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Nonrandom Structure in Thermally Unfolded Ribonuclease A

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A complete understanding of the factors important in directing the rapid and efficient transition of a thermally unfolded protein to its native conformation requires information on the structure of the unfolded form. Spectroscopic evidence suggests that the thermally unfolded form of ribonuclease A (RNAase A) contains nonrandom structure;[1-3] however, detailed information on the structure of this form has not been obtained.

In a previous NMR study of thermally unfolded RNAase A,[4] we reported that two of the four histidine residues occupy a solvent-exposed environment while the remaining two appear to be associated with nonrandom structure. Only a partial assignment of the NMR resonances to specific histidine residues was obtained in that study, leaving incomplete our knowledge of the structure of this form. Recently, the application of a paramagnetic probe that forms tight complexes with histidine residues, $(NH_3)_5Ru^{III}$, to RNAase A[5,6] enabled us to obtain a derivative labeled at histidine-105.[7] By examining the proton NMR spectrum of the thermally unfolded form of this derivative, we can now complete the assignment of the histidine C-2 resonances in thermally unfolded RNAase A and thereby obtain more information on the structure of this form.

Experimental Methods

The synthesis, isolation, and characterization of the derivative of RNAase A in which a single $(NH_3)_5Ru^{III}$ complex is formed with the N-3 nitrogen of histidine-105 has been described.[5-7] As expected from model compound data,[8] this complex is extremely stable at moderate temperatures (0–50 °C) from pH 1 to 8. It decomposes at temperatures greater than 60 °C or at pH values above pH 11.[15] The samples were prepared for NMR study by two cycles of a process in which they were heated at 40 °C in 99.7% ^2H_2O (Merck Sharp and Dohme Isotopes) at pH* 2 (uncorrected meter reading) for 15 min and then lyophilized. This procedure serves to remove nitrogen protons which absorb in the same region as the histidine C-2 protons and to decrease the contribution from the solvent.

Results and Discussion

In the previous 100-MHz NMR study,[4] it was shown that the histidine C-2 region of the proton NMR spectrum of thermally unfolded RNAase A consists of two equal-area peaks, each of which represents two histidine C-2 protons. The computer fits of the pH titration curves for these peaks to the Henderson–Hasselbach equation showed that the peak with pK = 5.96 at 69 °C should appear 0.01–0.02 ppm upfield from the peak with pK = 5.75 when the pH

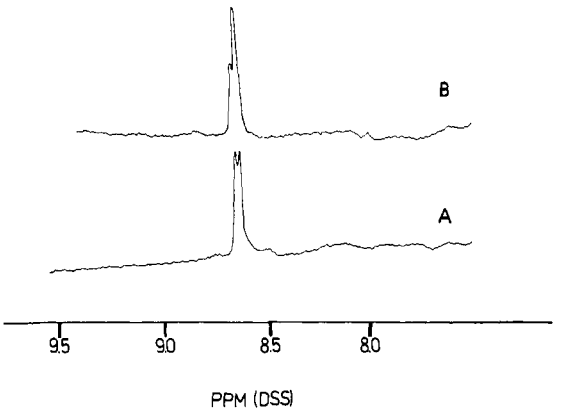

Figure 1. 200-MHz Fourier transform proton NMR spectra of unmodified RNAase A (A) and $(NH_3)_5Ru^{III}$-His-105 RNAase A (B) in ^2H_2O containing 20 mM glycine, pH* 2.3 (uncorrected meter reading), 0.2 M NaCl, 50 °C. The spectra were obtained by averaging transients of 32 768 data points for a spectral width of 3012 Hz. The acquisition time was 5.4 s and the digital resolution was 0.184 Hz/point. The spectra are the results of accumulating 1000 transients following 90° pulses. The delay between pulses, 5.4 s, was sufficient to permit complete recovery of the magnetization. The free induction decays were smoothed with an exponential function that resulted in 0.4-Hz line broadening. The chemical shifts are measured relative to the resonance of internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate. The protein concentration was ~10 mg mL^{-1}.

was less than 3 (Figure 1 in ref 4). Although these peaks could not be resolved at acidic pH at 100 MHz, they are easily resolved at 200 MHz (Figure 1). Also shown in Figure 1 is the effect of incorporating $(NH_3)_5Ru^{III}$ at histidine-105. The spectrum clearly shows that the downfield peak is reduced in amplitude by half when histidine-105 is labeled with $(NH_3)_5Ru^{III}$. Studies on a model compound, $(NH_3)_5Ru^{III}$-His, indicate that the resonances of the C-2 and C-4 protons of the histidine residue to which the ruthenium is bound are shifted away from the frequency at which they normally appear.[15]

To confirm that the histidine-105 C-2 proton can indeed be associated with the resonance whose pK value is 5.75 at 69 °C, we also obtained a spectrum of the labeled enzyme at pH 5.6, 69 °C. The results of the previous study had shown that both the resonances for the C-2 protons are shifted upfield due to deprotonation of the histidines under these conditions; however, the resonance with the lower pK value appears at higher field. In the spectrum at pH 5.6, 69 °C (data not shown), it was observed that the amplitude of the upfield peak was lower by a factor of 2 than that of the downfield peak. This result confirms the assignment of one of the two protons in the resonance whose pK is 5.75 at 69 °C to histidine-105. Since histidine-48 had already been assigned to this peak using selective deuteration techniques,[4] we conclude that histidines-48 and -105 have a pK of 5.75 in thermally unfolded RNAase A at 69 °C. By elimination histidines-12 and -119 have a pK of 5.96 at this temperature.

In the previous study,[4] the pK of histidine in the tripeptide Gly-His-Gly was found to be 6.03 at 69 °C. Assuming that this value is representative of a fully solvated histidine residue, it can be concluded that histidines-12 and -119 are freely exposed to solvent in thermally unfolded RNAase A; however, histidines-48 and -105 appear to be involved with some type of nonrandom structure. That the appearance of two peaks with differing pK values is due to nonrandom structure was demonstrated in the previous study[4] by the observation that the addition of guanidine hydrochloride to the thermally unfolded form

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resulted in a single peak in this region. In high concentrations of guanidine hydrochloride, protein structures are believed to be described closely as random coils.[9,10] The fact that the pK values for histidines-48 and -105 are lower than that of freely solvated histidine suggests that these two residues may be associated with hydrophobic clusters, which would be expected to stabilize the neutral form of histidine. The possibility that nearest neighbors lead to the observed decreases in pK values was ruled out previously.[4]

Support for the existence of hydrophobic clusters in thermally unfolded RNAase A comes from the work of Matheson and Scheraga.[11] A photoreactive reagent was used to probe the solvent accessibility of amino acid side chains in thermally unfolded RNAase A. Amino acid analysis showed that a group of ~20 amino acids, many of them hydrophobic, were resistant to modification at pH 5, 78 °C. It was suggested that a nonrandom structure arising from hydrophobic bonding among the residues of the unfolded protein could account for the unreactive core.

The postulated involvement of histidines-48 and -105 with hydrophobic clusters in thermally unfolded RNAase A may be related to the folding of this protein. Matheson and Scheraga[12] have proposed potential nucleation sites for the folding of a number of proteins, assuming that these nucleation sites involve clusters of hydrophobic amino acids. They predicted that the most stable hydrophobic cluster in RNAase A is contained in the sequence from residues 106 to 118. From the NMR evidence presented above, histidine-105 appears to be involved with this cluster; however, histidine-119 is not. In addition, histidine-48 may be associated with a second cluster in the thermally unfolded form. Although the relationship of these hydrophobic clusters to protein folding is not yet clear, the existence of such nonrandom structures in the thermally unfolded protein suggests that they may well be involved in directing the folding process. Since both X-ray[13] and NMR[14] evidence show that histidine-105 is well exposed to solvent in the native conformation, it appears that the folding of RNAase A may require the disruption of this nonrandom structure in the thermally unfolded protein.

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