Contribution of Histidine Residues to the Conformational Stability of Ribonuclease T1 and Mutant Glu-58 → Ala

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ABSTRACT: The pK values of the histidine residues in ribonuclease T1 (RNase T1) are unusually high: 7.8 (His-92), 7.9 (His-40), and 7.3 (His-27) [Inagaki et al. (1981) J. Biochem. 89, 1185–1195]. In the RNase T1 mutant Glu-58 → Ala, the first two pK values are reduced to 7.4 (His-92) and 7.1 (His-40). These lower pKs were expected since His-92 (5.5 Å) and His-40 (3.7 Å) are in close proximity to Glu-58 at the active site. The conformational stability of RNase T1 increases by over 4 kcal/mol between pH 9 and 5, and this can be entirely accounted for by the greater affinity for protons by the His residues in the folded protein (average pK = 7.6) than in the unfolded protein (pK = 6.6). Thus, almost half of the net conformational stability of RNase T1 results from a difference between the pK values of the histidine residues in the folded and unfolded conformations. In the Glu-58 → Ala mutant, the increase in stability between pH 9 and 5 is halved (~2 kcal/mol), as expected on the basis of the lower pK values for the His residues in the folded protein (average pK = 7.1). As a consequence, RNase T1 is more stable than the mutant below pH 7.5, and less stable above pH 7.5. These results emphasize the importance of measuring the conformational stability as a function of pH when comparing proteins differing in structure.

Several recent theoretical [see, e.g., Harvey (1989), Glackin et al. (1989), Gilson and Honig (1988), and Burley and Petsko (1988)] and experimental [see, e.g., Pace et al. (1990), Anderson et al. (1990), Perry et al. (1989), Fairman et al. (1989), and Nicholson et al. (1988)] studies have considered the contribution of electrostatic interactions to the structure and function of globular proteins. The facility we have developed for making small changes in the amino acid sequence of a protein allows experimental studies that will be useful in investigating the electrostatic interactions of the charged groups. Changes involving histidine residues will be especially informative because the pK values of individual histidine residues can be readily measured using NMR (Alexandrescu et al., 1989). The three histidine residues in ribonuclease T1 (RNase T1) are particularly interesting because they all have high pKs with an average value of 7.7 (Inagaki et al., 1981). In contrast, the average pK of the four histidines in RNase A is 6.3 [see Table II in Matthew and Richards (1982)], and the average pK of the four histidines in staphylococcal nuclease is 6.0 (Alexandrescu et al., 1989).

We have studied in the pH dependence of the conformational stability of RNase T1 and shown that the maximum stability of 8.8 kcal/mol occurs near the isoelectric point at pH 4.5 (Pace et al., 1990). We suggested that pK differences between groups in the folded and unfolded conformations make a significant contribution to the conformational stability. To investigate this further, we have measured the pKs of the histidine residues in RNase T1 and a mutant in which Glu-58 is replaced by Ala. Glu-58 is in close proximity to His-40 (3.7 Å) and His 92 (5.5 Å) at the active site and is thought to be largely responsible for the high pKs of these histidines. We have also measured the pH dependence of the conformational stability for both of these proteins between pH 5 and 9.

Glu-58 → Ala mutant was previously shown to be about 0.8 kcal/mol less stable than wild-type RNase T1 at pH 7 in the absence of salt (Shirley et al., 1989). The present results show that the difference in stability depends on pH: at low pH, wild-type RNase T1 is more stable, and at high pH, the Glu-58 → Ala mutant is more stable.

EXPERIMENTAL PROCEDURES

Wild-type RNase T1 and the Glu-58 → Ala mutant were prepared from a gene expressed in Escherichia coli (Shirley et al., 1989), and protein concentrations were determined from absorbance measurements at 278 nm using an absorption coefficient of 1.55 for a 1 mg/mL solution (Shirley & Larents, 1990). "Ultrapure" urea was purchased from Schwarz/Mann Biotech, sodium acetate was from Fisher, and all other buffers and chemicals were from Sigma. Urea stock solutions were prepared as described (Pace, 1986).

Urea denaturation curves were determined by measuring the intrinsic fluorescence intensity (278-nm excitation, 320-nm emission) of RNase T1 solutions in 1-cm quartz cuvettes thermostated at 25 ± 0.1 °C with a Perkin-Elmer MPF 44B spectrophotometer. The solutions were incubated for a minimum of 6 h at 25 °C prior to the measurements. The pH of several solutions near the midpoint of the transition region was measured at room temperature with a Radiometer PHM 26 pH meter after the fluorescence measurements, and the average of these values is the pH value given for each urea denaturation curve. The individual pH readings never differ from the average by more than ±0.03. The procedure for determining and analyzing urea denaturation curves has been described previously (Pace et al., 1989).

NMR spectra were measured on a Varian XL-400 multinuclear spectrometer operating at a frequency of 400 MHz. The probe temperature was 21 or 36 °C. Typical acquisition parameters were 5300-Hz sweep width, 3.0-s acquisition time, 3-s delay between pulses, two steady-state pulses, 3-μs pulse width (pulse width 90 = 12.4 μs), and Walz decoupling during the delay period only (4 dB). The NMR samples were dis-
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solved in D$_2$O at concentrations of approximately 2.3 and 1.1 mM for wild-type RNase T1 and the Glu-58 → Ala mutant, respectively. The labile protons were exchanged with deuterium atoms by incubation of the samples in D$_2$O at pH 8.5 for 15 min at 60 °C. Both proteins are unfolded under these conditions (Shirley et al., 1989). All samples contained the internal reference sodium 3-(trimethylsilyl)-1-propanoate. The pH of the samples was adjusted by addition of 0.1 M DCl or KOD. The pH values were measured at room temperature in the 5-mm NMR tube using a 4 × 253 mm Cole-Palmer combination electrode and an Orion 601A pH meter. The titration curves were fit by using a nonlinear, least-squares program to obtain the pK values (Johnson & Frazier, 1985). The standard errors ranged from ±0.02 to ±0.18, so we estimate that the pK values should be accurate ±0.2.

RESULTS

Typical urea unfolding curves for RNase T1 (Thomson et al., 1989) and the Glu-58 → Ala mutant (Shirley et al., 1989) have been published. RNase T1 unfolding closely approaches a two-state mechanism, so urea unfolding curves can be analyzed to give the fraction of unfolded protein, $F_u$, and the free energy change for unfolding, $\Delta G$, as a function of urea concentration (Thomson et al., 1989). Figure 1 shows $F_u$ as a function of urea concentration for RNase T1 and mutant Glu-58 → Ala in the pH range 5–9. It is evident that the stability of wild-type RNase T1 is much more pH dependent than the stability of mutant Glu-58 → Ala. This can be seen even more clearly in Figure 2.

Unfolding data such as those shown in Figure 1 were used to calculate $\Delta G$ as a function of urea concentration, and, in all cases, $\Delta G$ was found to vary linearly with urea molarity. A least-squares analysis (the proc reg subroutine in the SAS/STAT software available from SAS Institute Inc., Cary, NC) was used to fit the data to the equation:

$$\Delta G = \Delta G(H_2O) - m \text{urea}$$

where $\Delta G(H_2O)$ is the value of $\Delta G$ in the absence of urea and $m$ is a measure of the dependence of $\Delta G$ on urea molarity (Pace, 1986). These parameters are given in Table I along with $\Delta G(J_2)$, the midpoint of the urea unfolding curve. The $\Delta G(J_2)$ value is plotted as a function of pH in Figure 2. In a previous study (Pace et al., 1990), the pH dependence of $\Delta G(J_2)$ was measured in the absence of salt, and the results are shown by the solid line in Figure 2. For the experiments reported here, it was necessary to add 0.2 M NaCl so that higher protein concentrations could be obtained for the NMR experiments. It can be seen that RNase T1 is more stable in the presence of 0.2 M NaCl. This results mainly from the binding of Na$^+$ ions at a nonspecific cation binding site on the protein (Pace & Grimsley, 1988). The results with mutant Glu-58 → Ala show that this protein is more stable than wild-type RNase T1 above pH 7.5 and less stable below pH 7.5. We will see below that this is the expected result based on the pK values of the His residues in the two proteins.

The titration curves of His-40 in RNase T1 and mutant Glu-58 → Ala are shown in Figure 3. Our titration curves for His-27, -40, and -92 are similar to those reported by

\[\text{Table I: Parameters Characterizing the Urea Unfolding of RNase T1 and Mutant Glu-58 → Ala}\]

<table>
<thead>
<tr>
<th>pH and buffer</th>
<th>$m^2$ (cal mol$^{-1}$ M$^{-2}$)</th>
<th>$\Delta G(H_2O)$ (kcal/mol)</th>
<th>(urea)$_{1/2}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td></td>
<td></td>
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<tr>
<td>4.97, acetate</td>
<td>1349</td>
<td>9.40</td>
<td>6.97</td>
</tr>
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<td>1306</td>
<td>9.14</td>
<td>7.00</td>
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<td>6.02, MES</td>
<td>1252</td>
<td>8.24</td>
<td>6.58</td>
</tr>
<tr>
<td>6.51, MES</td>
<td>1285</td>
<td>7.93</td>
<td>6.18</td>
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<tr>
<td>7.13, MOPS</td>
<td>1355</td>
<td>7.41</td>
<td>5.47</td>
</tr>
<tr>
<td>7.47, MOPS</td>
<td>1330</td>
<td>6.69</td>
<td>5.03</td>
</tr>
<tr>
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<td>1350</td>
<td>5.92</td>
<td>4.38</td>
</tr>
<tr>
<td>8.47, HEPPSO</td>
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<td>5.62</td>
<td>4.08</td>
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<td>8.90, DiGly</td>
<td>1432</td>
<td>5.67</td>
<td>3.96</td>
</tr>
<tr>
<td>Mutant Glu-58 → Ala</td>
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<tr>
<td>5.03, acetate</td>
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<td>8.01, HEPPSO</td>
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<td>9.57, DiGly</td>
<td>1373</td>
<td>6.39</td>
<td>4.66</td>
</tr>
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</table>

*The curves were determined at 25 °C in solutions containing 0.2 M NaCl and 30 mM of the buffer indicated. A least-squares analysis of plots $\Delta G$ vs urea molarity was used to determine the parameters in eq 1. (urea)$_{1/2}$ = midpoint of the unfolding curve = $\Delta G(H_2O)$/m. On the basis of analysis of numerous urea unfolding curves for RNase T1 (Thomson et al., 1989; Pace et al., 1990), we estimate standard errors of approximately ±50 cal mol$^{-1}$ M$^{-2}$ in $m$, ±0.05 M in (urea)$_{1/2}$, and ±0.3 kcal/mol in $\Delta G(H_2O)$.
An unperturbed imidazole side chain in a peptide is expected to have a pK ≈ 6.6.¹ The pKs of the three histidine residues in RNase T1 are substantially higher than this (Table III). Long before the structure of RNase T1 was known, Ruterjans and Pongs (1971) suggested that His 40 "...is interacting with a carboxylate anion of an acidic amino acid residue...". The crystal structure confirmed this prediction and showed that both His-40 and His-92 are in close proximity to Glu-58 at the active site of RNase T1 (Table III). The exact distance between the side chains in the free enzyme is not known because all of the crystal structures have been determined with nucleotides or vanadate bound at the active site, and these groups interact with these side chains (Kostrewa et al., 1989).

However, it is clear that these side chains will be in close proximity and could be hydrogen-bonded in the free enzyme as His-40 and Glu-58 are in the 2'-GMP complex (Arnti et al., 1988). His-27 is also hydrogen bonded to a neighboring carboxyl group, and the interaction could be classed as a salt bridge or ion pair (Rashin & Honig, 1984; Barlow & Thornton, 1983). Note that all three of the His residues are largely buried (Table III); this would ordinarily tend to lower rather than raise the pK values.

Replacing Glu-58 with Ala decreases the pK of His-40 from 7.8 to 7.0 and the pK of His-92 from 7.7 to 7.3 (Table III). This confirms the suggestion made by Ruterjans and Pongs (1971). Also, the group with a pK of 4.3 that is observed to cause all of the crystal structures have been determined with nucleotides or vanadate bound at the active site, and these groups interact with these side chains (Kostrewa et al., 1989).

Molecules, including protons, that bind more tightly (higher pKs) to the folded conformation than to the unfolded conformations of a protein will shift the equilibrium toward the folded state and thereby increase the conformational stability. Consequently, the His residues in RNase T1 should make a major contribution to the conformational stability, and our results show that they do. The conformational stability of RNase T1 is shown plotted as a function of pH in Figure 4.

¹ Histidine pK values corrected to H2O AT 25 °C are 6.5 for N-acetyl-L-histidine methylamide (Tanokura et al., 1978), 6.6 for Gly-His-Gly (Tanokura et al., 1976), 6.7 for Gly-Gly-His-Gly-Gly (Gurd et al., 1973), 6.6 for the two freely accessible His residues in thermally unfolded RNase A (Matthews & Westmoreland, 1975), 6.3 for His-18 in unfolded (5 M urea) (Sali et al., 1988), and 6.6 for His-11 in unfolded (6 M urea) T4 lysozyme (Anderson et al., 1990). The corrections were made by using ΔH = 7 kcal/mol and pK(D2O) = pK(H2O) = 0.1 (Tanokura et al., 1978), and the corrections for urea as described by Sali et al. (1980) and by Anderson et al. (1990). On the basis of these data, the best estimate for the pK of an unperturbed His residue in H2O AT 25 °C is 6.6 ± 0.1. Perutz et al. (1985) reached the same conclusion, and Matthews and Richards (1982) suggest 6.00 for His N⁺ and 6.60 for His N⁻.
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\[ \Delta G(H_2O) \text{ kcal/mole} = \Delta G(H_2O)(pH < pK) - RT \sum \ln \left[ \frac{1 + K_{ID}/(H^+)}{1 + K_{IN}/(H^+)} \right] \]  

where \( K_{ID} \) and \( K_{IN} \) are the dissociation constants of groups \( i \) in the denatured and native conformations, respectively. \((H^+)\) is the hydrogen ion activity, and \( \Delta G(H_2O)(pH < pK) = \Delta G(H_2O) \) at low pH where the groups being considered are fully protonated. This equation assumes that the pH dependence of \( \Delta G(H_2O) \) results entirely from the differences in pKs between groups in the folded and unfolded conformations. The three pKs given in Table III were used for the \( K_{IN} \) values, and the values of \( \Delta G(H_2O)(pH < pK) = 9.44 \pm 0.12 \text{ kcal/mol} \) and \( pK_D = 6.57 \pm 0.04 \) (for all three groups) were obtained by best-fitting the experimental data to eq 2. It is reassuring that the value of \( pK_D \) is identical with the pK value expected for His residues in an unfolded protein. Thus, the increase in stability between pH 9 and 5 for RNase T1 results almost entirely from the presence of the three His residues with elevated pK values in the folded protein, and they account for almost half of the net conformational stability near pH 5 where the protein is most stable.

In line with this reasoning, \( \Delta G(H_2O) \) should be less dependent on pH in the Glu-58 \( \rightarrow \) Ala mutant where the pK values of His-92 and -40 are lower than they are in wild-type RNase T1 (Table III), and the results in Figure 4 show that this is indeed the case. The dashed line shows the pH dependence expected with \( pK_D = 6.57 \) (from the analysis above), \( \Delta G(H_2O)(pH < pK) = 8.51 \text{ kcal/mol} \), and the pK values given in Table III for the mutant. The fit to the experimental data is reasonably good, and this confirms the idea that the pH dependence of \( \Delta G(H_2O) \) in this pH range depends mainly on the difference between the pK values of the histidine residues in folded and unfolded RNase T1.

There is great interest in studying proteins differing by a single amino acid in order to gain a better understanding of the forces that contribute to the conformational stability (Alber, 1989). Our results emphasize an important point that must be kept in mind in these studies. If the mutation changes the pK of an ionizable group, then it is important that the stability of both proteins be measured as a function of pH. RNase T1 is more stable than the Glu-58 \( \rightarrow \) Ala mutant below pH 7.5 and less stable above pH 7.5. This type of behavior may occur with any amino acid substitution that leads to changes of the pKs of groups in either the folded or the unfolded conformation.

**REFERENCES**


Rates and Energetics of Tyrosine Ring Flips in Yeast Iso-2-cytochrome c

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ABSTRACT: Isotope-edited nuclear magnetic resonance spectroscopy is used to monitor ring flip motion of the five tyrosine side chains in the oxidized and reduced forms of yeast iso-2-cytochrome c. With specifically labeled protein purified from yeast grown on media containing [3,5-13C]tyrosine, isotope-edited one-dimensional proton spectra have been collected over a 5–55 °C temperature range. The spectra allow selective observation of the 10 3,5 tyrosine ring proton resonances and, using a two-site exchange model, allow estimation of the temperature dependence of ring flip rates from motion-induced changes in protein line shapes. For the reduced protein, tyrosines II and IV are in fast exchange throughout the temperature range investigated, or lack resolvable differences in static chemical shifts for the 3,5 ring protons. Tyrosines I, III, and V are in slow exchange at low temperatures and in fast exchange at high temperatures. Spectral simulations give flip rates for individual tyrosines in a range of one flip per second at low temperatures to thousands of flips per second at high temperatures. Eyring plots show that two of the tyrosines (I and III) have essentially the same activation parameters: $\Delta H^\circ = 28$ kcal/mol for both I and III; $\Delta S^\circ = 42$ cal/(mol-K) for I, and $\Delta S^\circ = 41$ cal/(mol-K) for III. The remaining tyrosine (V) has a larger enthalpy and entropy of activation: $\Delta H^\circ = 36$ kcal/mol, $\Delta S^\circ = 72$ cal/(mol-K). Tentative sequence-specific assignments for the tyrosines in reduced iso-2 are suggested by comparison to horse cytochrome c. For oxidized iso-2, five resonances are observed at high temperatures, suggesting flip rates for all five tyrosines sufficient to average static chemical shift differences. At lower temperatures, there is evidence of intermediate and slow flipping for some of the rings, but spectral simulations have not been possible because of the complexity of the isotope-edited spectra.

The investigation of internal motions in proteins provides an important link between experiment and theory for protein-mediated phenomena. Measurement and modeling of fundamental intramolecular rate processes is of importance in understanding the internal movement and rearrangement of protein segments that occurs together with enzyme catalysis, allostericy, and molecular recognition. Two experimental approaches are particularly valuable: exchange of labile protons with solvent protons (HX) and the rotational motion of aromatic side chains (ring flips) (Wagner, 1983). HX allows measurement of the location and equilibrium constants for protein breathing modes but only infrequently gives a direct measure of rates (Englander & Kallenbach, 1984). On the other hand, accurate rate information is obtained for protein fluctuations involving aromatic ring flips (Campbell et al., 1976; Wuthrich & Wagner, 1975; Wuthrich, 1979). The fluctuations in local environment probed by aromatic ring flips are in the time range of important biochemical processes (1 ms to 1 s) such as enzyme catalysis, protein conformational changes, and protein folding.

Despite its fundamental importance, protein internal dynamics as probed by aromatic ring motion have been studied in detail for a limited number of systems: principally BPTI (Snyder et al., 1975; Wagner, 1983, 1987; Wagner et al., 1976), alkaline phosphatase (Hull & Sykes, 1975), and horse cytochrome c (Campbell et al., 1976). This is largely because of the difficulty of obtaining suitable NMR spectra. The detailed line-shape analysis needed to extract rate constants

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† Abbreviations: iso-2, iso-2-cytochrome c from the yeast Saccharomyces cerevisiae; BPTI, basic pancreatic trypsin inhibitor; NMR, nuclear magnetic resonance; D2O, deuterium oxide; pH, uncorrected pH meter reading for a deuterium oxide solution; HX, labile proton exchange with either deuterium or tritium; s, seconds; ms, milliseconds; k, rate constant for ring flips (s⁻¹); Hz, hertz (s⁻¹).