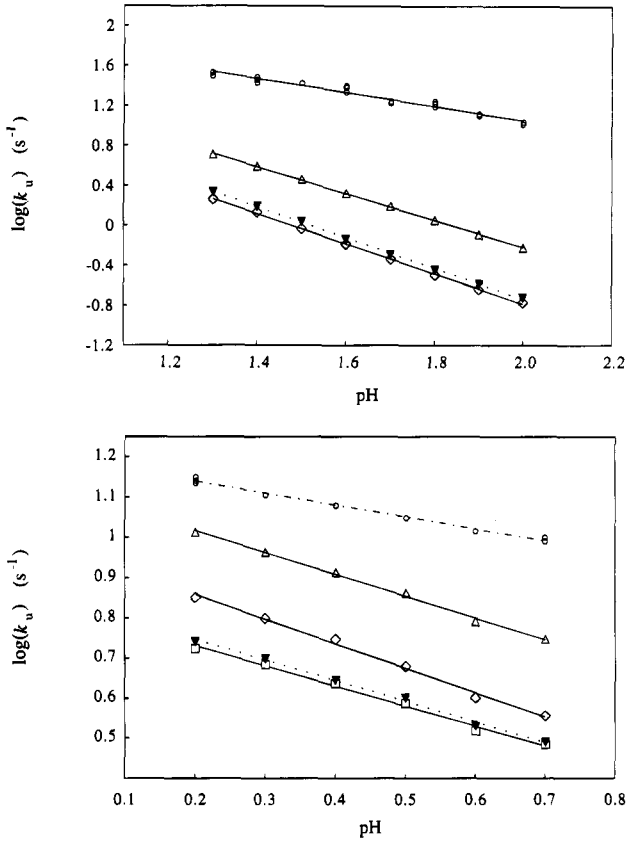

FIGURE 4: pH dependence of the unfolding rate constant for barnase at μ = 50 mM (top panel) and μ = 600 mM (bottom panel). Wild-type (▼), Asp→Ala 54 (△), Asp→Asn 54 (□), Glu→Gln 73 (◇), Asp→Asn 75 (○). The derivative of the lines which are fitted to the data are given in Table 1.

ever, it is clear that Asp 93 becomes protonated at about pH 4 in the denatured protein (Figure 3).

At lower pH values, $\Delta\Delta Q_{D\text{-}N}^{WT\text{-}Asn93}$(pH) starts to decrease, so that below pH 0.7, $\Delta Q_{D\text{-}N}^{WT}$(pH) ≈ $\Delta Q_{D\text{-}N}^{Asn93}$(pH) (Figure 3). The convergence of the wild-type and mutant values reflects the titration of Asp 93 in the native state. When the residue is fully protonated in the native state as well as in the denatured state, it no longer contributes to $\Delta Q_{D\text{-}N}$, and hence the wild-type protein and the Asp→Asn 93 mutant show the same degree of ionization in both the native and the denatured state. Since the protonation appears to be completed around pH 0.5, the pK_A value for Asp 93 in the native protein is about 1.5 at μ = 600 mM (cf. Figure 3) (Table 2). Interestingly, $\Delta Q_{D\text{-}N}$ increases below pH 0.5. The phenomenon is reproducible and unlikely to be a systematic error. An explanation may be that peptide bonds and amide groups start to become protonated in the denatured state at these extremely acid conditions. Another possibility is that low pH values and low temperatures induce conformational changes in the native or denatured protein which, in turn, affect the curvature of the free-energy profile.

pH Dependence of the Unfolding Rate Constants. The purpose of the time-resolved unfolding experiments was to obtain information about the ionization state of particular acidic residues in the native protein under denaturing conditions at very low pH values. The unfolding rate constants for wild-type barnase and its mutants (Asp→Ala 54, Asp→Asn 73, Asp→Asn 75, Asp→Asn 54, and Asp→Asn 93) are plotted versus pH at μ = 50 mM and μ = 600 mM in Figure 4. For simplicity, the pH dependence of log k_u was assumed to be linear in the narrow pH range of the experiments, pH 0.2−1 at μ = 600 mM, and pH 1.3−2 at μ = 50 mM. The values of $\Delta Q_{‡\text{-}N}$ obtained from the slopes of linear functions fitted to the unfolding data (eq 6) are shown in Table 1. With the exception of Asp→Asn 75, the mutants display a pH dependence similar to that of the wild-type protein. The similarity between mutant and wild-type kinetics suggest that the mutated carboxyl groups are fully protonated in the wild-type protein under these conditions and, therefore, do not contribute to $\Delta Q_{‡\text{-}N}$. The decreased pH dependence of Asp→Asn 75, on the other hand ($\Delta\Delta Q_{D\text{-}N}^{WT\text{-}Asn75}$(pH) ≈ 0.2 at μ = 50 mM and ~0.8 at μ = 600 mM, Table 1), shows that replacement of this carboxyl group is accompanied by a loss of fractional charge in the native protein. That is, Asp 75 is not yet fully protonated in the pH range of the unfolding experiments, pH 0.2−2. It must be pointed out, however, that not only do the titration properties of the truncated residue determine the pH dependence of the kinetics but also its interactions in the transition state (which may well be different from its interactions in the denatured state). In other words, if the titration behavior of a residue in the transition state is identical to that in the native state, the mutant protein will show the same pH dependence as the wild-type. With the acidic residues above, however, the interactions in the transition state are found to be substantially weakened [cf. ϕ-values in Matouschek et al. (1992)] and, hence, the observed kinetics representative of their titration behavior in the native state.

Titration of the Acidic Residues of Native Barnase As Measured by NMR. The proton resonances that are pH-dependent and for which $\Delta\delta \geq 0.1$ ppm in the pH range 5.9−2.2 are listed in Table 2 together with the pK_A value

Table 1: Thermodynamic and Kinetic Quantities of Barnase and Its Charge Mutants

	$\Delta G_{D\text{-}N}$[a] kcal/mol	midpoint[b] (pH, I = 50 mM)	midpoint[c] (pH, I = 600 mM)	$\Delta Q_{\ddagger\text{-}N}$[d] (pH 1.3−2.0)	$\Delta Q_{\ddagger\text{-}N}$[e] (pH 0.2−0.7)	pK_A[f]
Wild type	10.2 ± 0.2	2.12 ± 0.05	1.62 ± 0.05	1.54 ± 0.020	0.51 ± 0.018	
Asp→Ala 54	6.9 ± 0.2[h]			1.35± 0.011	0.54 ± 0.016	2.2 ± 0.3
Asp→Asn 54	7.6 ± 0.2[h]	2.4 ± 0.1[i]	1.89 ± 0.03[i]		0.50 ± 0.021	2.2 ± 0.3
Glu→Ala 73	7.7 ± 0.2[g]	2.35 ± 0.01[i]	1.92 ± 0.01[i]	1.50 ± 0.014	0.61 ± 0.024	2.1 ± 0.1
Asp→Asn 75	5.2 ± 0.2[i]	3.0 ± 0.1[i]	2.75 ± 0.07[i]	0.70 ± 0.040	0.29 ± 0.009	broad

[a] The free energy of unfolding at pH 6.3 and μ = 50 mM. The value for wild-type barnase is obtained from thermal unfolding experiments and the values for the mutant by subtraction of $\Delta\Delta G_{D\text{-}N}$ values obtained from urea denaturation experiments. [b,c] The pH for the midpoint of the unfolding transition at 25 °C, determined by fluorescence- or CD-monitored equilibrium experiments at constant ionic strengths of 50 and 600 mM, respectively. [d,e] The difference in degree of ionization (protonation) determined from the pH dependence of the unfolding kinetics (eq 6) under fully denaturing conditions at μ = 50 mM and μ = 600 mM, respectively. [f] The pK_A values of the acidic residues determined by NMR (see Table 2). [g] Data from Meiering et al. (1992). [h] Data from Serrano et al. (1992). [i] Data from Alain Tissot, Diplomarbeit this lab (1993).

Table 2: pH Dependence of Selected[a] Proton Resonances in Native Barnase

group[b]	residue (location)	H	pK_A[e] (error)	$\Delta\delta$	interaction[c]	residue (location)	H	pK_A[e]	$\Delta\delta$
A	Asp 22 (loop 1)	NH	3.3 (0.1)	0.17					
		CβH	3.3 (0.1)	0.18					
	Asp 44 (helix 3)	NH	3.6 (0.1)	0.12					
		CβH	3.35 (0.05)	0.19					
	Glu 29 (helix 2)	CγH	3.75 (0.05)	0.13					
	Glu 60 (loop 3)	NH	3.4 (0.1)	0.16					
		CγH	3.0 (0.1)	0.15					
B	Asp 8 (helix 1)	NH	3.1 (0.1)	0.30	N$^{\eta 2}$ Arg 110	Arg 110 (C-terminus)	CδH	3.3 (0.1)	0.27
		CβH	2.9 (0.1)	0.32					
		CβH	3.2 (0.2)	0.10					
	Asp 12 (helix 1)	CβH	3.8 (0.1)	0.16	N$^{\eta 1}$ Arg 110				
		CβH	3.5 (0.2)	0.17	N$^{\epsilon}$ Arg 110				
	Arg 110 (C-terminus)	NH	3.3 (0.1)	0.17					
C	Asp 54[d] (β1)	NH	2.2 (0.3)	0.17	NH Lys 27	Lys 27[d] (helix 2)	NH	2.1 (0.1)	0.34
	Asp 86 (β3)	CβH	4.2 (0.1)	0.31	NH$_2$ Asn 77	Asn 77 (loop 4)	CβH	3.9 (0.1)	0.12
	Asp 75 (β2)	NH	3.1 (0.2)	0.12	N$^{\eta 2}$ Arg 83, N$^{\epsilon}$ Arg 83	Arg 83 (loop 4)	N$^{\epsilon}$H	2.8 (0.1)	0.10
D	Glu 73[d] (β3)	NH	2.1 (0.1)	0.23	OH	Tyr 103			
	Asp 101[d] (loop 5)	CβH	2.0 (0.2)	0.13	NH Thr 105, OH Thr 105, OH Thr 99, NH Gln 104	Thr 105[d] (loop 5)	NH	2.3 (0.1)	0.86
	Asp 93 (β3−β4)		<2.0		N$^{\epsilon}$Arg 69, N$^{\eta 1}$Arg 69, N$^{\eta 2}$Arg 69, OH Ser 91	Arg 69 (loop 3)		<2.0	

[a] Only those resonances for which $\Delta\delta \geq 0.1$ are given in the table. Some resonances show a broad pH dependence, and this is reflected in a pK_A error of >0.1. This phenomenon is discussed in the text. [b] See Discussion and Figure 5. [c] The moiety and residue involved in hydrogen bonding or ion pairing with the carboxylate group. [d] In general the base line, δ_{acid}, is not well defined for these data. This will lead to a possible overestimation of the pK_A for the particular residue. [e] pK_A values were determined by a fit of the data to eq 7.

determined from a fit of the data to the theoretical titration curve (eq 7). The 12 acidic residues fall into four groups (Table 2 and Figure 5):

(A) Barnase contains four solvent-exposed acidic residues whose carboxyl groups are not involved in discrete hydrogen bonding or salt-bridge interactions. These are Asp 22, Asp 44, Glu 29, and Glu 60. For these four residues, the methylene protons adjacent to the carboxylate group are degenerate in the TOCSY spectra and show clear titration curves giving pK_A values of 3.3, 3.35, 3.75, and 3.4, respectively. For Asp 22, Asp 44, and Glu 60 the titration of the methylene protons is mirrored by the corresponding amide protons albeit with a broader pH dependence. For Asp 44 and Glu 60, this broader titration behavior results in marginally higher determined pK_A values (Table 2).

(B) Asp 8, Asp 12, and Arg 110 form a salt-linked triad in native barnase between the first helix and the C-terminus. This salt bridge is largely solvent exposed and has been previously identified to contribute only 0.3−1.0 kcal mol^{-1} to the stability of the native state (Horovitz et al., 1990). The titration curves for the protons of Asp 8, Asp 12, and Arg 110 show only moderately perturbed pK_A values and are likely to represent a mixture of the titrations of the two acidic residues along with that of the C-terminus (Table 2 and Figure 5).

(C) The four residues Asp 54, Asp 86, Asp 75, and Glu 73, which are clustered around the active site of barnase, are predicted theoretically to show coupled titrations (J. Warwicker, private communication). Evidence for this coupling is seen in the titration curves for Asp 54, Asp 75, and Asp 86 either through curves exhibiting multiple titrations or broad titration behavior.

Asp 54. The β protons of Asp 54 have $\Delta\delta$ < 0.1 ppm and are not included in Table 2 or Figure 5. The carboxylate group, however, makes hydrogen bonds with the backbone amide of Lys 27, and this amide proton should directly reflect the titration of Asp 54. Lys 27 NH shows a clear titration of large amplitude with a pK_A value of ≤2.1 (the baseline, δ_{acid}, is not defined here, and this may result in an overestimation of the pK_A; Figure 5). A second titration of smaller amplitude may be postulated from this curve with an approximate pK_A value of 4.2. These two titrations can also be clearly seen in the pH dependence of the amide resonance of Asp 54 (Figure 5).

Asp 86. The methylene protons of Asp 86 and its hydrogen-bonded counterpart Asn 77 show well behaved titrations (Figure 5). In contrast, the amide proton of Asp 86 shows a large amplitude change, 0.47 ppm, but poorly defined titration behavior, and this may reflect the coupling referred to above.

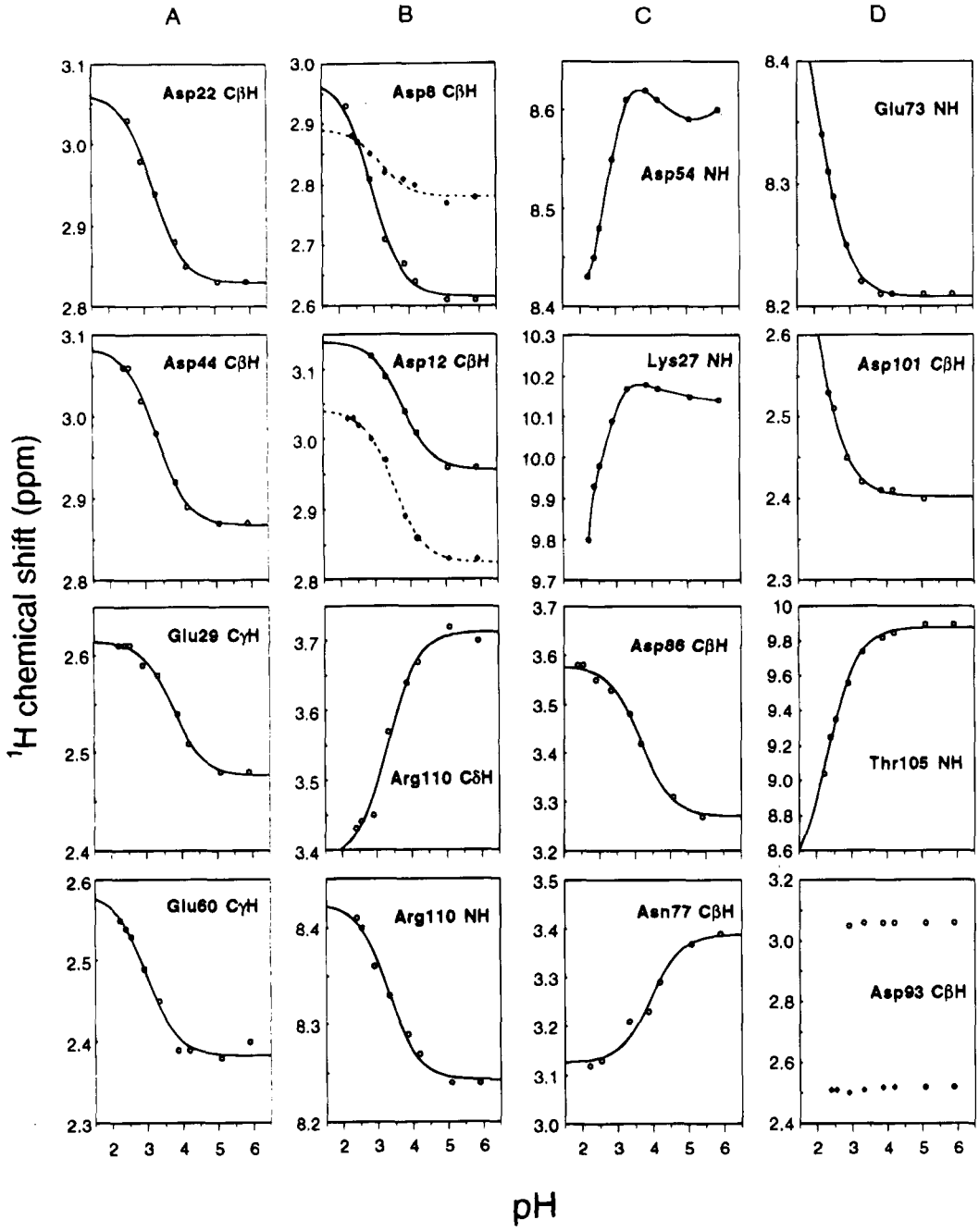

FIGURE 5: pH dependence of the chemical shifts for selected proton resonances in native barnase. Each column shows the titration curves of related residues (see text). For example, Asp 8, Asp 12, and Arg 110 (column B) are involved in a salt-bridge system linking the C-terminus with the first major helix of the native protein, and thus the titration of these residues may be coupled. With the exception of Asp 54 NH, Lys 27 NH, and Asp 93 CβH, all curves show a fit of the data to the theoretical titration eq 7. Asp 54 NH and Lys 27 NH show possible multiple titrations, and the data have been fitted with a smooth function (see Table 2 for pK_A values).

Asp 75. The ambiguous (coupled) pH dependence seen for Asp 86 NH is also seen for the amide proton of Asp 75. The β proton resonances for this residue are not seen in the TOCSY spectrum; however, clear evidence for the titration of this group is seen in the pH dependence of its hydrogen-bonded counterpart, Arg 83, whose N$^\epsilon$H proton shows a pK_A value of 2.8. No evidence for coupling is seen for Glu 73 and this residue is placed in group D.

(D) Both Glu 73 and Asp 101 (Thr 105) protons display sharp isolated titrations with pK_A values of $\leq$2.1 and $\leq$2.2, respectively. These titrations are concomitant with the unfolding of the protein.

The two residues involved in the semiburied salt bridge, Asp 93 and Arg 69 (Figure 7), show no changes in chemical shift over the pH range under scrutiny, implying a pK_A value of well below 2.0 for Asp 93.

In addition, there may be changes in proton chemical shift ($\Delta\delta$) that are not induced directly by ionization events that could also contribute to ill-defined titration behavior. These effects have been identified and discussed by Oda and co-workers in comparing the titration of side chain ^{13}C carbonyl chemical shifts with their corresponding ^{1}H titration curves (Oda *et al.*, 1994).

DISCUSSION

pH Dependence of Protein Stability. The stability of proteins in the presence of chemical ligands (denaturants) is related to the number of accessible ligand binding sites in

the native and denatured conformations, respectively (Tanford, 1968). Conformations of the protein with a large number of binding sites will be stabilized relative to those with fewer binding sites, and the degree of stabilization is determined by the concentration of denaturant. With weak-binding and less specific ligands like urea or guanidine hydrochloride, it is easy to visualize that an extended polypeptide chain provides more solvent-exposed binding sites than does the folded protein, and that the denatured state becomes the most stable conformation at sufficiently high concentrations of these denaturants. The same principle applies to the binding of protons. In this case, however, there are a few practical differences which are of advantage: (i) in contrast to other denaturants, the binding of protons is specific and the binding sites are known, i.e., ionizable residues; (ii) the binding of protons is comparatively strong and can be described by individual pK_A values which (often) can be determined experimentally. (iii) The pK_A values report on the specific properties of the binding sites and their environment (Bashford & Karplus, 1990). Accordingly, the pH dependence of a protein's stability can be precisely calculated if the number of conformations that are involved and their pK_A values are known (eqs 4 and 5) or, vice versa, information about the titration properties can be obtained from experimental observations of the stability (Yang & Honig, 1993; Oliveberg *et al.*, 1994).

Comparison of the Calculated and Observed pH Dependence of $\Delta G_{D\text{-}N}$ Reveals Anomalous pK_A Values of the Denatured State. The pH dependence of $\Delta G_{D\text{-}N}$ expected from the pK_A values of the native state (Table 2) was calculated according to eqs 3 and 4, assuming model compound pK_A values in the denatured state ($pK_A^{Asp} = 3.9$ and $pK_A^{Glu} = 4.1$) (Creighton, 1993). The calculated stability ($\Delta G_{D\text{-}N}^{calc}$) is compared with the observed stability ($\Delta G_{D\text{-}N}^{exp}$) in Figure 6. It is seen in Figure 6 that $\Delta G_{D\text{-}N}^{calc}$ behaves differently from the observed stability, $\Delta G_{D\text{-}N}^{exp}$: $\Delta Q_{D\text{-}N}^{calc}$ starts to increase at a higher pH value than does $\Delta Q_{D\text{-}N}^{exp}$; $\Delta Q_{D\text{-}N}^{calc}$ reaches a maximum value of 5.8 at pH 3.3 whereas $\Delta Q_{D\text{-}N}^{exp}$ shows a maximum of 4 at pH 2.9, and $\Delta Q_{D\text{-}N}^{calc}$ decreases much more rapidly at lower pH values than does $\Delta Q_{D\text{-}N}^{exp}$, so that at pH 1.3 $\Delta Q_{D\text{-}N}^{calc} = 1$ and $\Delta Q_{D\text{-}N}^{exp} = 2.3$.

The most straightforward way to account for the apparent offset of the results between pH 2 and 5 is to decrease the pK_A values of the denatured state in the equations. By assuming pK_A values of 3.5 and 3.7 for the Asp and Glu,-respectively, the simulations have good agreement with the experimental data (Figure 6). In other words, the results suggest that the pK_A values of the denatured state appear to be shifted on average −0.4 units from those of model compounds. An alternative explanation of the discrepancy in Figure 6 would be that some acidic residues in the native state show atypically high pK_A values, and that the titration of these groups, which contributes to a negative value of $\Delta Q_{D\text{-}N}$, counteracts the increase in $\Delta Q_{D\text{-}N}$ caused by protonation of the denatured state. This idea, however, is not consistent with the NMR data which show that the vast majority (if not all) of the acidic residues titrate with pK_A values below 4. The only exception, the broad coupled titrations of Asp 54, Asp 75, and Asp 86 (Figure 5), involve, at most one proton around pH 4.5−5.0 and affects $\Delta Q_{D\text{-}N}^{calc}$ only marginally (simulations not shown).

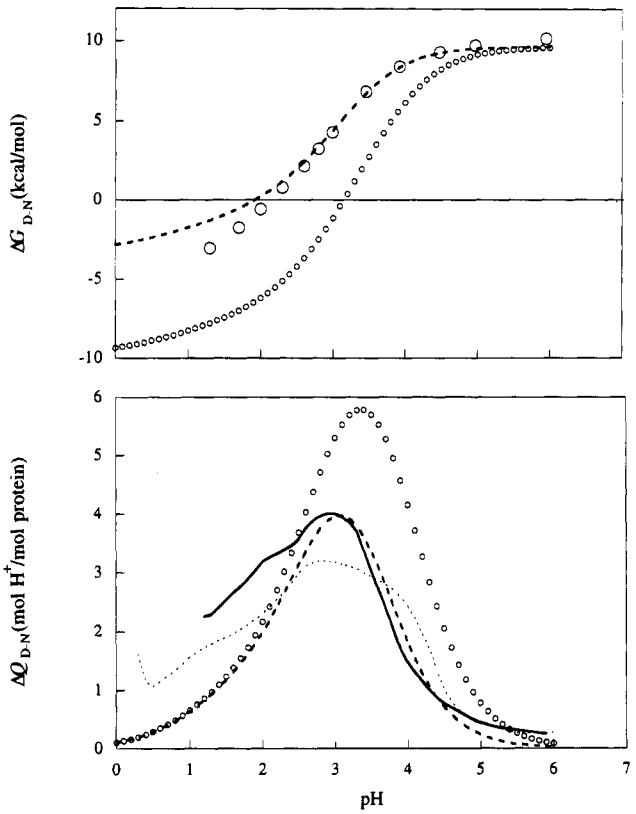

FIGURE 6: Protein-stability simulations showing the effects of pK_A shifts in the denatured state. (Upper panel) Free energy simulation (eqs 3 and 4) using the observed pK_A values of the native state (Table 2) and model-compound pK_A values for the denatured state, i.e., $pK_A^{Asp} = 3.9$ and $pK_A^{Glu} = 4.1$ (small open circles); corresponding simulation using $pK_A^{Asp} = 3.5$ and $pK_A^{Asp} = 3.7$ for the denatured state (dashed line); the experimentally determined free energy of unfolding at $\mu = 50$ mM (large open circles). (Lower panel) Simulation of $\Delta Q_{D\text{-}N}$ according to eq 3 using model-compound pK_A values for the denatured state, i.e., $pK_A^{Asp} = 3.9$ and $pK_A^{Asp} = 4.1$ (small open circles); simulation of $\Delta Q_{D\text{-}N}$ using shifted down pK_A values for the denatured state, i.e., $pK_A^{Asp} = 3.5$ and $pK_A^{Asp} = 3.7$ (dashed line); experimentally determined free energy of unfolding at $\mu = 50$ mM (solid line) and at $\mu = 600$ mM (thin dotted line).

Although the pK_A values obtained here, $pK_A^{Asp} = 3.5$ and $pK_A^{Glu} = 3.7$, represent no more than *mean values* for the denatured state, they can be successfully used in relating ionization equilibria of the native state to overall stability. A more detailed analysis of the electrostatic interactions within the denatured state, including shifts of individual residues, is currently being undertaken by mutational analysis. Nevertheless, it may be of interest to consider briefly what structural constraints in the denatured state may give rise to anomalous titration properties. According to the *framework model* for protein folding (Kim & Baldwin, 1982), where partially structured conformations are assumed to represent collections of elements of native-like (secondary) structure with some intrinsic stability, the overall pK_A shift may be attributed to an average value of (i) some residues localized in structural elements showing highly perturbed titration properties and (ii) other residues, which are localized in nonstructured parts of the peptide chain, showing model-compound values. Alternatively, the pK_A shift results not only from interactions with neighboring groups but could be due to a nonspecific increase in the overall charge density, or the electric field, following a so-called *hydrophobic collapse* (Kim & Baldwin, 1982). In its simplest form, the

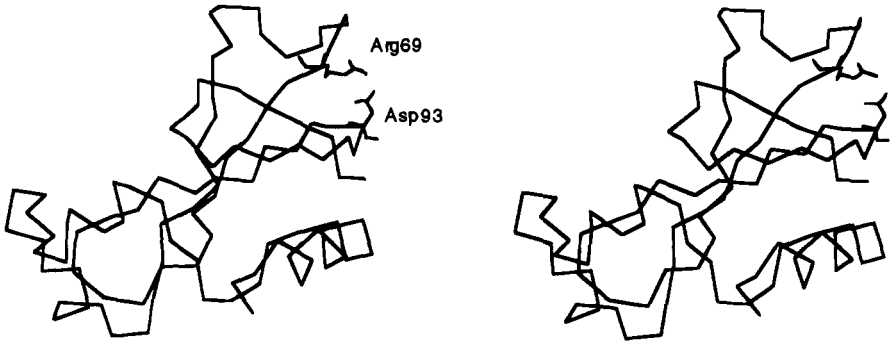

FIGURE 7: Stereopicture showing the semiburied Asp 93−Arg 69 salt bridge. The solvent-accessible areas of Asp 93 and Arg 69 are 69 and 42 Å^2, respectively.

denatured state may here be modeled as a compact porous sphere in which titrating residues, charges, and solvent are mixed randomly (Stigter & Dill, 1990). The observed pK_A values would in this system reflect the coupled, concurrent titration of all the acidic residues displaying the same (or similar) ionization state.

Titration Behavior of the Native State. Although the pK_A values of the denatured state have pronounced effects on the pH dependence of the stability above pH 2 (see above), they have no significant effect on $\Delta Q_{D\text{-}N}$ at lower pH values; below pH 2, the denatured state is fully protonated and Q_D is constant, and any change in $\Delta Q_{D\text{-}N}$ can, hence, be attributed solely to the titration of the native state, i.e., changes in Q_N. Accordingly, since $\Delta Q_{D\text{-}N}^{calc} < \Delta Q_{D\text{-}N}^{exp}$, the native protein remains ionised at pH values much lower than directly predicted from its pK_A values. Deviations in this direction, however, are not unexpected. First, the lack of base lines (chemical shifts for the protonated residues) in the NMR titration curves may well result in an overestimate of the pK_A values for some of the residues. Second, the residues may display nonideal titration curves because of charge interactions with other partly ionized groups. Such pH-dependent interactions, the magnitude of which forms an essential part in more rigorous calculations of ionization equilibria (Bashford & Karplus, 1990; Warshel & Åqvist, 1991; Yang & Honig, 1993; Antosiewicz et al., 1994; Oda et al., 1994), have been omitted in this minimal approach. Deviations from ideal behavior have been observed recently in a NMR study of ribonuclease HI (Oda et al., 1994). With barnase, preliminary results from linearised Poisson−Boltzmann calculations imply that, except for the salt-linked triad (Asp 8, Asp 12, and Arg 110), Asp 54, Glu 73, Asp 75, and Asp 86 show an interaction energy of half a pK_A unit or more (J. Warwicker, personal communication). From NMR data, however, only Asp 75 and Asp 86 display clearly a coupled titration (cf. Yang & Honig, 1993; Oda et al., 1994). In addition, the unfolding kinetics reveal that the broad titration of Asp 75 extends to pH values well below 1. As the protonation of Asp 54 and Asp 73 appear complete at such low pH (Figure 4), it is plausible that the main part of the deviation between $\Delta Q_{D\text{-}N}^{calc}$ and $\Delta Q_{D\text{-}N}^{exp}$, at low pH values is accounted for by the coupled titration of Asp 75 and Asp 86.

The Mutation of Asp→Asn in the Ionic Interaction between Asp 93 and Arg 69 Destabilizes the Protein by 4 kcal/mol. It follows from the very low pK_A value of Asp 93 that its titration can be followed only under conditions of high ionic strength, in this case 600 mM. At high concentrations of anions, however, the ionic interaction between Asp 93 and Arg 69 is likely to be screened, and, hence, the pK_A value observed at μ = 600 mM could be substantially shifted from that at low ionic strength. In order to estimate the effect of salt, we assume that the dependence on ionic strength and pH of energetic factors other than the electrostatic (ionic) interaction between Asp 93 and Arg 69 is relatively small. Hence, the stabilization upon mutation of Asp → Asn at pH values where Asp 93 is fully protonated (and the ionic interaction broken) is assumed to be the same at μ = 600 mM and μ = 50 mM, i.e., 0.5 kcal/mol ($\Delta\Delta G_{D\text{-}N}^{WT\text{-}D93N,nonionic}$) (Figures 1 and 2). The decreased stability of the mutant protein at pH 6 ($\Delta\Delta G_{D\text{-}N}^{WT\text{-}D93N}$) reflects thus the (total) cost of disrupting the salt link on top of the 0.5 kcal/mol intrinsic stabilization. Accordingly, an apparent energy of the ionic interaction between Asp 93 and Arg 69 would be approximately 3 + 0.5 kcal/mol at μ = 600 mM and 4 + 0.5 kcal/mol at μ = 50 mM, corresponding to pK_A shifts of 2.6 and 3.3 units, respectively. The pK_A value of Asp 93 would then be 1.4 at μ = 600 mM and 0.7 at μ = 50 mM. It is important to point out that although the pK_A shifts of salt-bridge partners are frequently interpreted as the strength of the ionic interaction, this may not always be the case: it is equally valid that the protonated form of the salt bridge is destabilizing because the protonation of the carboxylate leads to steric repulsion or poor hydrogen bond formation. In this study, however, the translation of apparent ionic interaction energies into a pK_A shifts appears valid and is consistent with the $\Delta Q_{D\text{-}N}$ values in Figure 3.

It has been demonstrated by Anderson et al. (1990), that the strong Asp 70−His 31 salt bridge in T4 lysozyme gives rise to a distinct maximum in stability around pH 5, and that this peak is absent with mutant protein. The contribution to stability is largest at pH values between the pK_A values of the salt-bridge partners in the denatured state, approximately 4 for Asp 70 and 6−7 for His 31 (Andersson et al., 1990), and outside this pH range the salt bridge becomes destabilizing for the protein. The Asp 93−Arg 69 salt bridge in barnase, although of similar strength, shows maximal stability over a much broader range, pH 4−9, the approximate pK_A values of Asp and Arg in the denatured state.

It can be noted, that although the pH where $\Delta\Delta G_{D\text{-}N}^{WT\text{-}Asn93}$ = 0 coincides with the pK_A value for Asp 93, this isoenergetic point is not directly linked to any pK_A value, but is determined by the magnitude of $\Delta\Delta G_{D\text{-}N}^{Asn93,nonionic}$. Consistent with the results in this study, it has been reported that the corresponding nonionic contribution of the surface-exposed partners in the salt-linked triad, Asp 12−Asp 8−Arg 110, is also destabilizing, so that when all three residues are

mutated the protein becomes 0.2 kcal/mol more stable (Horovitz et al., 1990). On the basis of the high calculated values for the desolvation penalties of charged and polar residues, it has been argued that complete substitution of buried salt bridges with aliphatic residues, which fit snugly and do not create cavities, would in most (if not all) cases be substantially stabilizing, on average as much as 3 kcal/mol (Hendsch & Tidor, 1994). A weakness in this analysis is that the calculated values are highly dependent on the uncertain assumption of the protein dielectric constant, and thereby the (electrostatic) desolvation penalty (cf. Antosiewicz et al., 1993).

It is apparent from this study, that pK_A values obtained from fitting of ideal titration curves to NMR titration data can be misleading in stability calculations: in addition to overestimated pK_A values, multiple small deviations from nonideal titration behavior add up to give a significant error in estimates of the total charge. These errors seem particularly pronounced at the extreme of pH values, where tailing titration curves are difficult to resolve experimentally because of the difficulty in measuring the base lines. An attractive way of presenting titration data, however, has recently been shown by Oda et al. (1994), where exemplary experimental data not only are presented as pK_A values but are also theoretically reproduced on the basis of coupled titrations (cf. Yang & Honig, 1993). An alternative, and perhaps more practical, theoretical approach is to reproduce the pH dependence of the free energy [see, for example, Antosiewicz et al. (1993)]. The advantage of this method is that experimental stability profiles can be obtained relatively easily and under conditions of ionic strength where highly concentrated NMR samples precipitate. In addition, residues of interest and their interacting partners can be mutationally and theoretically truncated and the effects on the observed pH dependence compared. To this end we are now characterizing the pH vs stability profiles of a series of barnase mutants and multiple-mutant cycles, in an attempt to produce a more detailed picture of complex protein-titration behavior.

REFERENCES

Andersson, D. E., Becktel, W. J., & Dahlquist, F. W. (1990) *Biochemistry 29*, 2403−2408.

Antosiewicz, J., McCammon, J. A., & Gilson, M. K. (1994) *J. Mol. Biol. 238*, 415−436.

Arcus, V., Vuilleumier, S., Freund, S. M. V., Bycroft, M., & Fersht, A. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9412−9416.

Bashford, D., & Karplus, M. (1990) *Biochemistry 29*, 10219−10225.

Bax, A., & Davies, D. G. (1985) *J. Magn. Reson. 65*, 355−360.

Bycroft, M., Matouschek, A., Kellis, J. T., Jr., Serrano, L., & Fersht, A. R. (1990a) *Nature 346*, 488−490.

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

Creighton, T. E. (1993) *Proteins*, W. H. Freeman and Company, New York.

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

Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Y., & Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311−315.

Dyson, H. J., Tennant, L. L., & Holmgren, A. (1991) *Biochemistry 30*, 4262−4268.

Fersht, A. R. (1972) *J. Mol. Biol. 64*, 497−509.

Fersht, A. R. (1993) *FEBS Lett. 325*, 5−16.

Forman-Kay, J. D., Clore, G. M., & Gronenborn, A. M. (1992) *Biochemistry 31*, 3442−3452.

Goto, Y., & Fink, A. L. (1989) *Biochemistry 28*, 945−952.

Griko, Y. V., Freire, E., & Privalov, P. I. (1994) *Biochemistry 33*, 1889−1899.

Hartley, R. W. (1969) *Biochemistry 8*, 2929−2933.

Haruyama, H., Qian, Y.-Q., & Wüthrich, K. (1989) *Biochemistry 28*, 4312−4317.

Hendsch, Z. S., & Tidor, B. (1994) *Protein Sci. 3*, 211−226.

Horovitz, A., Serrano, L., Avron, B., Bycroft, M., & Fersht, A. R. (1990) *J. Mol. Biol. 216*, 1031−1044.

Inoue, M., Yamada, H., Hashimoto, Y., Yasichochi, T., Hamaguchi, K., Miki, T., Horiuchi, T., & Imoto, T. (1992) *Biochemistry 31*, 8816−8821.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459−489.

Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87−103.

Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359−373.

Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967−974.

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.

Mauguen, Y., Hartley, R. W., Dodson, E. J., Dodson, G. G., Bricogne, G., Chothia, C., & Jack, A. (1982) *Nature 29*, 162−164.

Meiering, E. M., Serrano, L., & Fersht, A. R. (1992) *J. Mol. Biol. 225*, 585−589.

Myer, Y. P. (1968) *Biochemistry 7*, 765−776.

Neri, D., Billeter, M., Wider, G., & Wüthrich, K. (1992) *Science 257*, 1559−1563.

Nozaki, Y., & Tanford, C. (1967) *J. Am. Chem. Soc. 89*, 742−749.

Oda, Y., Yamazaki, T., Nagayama, K., Kanaya, S., Kuroda, Y., & Nakamura, H. (1994) *Biochemistry 33*, 5275−5284.

Oliveberg, M., Vuilleumier, S., & Fersht, A. R. (1994a) *Biochemistry 33*, 8826−8832.

Pace, C. N., Laurents, D. V., & Ericson, R. E. (1992) *Biochemistry 31*, 2728−2734.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167−236.

Privalov, P. L., Griko, Y. V., Venyaminov, S. Y., & Kutyshenko, V. P. (1986) *J. Mol. Biol. 190*, 487−498.

Privalov, P. L., Tiktopulo, E. I., Venyaminov, S. Y., Griko, Y. V., Makhatadze, G. I., & Khechinashvili, N. N. (1989) *J. Mol. Biol. 205*, 737 - 750.

Ptitsyn, O. E. (1992) In *Protein Folding* (Creighton, T. E., Ed.) W. H. Freeman and Co., New York.

Redfield, A. G., & Kuntz, S. D. (1975) *J. Magn. Reson. 19*, 250−254.

Sanz, J. M., & Fersht, A. R. (1993) *Biochemistry 32*, 13584−13592.

Sayers, J. R., Schmidt, W., & Eckstein, F. (1988) *Nucleic Acids Res. 16*, 791−802.

Serrano, L., & Fersht, A. R. (1989) *Nature 342*, 296−299.

Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 783−804.

Shrager, R. I., Cohen, J. S., Heller, S. R., Sachs, D. H., & Schechter, A. N. (1972) *Biochemistry 11*, 541−547.

Stigter, D., & Dill, K. A. (1989) *J. Phys. Chem. 93*, 6737−6743.

Stigter, D., & Dill, K. A. (1990) *Biochemistry 29*, 1262−1271.

Tanford, C. (1968a) *Adv. Protein Chem. 23*, 121−282.

Tanford, C. (1968b) *Adv. Protein Chem. 24*, 1−95.

Tanford, C., & Kirkwood, J. G. (1957) *J. Am. Chem. Soc. 79*, 5333−5339.

Tanford, C., & Roxby, R. (1972) *Biochemistry 11*, 2192−2198.

Warshel, A., & Åqvist, J. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 267−298.

Yang, A.-S., & Honig, B. (1993) *J. Mol. Biol. 231*, 459−474.

Yang, A.-S., Gunner, M. N., Sampogna, R., Sharp, K., & Honig, B. (1993) *Proteins: Struct., Funct., Genet. 15*, 252−265.

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