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Histidine Residues at the N- and C-Termini of α-Helices: Perturbed pK_as and Protein Stability

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ABSTRACT: A single histidine residue has been placed at either the N-terminus or the C-terminus of each of the two α-helices of barnase. The pK_a of that histidine residue in each of the four mutants has been determined by ^{1}H NMR. The pK_as of the two residues at the C-terminus are, on average, 0.5 unit higher, and those of the residues at the N-terminus are 0.8 unit lower, than the pK_a of histidines in unfolded barnase at low ionic strength. The conformational stability of the mutant proteins at different values of pH has been measured by urea denaturation. C-Terminal histidine mutants are $\sim$0.6 kcal mol^{-1} more stable when the introduced histidine is protonated, both at low and high ionic strength. N-Terminal mutants with a protonated histidine residue are $\sim$1.1 kcal mol^{-1} less stable at low ionic strength and 0.5 kcal mol^{-1} less stable at high ionic strength (1 M NaCl). The low-field ^{1}H NMR spectra of the mutant proteins at low pH suggest that the C-terminal histidines form hydrogen bonds with the protein while the N-terminal histidines do not form the same. The perturbations of pK_a and stability result from a combination of different electrostatic environments and hydrogen-bonding patterns at either ends of helices. The value of 0.6 kcal mol^{-1} represents a lower limit to the favorable electrostatic interaction between the α-helix dipole and a protonated histidine residue at the C-terminal end of the helix. Part of this electrostatic interaction should be attributed to the difference in stability between the charged and the noncharged hydrogen bond of C-terminal histidines.

The properties of amino acid side chains in a protein depend on their local and global environments. Charged residues, in particular, are affected by long-range as well as local electrostatic effects. These can perturb the ionic properties of the residue, and conversely, the mutual interactions affect the stability of the protein. It has been suggested, for example, that α-helices have a macroscopic dipolar character arising from the parallel alignment of the dipolar peptide bonds of the helix (Wada, 1976). The effect of the α-helix dipole has been suggested to be equivalent to that of a −0.5 elemental charge at the C-terminus plus a +0.5 elemental charge at the N-terminus of the helix (Hol et al., 1978). The dipolar nature of α-helices has been invoked to explain the structure of ligand binding sites (Hol et al., 1978), the relative disposition of α-helices within proteins (Sheridan et al., 1982; Presnell & Cohen, 1989), and the clustering of positive and negative charges toward the C- and N-termini of helices (Richardson & Richardson, 1988). The interaction between α-helix dipoles and charged residues in small peptides (Shoemaker et al., 1985; Fairman et al., 1989) as well as in proteins (Perutz et al., 1985; Sali et al., 1988; Nicholson et al., 1988) has been considered to contribute to stability, but little quantitative data are available (Perutz et al., 1985; Sali et al., 1988; Nicholson et al., 1988).

One of the most detailed measurements on the effects of charge–helix dipole interactions has come from the raising of the pK_a of His18, a residue at the C-terminus of the first helix in barnase (Sali et al., 1988). The imidazole side chain of His18 resides at the negatively charged terminus of the helix and makes a hydrogen bond with the backbone carboxamide

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oxygen of Gln15 (Mauguen et al., 1982). This raises the pK_a of histidine by stabilizing the positively charged form. The total stabilization energy is about 1.5–2 kcal mol^{-1}. We have now, however, discovered a novel interaction between histidine and tryptophan side chains that increases in magnitude on protonation, and that part of the perturbation of the pK_a of His18 is attributable to an interaction with Trp94 (Loewenthal et al., 1992). This suggests that the field from the helix dipole is smaller than previously thought. Another question is whether or not the electrostatic interaction results from the macroscopic dipole or just the final hydrogen bond. We have investigated the possibilities further in this study by constructing four barnase mutants with histidine residues at the ends of each of the two significant helices at positions 6, 18, 26, and 34. In one case, His18, the residue is already present in wild-type barnase but a tertiary interaction with Trp94 is removed by converting it to Leu94 (Loewenthal et al., 1992). In the mutant Thr26 → His, Asp54 was also mutated (into asparagine) because its side chain is about 4 Å from the side chain of Thr26 in wild-type barnase. The pK_as of the four histidine residues have been determined by ^{1}H NMR, and the changes in stability of the mutant proteins have been determined by urea-mediated denaturation.

Materials and Methods

Materials

Tris [tris(hydroxymethyl)aminomethane], Bis-Tris ([bis-(2-hydroxyethyl)amino]tris(hydroxymethyl)methane), 2 N hydrochloric acid, and imidazole were obtained from Sigma (St. Louis, MO). SP-Trisacryl-M was purchased from IBF Biotechnics (Villeneuve La Garenne, France). Dialysis tubing was from Spectrum Medical Industries Inc. (Los Angeles, CA). Deuterated water was from Fluochem Ltd. (Derbs, U.K.). Urea was purchased from Bethesda Research Laboratories (Gaithersburg, MD). All other reagents were purchased from either Sigma or Amersham.

The plasmid pTZ18U and the helper phage M13KO7 used for the mutagenesis were from Pharmacia (Uppsala, Sweden). *Escherichia coli* BL21(DE3) pLysS cells were donated to us by Dr. F. W. Studier. The wild-type barnase gene, cloned into the plasmid pUC9 fused to the promoter and signal sequence of the *E. coli* alkaline phosphatase gene (Hartley, 1988), was subcloned into the plasmid pTZ18U (Serrano et al., 1990).

Methods

Mutagenesis. Site-directed mutagenesis was performed by the method of Sayers et al. (1988) as described (Serrano et al., 1990). Single-stranded DNA was obtained from the modified plasmid pTZ18U, harbored in *E. coli* TG2 cells after infection with the helper phage M13KO7. The mutants Gly34 → His, Thr6 → His, and Asp54 → Asn and the double mutant (Thr26 → His/Asp54 → Asn) were obtained using the following oligonucleotides:

Gly34 → His:
5′-TGCCACCCAGT*G*GAGGGCTTG-3′

Thr6 → His:
5′-CCCGTCAAAA*T*G*GTTGATAAC-3′

Thr26 → His:
5′-TGCTTCTGATTTA*T*G*AATGTAATT-3′

Asp54 → Asn:
5′-TGAGAAGATGTT*TCCGCCGATG-3′

where asterisks follow mismatched bases.

Mutant plasmids were identified by direct sequencing of their ssDNA according to the chain termination method (Sanger et al., 1977) using the Sequenase (Tabor & Richardson, 1987) kit supplied by United States Biochemical Corp. (Cleveland, OH). The mutants Trp94 → Leu and (Trp94 → Leu/His18 → Gly) were made by R. Loewenthal (Loewenthal et al., 1992).

Expression and Purification of Barnase. The mutant plasmids were used to transfect BL21(DE3) (F$^-$, *ompT*, *rBmB*$^-$) *E. coli* cells (Grodberg & Dunn, 1988; Studier & Moffatt, 1986). Cells were grown and the proteins were purified as described (Serrano et al., 1990).

pK_a Determinations. The pK_as of the histidine residues in the different mutant proteins were determined at 25 °C by ^{1}H NMR as described by Sali et al. (1988), using a 500-MHz AMX-Bruker spectrometer. The amide protons of the mutant proteins were completely exchanged by deuteriums at pD = 11 before the experiments were performed. After a spectrum had been recorded, the pD of the solution was measured with a Radiometer Copenhagen PM64 pH meter, calibrated against standard buffers in H_2O. The chemical shifts of the C2 proton of the histidine ring at different pHs were fitted to the equation of a single ionization equilibrium. The values of pK_a so obtained in D_2O were converted to values in H_2O by subtracting 0.20 unit to allow for the isotope effect, as found from standard calibration experiments (Loewenthal et al., 1991).

The values of pK_a for both histidine residues in unfolded barnase (His18 and His102) were determined at 25 °C in 7 M urea (perdeuterated) in D_2O. The pK_a of imidazole was also determined under identical conditions. The pK_a of imidazole in H_2O at 25 °C was calculated using the Henderson–Hasselbalch equation from the pH of imidazole solutions (5 mM ionic strength) which were prepared by mixing weighed amounts of imidazole (more than 99% pure, from Sigma) with known volumes of hydrochloric acid from a 2 N stock solution from Sigma.

Low-Field ^{1}H NMR Spectra. The low-field spectra of wild-type barnase and of the histidine mutant proteins were acquired with a 500-MHz AMX-Bruker spectrometer without presaturation of solvent using a 1–1 pulse sequence. The sample proteins were dissolved in H_2O at concentrations between 0.5 and 1 mM, and the pH was adjusted to 4.5 with HCl. The number of scans varied from 256 to 2048.

Urea Denaturation Experiments. The free energies of unfolding of the proteins were determined by urea denaturation (Serrano et al., 1990). For each protein, the determination was carried out at two different pH values (termed low pH and high pH). Low pH is either 5.9 (Mes) or 4.4 (sodium acetate, Tables II and III). High pH is 8.9 (Tris). All buffers were used at an ionic strength of 30 mM. At low pH, at least 95% of the histidine residue being studied was protonated; at high pH, 95% or more of the histidine ring was unprotonated. The proteins were dialyzed in the appropriate buffer (150 mM ionic strength) and diluted 5-fold in the unbuffered urea solutions before the unfolding experiment was performed. For the high salt experiments, the 150 mM buffer contained 4.85 M NaCl before dilution so that the final salt concentration was 1 M. The values of the free energies of unfolding in H_2O are calculated by multiplying the urea concentration of half unfolding by the slope of a plot of free energy versus urea concentration (Serrano et al., 1990). Using an averaged slope for the different proteins, this method yielded the value of the free energy of unfolding in water with an error of ±0.02 kcal/mol.

Results

pK_a of Histidine Residues in Unfolded Barnase. The titration in D_2O of the C2-H resonance of the two histidine

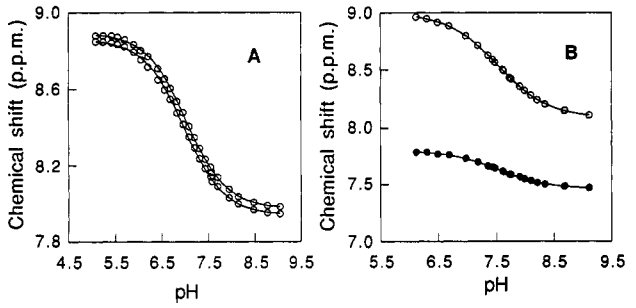

FIGURE 1: (A) ^1H NMR pH titration of the C2-H protons (circles) of the two histidines (His18 and His102) in wild-type barnase; (B) ^1H NMR pH titration of the C2-H and C4-H protons of imidazole (open and solid circles, respectively). At 1 mM solution of wild-type barnase (or imidazole) in D_2O and 7 M urea was titrated at 25 °C as described in Methods. The solid curves are the theoretical ionization curves.

Table I: pK_as of Histidine Residues and Their Positions in Barnase

histidine	location	pK_α[a]	ΔpK_a[b]
18	C-terminus	7.13 ± 0.01	+0.61 ± 0.04
34	C-terminus	6.91 ± 0.01	+0.39 ± 0.04
18/102	denatured barnase	6.52 ± 0.03[b]	0.00
6	N-terminus	5.82 ± 0.02	−0.70 ± 0.05
26	N-terminus	5.68 ± 0.01	−0.84 ± 0.04

[a] The pK_a values were determined by ^1H NMR as described by Sali et al. (1988) using a 500-MHz AMX-Bruker spectrometer. The pK_as were first determined in D_2O and then corrected for the H/D isotope effect by subtracting +0.20 pK_a unit (Loewenthal et al., 1991). [b] ΔpK_a is the difference between the pK_a values of a histidine residue and the pK_a value of histidine in unfolded barnase in H_2O (6.52).

residues in barnase (His18 and His102) under denaturing conditions (7 M urea) is shown in Figure 1A. The chemical shifts of the two histidine residues differ by 0.04 ppm throughout the pH range of the experiment and could be followed independently. The calculated pK_a values for the two histidines are 7.03 ± 0.01 and 6.98 ± 0.01 (±SE). No attempt has been made to assign values to the particular residues, but an average value of 7.00 ± 0.04 has been used as typical. We have determined the effect of 7 M urea on the pK_a of imidazole as follows. Under conditions identical to those in the experiments on denatured enzyme (7 M urea, Figure 1B), titration of the C2 and C4 protons gives a pK_a value of 7.53 ± 0.02. The pK_a of imidazole in water, determined as described in Methods, is 7.05 ± 0.01 (mean of seven determinations ± standard error). Assuming that the effect of urea and D_2O on the pK_a of imidazole and histidine residues in unfolded barnase is the same, the pK_a of the histidine residues of denatured barnase in H_2O is calculated to be 6.52 ± 0.07.

pK_a of Histidine Residues at Termini of Helices in Barnase Mutants. The titrations in D_2O of the C2-H resonance of the histidine residues in several barnase mutants are shown in Figure 2. In each case, the resonances of His18 and His102 were assigned by comparison with those of His18 and His102 in wild-type barnase (Sali et al., 1988), and the new resonance was attributed to the new histidine residue in the protein. The pK_as of histidine residues located at the C-terminus of either helix in barnase are higher than those of histidine residues in model compounds or unfolded proteins (Table I). Conversely, the pK_as of histidine residues located at the N-terminus are lower.

Calculation of Free Energy of Protonation of the Histidine Residues. The change in pK_a of a residue from its unperturbed value in the unfolded protein to its perturbed value in the folded structure is linked by a thermodynamic cycle (Figure 3) to the change in the free energy of unfolding as that residue

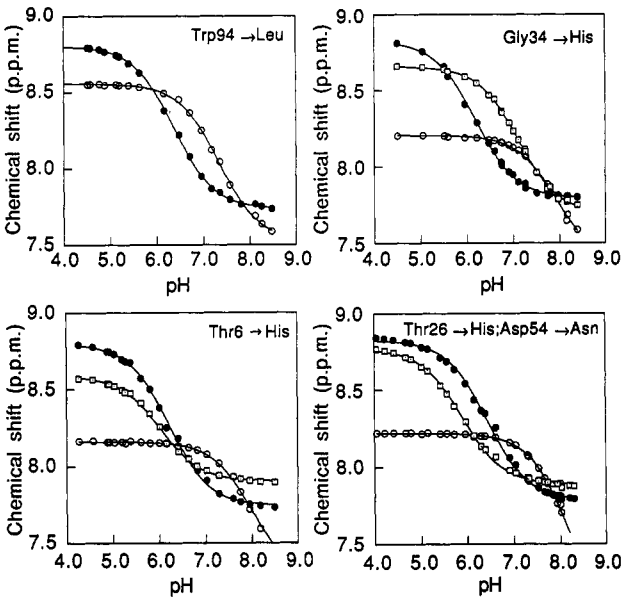

FIGURE 2: ^1H NMR pH titration of the C2-H of the histidine residues in several barnase mutants. The protons of a 1 mM solution of the mutant protein in D_2O were exchanged with deuterons at pD = 11, and the pH titration was performed at 25 °C as described in Methods. Solid circles, His102; open circles, His18; open squares, His34, His6, or His26. The solid curves are the theoretical ionization curves.

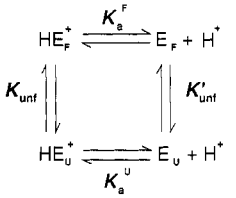

FIGURE 3: Thermodynamic cycle relating the protonated (HE^+) and unprotonated (E) forms of the protein in their folded (F) and unfolded (U) states. K_a is the dissociation constant of the ionizable residue, and K_{unf} and K'_{unf} are the equilibrium constants for the unfolding of protonated and unprotonated protein.

ionizes ($\Delta\Delta G_{unf}$) (Sali et al., 1988). The difference in pK_a between the folded and the unfolded protein (ΔpK_a) is related to the change in free energy of unfolding by eq 1. Mutants

$$\Delta pK_a = 1.364\Delta\Delta G_{unf} \quad (1)$$

with C-terminal histidine residues are calculated from ΔpK_a to be more stable at a low pH, where the imidazole ring is protonated, whereas N-terminal histidine residues should be more stable at high pH (Table IV).

Stability of the Proteins at Different pH Values and Salt Concentrations. Calculation of $\Delta\Delta G_{unf}$ Using Reference Mutants. The free energy of unfolding in H_2O of the four histidine mutants and their corresponding reference proteins (in which a noncharged residue has been substituted for the histidine one) are shown in Table II (30 mM ionic strength) and Table III (1 M ionic strength) at two different pH values. The two mutants with histidines at the C-terminus are more stable at low pH while the opposite is true for mutants with histidine at the N-terminus. The difference in stability between low and high pH, $\Delta\Delta G$ (low minus high pH), does not, however, give the true value of $\Delta\Delta G_{unf}$ (as defined above) since other groups, beside histidine, affecting the stability of the protein may also be titrating. The value of $\Delta\Delta G_{unf}$ for each histidine mutant can, however, be calculated by subtracting $\Delta\Delta G$ (low minus high pH) for a reference mutant lacking the histidine residue from the value obtained for the histidine mutant (Table IV). The values at low ionic strength (30 mM)

Table II: Free Energies of Unfolding[a] of Wild-Type Barnase and Several Mutants at Two pH Values (Ionic Strength = 30 mM)

histidine	protein (reference)	$\Delta G_{(low\ pH)}$	$\Delta G_{(high\ pH)}$ (kcal mol^{-1})	$\Delta\Delta G_{(low-high\ pH)}$
18	Trp94 → Leu	7.67[b]	7.41	0.26
	(Trp94 → Leu/ His18 → Gly)	8.11[b]	8.38	−0.27
34	Gly34 → His	5.53[c]	4.90	0.63
	(wt)	8.13[c]	8.05	0.08
6	Thr6 → His	5.48[c]	6.49	−1.01
	(wt)	8.13[c]	8.05	0.08
26	Thr26 → His; Asp54 → Asn	2.39[c]	3.78	−1.39
	(Asp54 → Asn)	5.48[c]	5.73	0.25

[a] ΔG of unfolding at both low and high pH were determined by urea denaturation equilibrium studies as explained in Serrano et al. (1990). Low pH is 5.9 (Mes)[b] or 4.4 (sodium acetate)[c]. High pH is 8.9 (Tris). All buffers were used at a 30 mM ionic strength. The proteins were dialyzed in the appropriate buffer (150 mM ionic strength) and diluted 5-fold in the different urea solutions before the unfolding experiment was performed.

Table III: Free Energies of Unfolding of Wild-Type Barnase and Several Mutants at Two Values of pH (Salt Concentration = 1 M)[a]

histidine	protein (reference)	$\Delta G_{(low\ pH)}$	$\Delta G_{(high\ pH)}$ (kcal mol^{-1})	$\Delta\Delta G_{(low-high\ pH)}$
18	Trp94 → Leu	10.0	9.30	0.70
	(Trp94 → Leu; His18 → Gly)	10.4	10.3	0.09
34	Gly34 → His	7.43	6.50	0.93
	(wt)	10.4	10.0	0.40
6	Thr6 → His	8.11	8.24	−0.13
	(wt)	10.4	10.0	0.40
26	Thr26 → His; Asp54 → Asn	6.41	6.51	−0.10
	(Asp54 → Asn)	8.92	8.45	0.47

[a] All experimental details are as in Table II, but the final buffer after dilution with the appropriate urea solution was 1 M salt (0.97 NaCl and 0.03 buffer).

Table IV: Changes in Free Energy of Unfolding on Ionization of Histidine Residues Located at Ends of Helices Relative to Reference Histidine

histidine	$\Delta\Delta G_{unf}$ on ionization measured directly[a]		$\Delta\Delta G_{unf}$ on ionization calculated from ΔpK_a[b]
	I = 30 mM	1 M salt	(I < 3 mM)
18	0.53	0.61	0.83
34	0.56	0.53	0.53
6	−1.08	−0.53	−0.95
26	−1.14	−0.57	−1.15

[a] Calculated as the difference between $\Delta\Delta G_{(low-high\ pH)}$ (see Table II) for each reporting mutant protein and that of a reference protein in which the histidine residue has been mutated into a noncharged one. The reference proteins are His6 → Thr (wild type) for His6, His34 → Gly (wild type) for His34, Asp54 → Asn;His-26 → Thr for His26 and Trp94 → Leu/His18 → Gly for His18. [b] Calculated from the formula $\Delta\Delta G_{unf}$ on ionization = 1.364ΔpK_a with the values of ΔpK_a in Table I.

agree with those calculated from ΔpK_a (ionic strength < 3 mM). The effect of a high ionic strength (1 M salt concentration) on the value of $\Delta\Delta G_{unf}$ is different for the C-terminal and the N-terminal histidine mutants. For C-terminal histidine mutants, $\Delta\Delta G_{unf}$ has the same value as at low ionic strength (~0.6 kcal/mol) while for N-terminal histidine mutants $\Delta\Delta G_{unf}$ is halved from −1.1 kcal/mol (at low ionic strength) to −0.5 kcal/mol.

Low-Field ^{1}H NMR Spectra of the Histidine Mutants. The low-field spectra at pH 4.5 of wild-type barnase and the four histidine mutants are shown in Figure 4. Two peaks are observed in the spectrum of wild-type barnase. Both of them correspond to imidazole N-H protons of His18 since they are absent in the spectrum of the mutant His18 → Gln (A. Hounslow and M. Bycroft, unpublished results). The two peaks collapse into a broader one in the mutant Trp94 → Leu

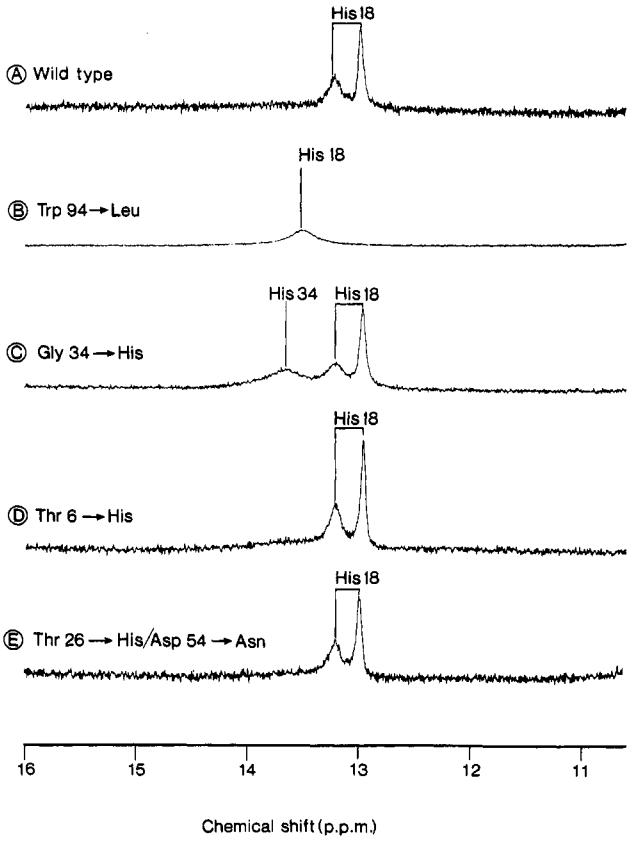

FIGURE 4: Low-field ^{1}H NMR spectrum of wild-type barnase and several mutant proteins in water. The pH of the samples was adjusted to 4.5 with HCl. (a) Wild-type barnase. The two low-field resonances are attributed to His18 since they are not present in the spectrum of the mutant His18 → Gln (A. Hounslow and M. Bycroft, unpublished results). (b) Trp94 → Leu. The two signals of His18 collapses into a broader one. (c) Gly34 → His. The new signal at lowest field is assigned to His34. (d) Thr6 → His. (e) Thr26 → His;Asp54 → Asn.

where Trp94 which is known to interact with His18 (Loewenthal et al., 1992) has been removed. In the spectrum of the mutant Gly34 → His, the two signals of His18 (in the presence of Trp94) are observed together with a new signal at lower field that is attributed to the new histidine (His34). The shape of this signal is similar to that of His18 in the mutant Trp94 → Leu. For the two N-terminal histidine mutants, only the two signals of His18 are observed. The low-field signals of wild-type barnase disappear when it is denatured by urea.

Discussion

A general prediction may be made about perturbations of pK_as of ionizable groups if the α-helix has a macroscopic electrostatic dipole with a positive end at the N-terminus. In the absence of other effects, the protonated form of ionizable residues at the C-termini should be more stable than the unprotonated form, and conversely, the unprotonated form should be preferred over the protonated form at the N-termini. The pK_as of C-terminal residues should be higher and those of N-terminal residues should be lower than the pK_a of equivalent residues in model compounds or in unfolded proteins. We have tested this hypothesis by placing histidine residues at each end of the two α-helices in barnase and have found that C-terminal histidines do have a higher pK_a than histidines in the denatured protein. The opposite is true for N-terminal histidines. Attribution of the differential stability of charged residues at the ends of α-helices to a purely through-space electrostatic interaction with the helix dipole may be, however, an oversimplification.

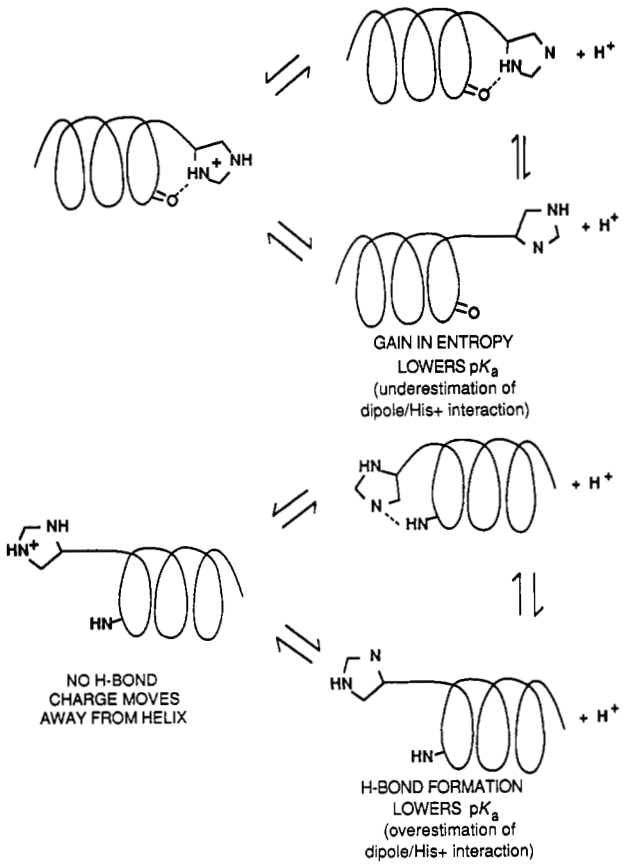

FIGURE 5: Scheme showing the hydrogen-bonding patterns at the N and C-termini of α-helices. As shown in the figure, changes in the hydrogen-bonding pattern on deprotonation of the histidyl residues may contribute to alteration of their pK_as.

Different Hydrogen-Bonding Patterns in N- and C-Termini of Helices. The two ends of a helix differ in their hydrogen-bonding properties. C-Termini provide acceptor groups (the amide carbonyls) that are suitable for binding positively charged ions. N-Termini contain donor groups (the amide protons) that can interact with negatively charged groups. This introduces a difference between histidine residues at the C- and N-termini of helices (Figure 5). C-Terminal histidines may form hydrogen bonds with an exposed backbone carbonyl oxygen of the last turn of the helix (cf. His18 with Gln15). The positively charged form of the imidazole ring may thus move as close as possible to the favorable pole of the helix. In contrast to this, the protonated N-terminal histidine is a hydrogen-bond donor only and cannot make a hydrogen bond with the first turn of the helix since only hydrogen-bond donor groups from the backbone >NHs are exposed. The side chain is not constrained and so can move away from the positively charged pole and minimize the electrostatic repulsion between the two (Figure 4). We have tested this model of hydrogen bonding for histidine residues at low pH by comparing the low-field ^{1}H NMR spectra of the different histidine mutants. Fast exchange of the low-field histidine resonances precluded the study at high pH (data not shown). We have found signals in the ^{1}H NMR spectra of the mutants containing Trp94 → Leu and Gly34 → His that can be assigned to N-H protons of the protonated imidazole rings in His18 and His34, respectively (Figure 4). Low-field histidine signals are indicative of imidazole N-H protons in slow exchange and have been interpreted before as resulting from the involvement of these protons in hydrogen bonds (Robillard & Shulman, 1972). We do not observe any new low-field signal in the spectra of the mutant Thr6 → His or the double mutant Thr26 → His/Asp54 → Asn, consistent with the N-terminal histidine residues (His6 and His26) not making hydrogen bonds to the helix at low pH. The different hydrogen-bonding pattern at the two ends of the helices is also suggested by the different effect of salt on the stability of the mutant proteins. The interaction of C-terminal histidine residues is not screened by 1 M NaCl while that of N-terminal residues is partly screened (see Table IV). The latter is expected perhaps since positive N-terminal histidines should move away from the helix and so allow ions to enter between the imidazole ring and the positive end of the helix.

Helix-Terminal Histidines and Protein Stability. The electrostatic interaction of protonated histidine residues with the rest of the protein influences its stability. This effect can be studied by measuring the stability of the protein at two extremes of pH, where either the protonated or neutral form of the imidazole ring predominates. The difference in stability between low and high pH is then compared with that of a reference protein lacking the histidine residue. Proteins with histidines at the C-terminus should be more stable at low pH while proteins with N-terminal histidines should be less stable. Our results confirm this prediction, and the difference in stability agrees with the observed perturbation of the pK_as. We find that a positively charged histidine residue at a C-terminus stabilizes the protein by 0.6 kcal mol^{-1} with respect to the unprotonated histidine and that, at the N-terminus, a protonated histidine destabilizes the protein by 1.1 kcal mol^{-1} with respect to the unprotonated form.

Quantitative Relationship between Electrostatic Dipole and Perturbation of pK_a. The stability of histidine residues at the end of helices does not only depend on the electrostatic interaction with the helix dipole. Differences in solvation energy between the folded and unfolded state and differences in entropic freedom between the protonated and unprotonated forms of histidine also play a role. These factors complicate previous analyses of the effects of helices on the pK_a of ionizable residues at their ends (Sali et al., 1988; Nicholson et al., 1988; Åqvist et al., 1991). Consider first a histidine residue at the C-terminus (Figure 5 (top)). At low pH, the protonated form of the histidine is constrained by both the hydrogen bond and the dipole moment. At high pH, the uncharged form of the histidine is constrained just by the hydrogen bond. If, as at the very top of Figure 5, the histidine makes a hydrogen bond with the C-terminus at both high and low pH, then, all other things being equal, the perturbation of pK_a may be attributed to electrostatic effects of the helix dipole, both long range effects and effects due to the direct hydrogen bond. It could be possible, however, that the gain in entropy on breaking the hydrogen bond at high pH more than compensates for the intramolecular hydrogen bond. If this happens, then it must be a favorable event compared with retaining the hydrogen bond and so will be a factor favoring deprotonation. If the side chain moves away from the protein on deprotonation, then this will lower the pK_a because the uncharged side chain has a more favorable location. In such case, the combination of an equilibrium between two states and an ionization still will appear as a single, macroscopic pK_a (Fersht, 1985). Solvation effects have also to be considered. It is likely, when the imidazole ring remains close to the protein, that the mass of the protein inhibits solvation. Poor solvation destabilizes the protonated form of histidine in the folded protein and so will lower the pK_a. Both the entropic and solvation factors conspire to lower the pK_a of the C-terminal histidine, and so the increase in pK_a of 0.5 unit is thus a minimum estimate of the effects of electrostatic interactions, both from the macroscopic dipole

Table V: Distances between the CA of C-Terminal and N-Terminal Residues in Barnase and Charges within 10 Å

amino acid	charged group	distance[a] (Å)
His18	Lys19	7
Gly34	-	
Thr6	Asp8	7.5
	Asp12	8.5
	Asp86	9
	Lys98	9
	Arg110	9.5
Thr26	Lys27	7.5
	Glu29	5
	Arg72	10
	Glu73	9.5
	Asp75	9.5
	Arg83	9

[a] Averaged distances between the CA of the C-terminal and N-terminal residues in barnase and side-chain heteroatoms of charged residues within a 10-Å radius. Barnase crystallizes as a trimer. The distances shown are the average for the three monomers or, when the side chain is only defined in two of the monomers, the average for the two.

and the direct hydrogen bond between the imidazole and backbone carbonyl oxygen. A contribution from other electrostatic interactions between the C-terminal histidines and charged residues elsewhere in the protein (Table V) can be ruled out on the basis of the lack of a screening effect of salt on $\Delta\Delta G_{unf}$ (Table IV).

Effects at the N-terminus are more complicated. Protonated histidine cannot make a hydrogen bond with the N-terminal NH groups. If there is an unfavorable electrostatic interaction of the dipole with the charge on the side chain, then it can rotate away from the dipole and minimize the repulsion. This leads to an underestimation of the electrostatic effect of the dipole. However, deprotonation at high pH may allow the formation of a hydrogen bond with the N-terminus. If this occurs, it will be de facto a stabilizing factor and so will lower the pK_a leading to an overestimation of the effect of the dipole. Again, the combination of an equilibrium and an ionization appears as a single, macroscopic pK_a.

The measurements we report here represent the observed, experimental effects of the perturbation of histidine pK_a values with locations at the ends of the helices. Importantly, the same empirical results have been obtained for two helices that differ in length and do not show sequence homology, which suggests that the findings are general.

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