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Nuclear Magnetic Resonance Study of the Thermal Denaturation of Ribonuclease A: Implications for Multistate Behavior at Low pH

(reversible unfolding/histidine/fast and slow chemical exchange)

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ABSTRACT **The thermal denaturation of ribonuclease A has been studied by use of Fourier transform nuclear magnetic resonance by monitoring the imidazole C-2 proton resonances of the histidine residues as a function of temperature at pH 1.3. As the temperature is raised, a slow chemical exchange process results in the disappearance of the peaks corresponding to the native conformation and the appearance of a single peak corresponding to histidine in the denatured state. The disappearance of the native peaks is not simultaneous, implying that at least two regions of the molecule denature at different temperatures. Also, fast chemical exchange processes result in small chemical shifts that appear to be related to local conformational changes. The observed phenomena have been shown to be reversible by the measurement of absorbance at 278 nm, enzyme activity, and nuclear magnetic resonance spectroscopy. The results of this equilibrium study support a multistate denaturation mechanism for ribonuclease A at pH 1.3.**

The denaturation of bovine pancreatic ribonuclease A (EC 2.7.7.16), for which the amino-acid sequence (1) and the three-dimensional structure in the solid state (2) are known, has been the subject of much study (3, 4). The thermal denaturation in particular has been studied by a wide variety of techniques including circular dichroism (5), electron paramagnetic resonance of covalently bound spin labels (6), the action of proteolytic enzymes on RNase A during the course of denaturation (7), thermocalorimetry (8), ultraviolet difference spectroscopy (9), and temperature-jump and pH-jump kinetic experiments (10–13).

A large body of data accumulated on the thermal denaturation of RNase A at low pH was consistent with a two-state mechanism, except for one study of the slow stage (seconds) of the unfolding kinetics (14). Recently, fast kinetic studies after tyrosine absorbance during temperature jumps have shown that there is an additional fast stage of the unfolding kinetics and provided evidence for the presence of intermediate, partly-folded states (10). The apparent contradiction between previous equilibrium results and the fast kinetic studies could be explained in terms of a model of nucleation-dependent sequential protein folding (15).

We report here an equilibrium study using Fourier transform nuclear magnetic resonance (NMR) that, in contrast to previous equilibrium studies, appears to detect intermediates in the thermal unfolding of RNase A at pH 1.3 by examination of the histidine proton resonances. The behavior of the four histidine residues, which are useful as local probes of RNase A conformation during the denaturation, implies that at least two regions of the molecule denature at different temperatures. Additionally, small local conformational changes appear to accompany the process.

Abbreviations: $(Me_3Si)_2O$ hexamethyldisiloxane; Me_2SO, dimethylsulfoxide.

MATERIALS AND METHODS

Bovine pancreatic ribonuclease A (Worthington Biochemical Corp., lot RAF 1FC) was obtained as a phosphate-free, lyophilized powder and used without further purification. D_2O (99.8%) was purchased from Stohler Isotope Chemicals and was distilled once under reduced pressure. Concentrated solutions of DCl and NaOD in D_2O (Stohler Isotope Chemicals) were diluted to 1.0 M stock solutions for pH adjustment.

The enzyme was lyophilized from dilute, salt-free solution in D_2O to minimize aggregate formation (16). Usually, 75 mg of twice-lyophilized enzyme was dissolved in 0.5 ml of a D_2O solution that was 0.2 M in NaCl (Schwartz/Mann, highly purified). RNase A samples were placed in 5-mm NMR tubes (Wilmad Glass Co., type 507-PP) followed by insertion of a thin, coaxial capillary containing an external reference. Either neat dimethylsulfoxide (Me_2SO) or hexamethyldisiloxane [$(Me_3Si)_2O$] was used as an external reference for chemical shift measurements to avoid any interactions between an internal standard and the protein. Empirically it was found that neither of these compounds has a measurable temperature-dependent chemical shift with respect to each other or to tetramethylsilane over the range studied.

RNase A concentrations were determined at 278 nm in a Cary model 14 spectrophotometer with a molar extinction coefficient of 9800 (17). Assays of enzyme activity of RNase A were performed by the differential method of Crook *et al.* (18). Relative activities of RNase A samples from denaturation runs were measured from the initial velocity of the reaction.

Proton magnetic resonance spectra were recorded on a Varian Associates XL-100 spectrometer operating at 100 MHz and equipped with Fourier transform accessory and 620i computer. The spectrometer was field-frequency locked to the deuterium resonance of solvent D_2O, and Fourier transform NMR spectra were collected by use of the software provided by Varian with a repetitive 90° pulse sequence. The spin-lattice relaxation time T_1 was measured with a $(180°—t—90°—T)_N$ pulse sequence, where T is a time long compared to T_1 and t is a variable delay time (19).

The temperature of the probe at the sample was regulated

by a variable-temperature controller (Varian Associates V-4343). We measured the temperature by replacing the sample with an NMR tube containing a copper-constantan thermocouple immersed in ethylene glycol and measuring the potential with a Leeds and Northrop millivolt potentiometer. With this system it was found that the temperature could be set to within ±0.2° and that this value usually drifted less than 0.3° over the period of a single spectrum accumulation, typically 0.5 hr. The temperature was measured before and after each spectrum was obtained and the average value was reported.

Measurements of pH were made in the NMR tube with a Beckman model 76A pH meter equipped with a long, thin combination electrode (180 × 3 mm, Instrumentation Laboratory, Inc.). The temperature of the sample was maintained the same during pH measurement as during spectrum accumulation by rapid transferral of the sample from the probe to a constant-temperature bath. The pH was measured before and after each spectrum was obtained and the average value was reported. The pH readings usually differed by less than 0.03 pH unit, and a difference larger than 0.05 pH unit was considered unacceptable. All pH values quoted are meter readings uncorrected for the effect of D_2O.

RESULTS

The four histidine C-2 proton resonances of RNase A have been assigned to histidines 12, 48, 105, and 119 in the primary amino-acid sequence (20). The chemical shifts of these protons reflect their electromagnetic environments and are a sensitive function of pH from pH 4 to 8 due to titration of an imidazole nitrogen. Since this study was performed at pH 1.3, a value below any previously studied (20, 21), the pH-titration curves were extended to pH 1 in order to assign the resonances (Fig. 1). Below pH 3 the imidazole rings of the four histidines are fully protonated. The small changes in the His 12, His 48, and His 105 shifts at low pH might be correlated with the appearance of a histidine resonance in a denatured environment and therefore reflect the acid denaturation process, which begins at about pH 3 at 37.5°.

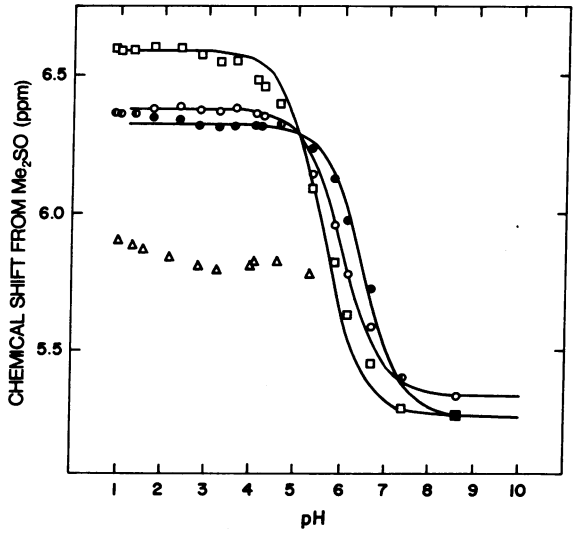

FIG. 1. Chemical shift values of the imidazole C-2 proton peaks of (○) His 12, (△) His 48, (●) His 105, and (□) His 119 as a function of pH at 37.5°. *Half-filled circles* represent overlapping points for His 12 and His 105. *Solid lines* represent best fits by the computer program MODELAIDE of a single Henderson–Hasselbach equation to histidine titration curves. The pK values obtained are as follows: pK (His 105) = 6.52, pK (His 119) = 5.62, and pK (His 12) = 6.03. A closer fit to the His 119 titration curve requires the use of multiple Henderson–Hasselbach equations (21).

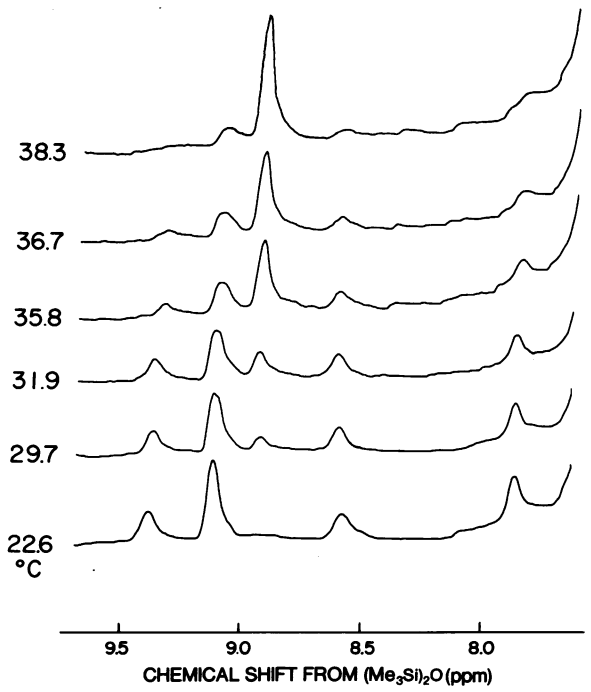

FIG. 2. Fourier transform NMR spectra of RNase A showing the histidine region as a function of temperature at pH 1.3. Each spectrum is the result of the accumulation of 600 transients. In the spectrum at 31.9° the peaks in order of increasing shift from $(Me_3Si)_2O$ are the C-4 proton peak of His 105 and the C-2 proton peaks of His 48, denatured histidine, His 12 + 105, and His 119.

Fig. 2 shows a series of NMR spectra of the histidine region of the RNase A spectrum as a function of temperature at pH 1.3. A plot of the relative areas of the imidazole C-2 proton peaks is shown in Fig. 3. The areas of the His 119, composite His 12 and His 105, and His 48 peaks show about the expected 1:2:1 relationship at low temperatures. As the temperature is raised from about 30° to 40° the areas of the His 119, His 48, and His 12 + 105 peaks decrease, while simultaneously a new peak appears whose area increases. At 40° this peak, which represents histidine in a denatured conformation, is the only one present. The areas of the peaks of His 48 and His 119 do not disappear simultaneously as a function of temperature. This fact cannot be explained by differential broadening of the His 119 peak, as the line widths of the His 119 and His 48 peaks are the same within experimental error at all temperatures. Since the area of a resonance is proportional to the concentration of protons in a particular electromagnetic environment, the data of Fig. 3 imply that RNase A undergoes a major conformational change in the 30° to 40° range. The total area of histidine C-2 proton peaks remains constant during denaturation to within 10% measuring error. This fact implies that only the histidine C-2 protons are represented by these peaks and that all four protons are

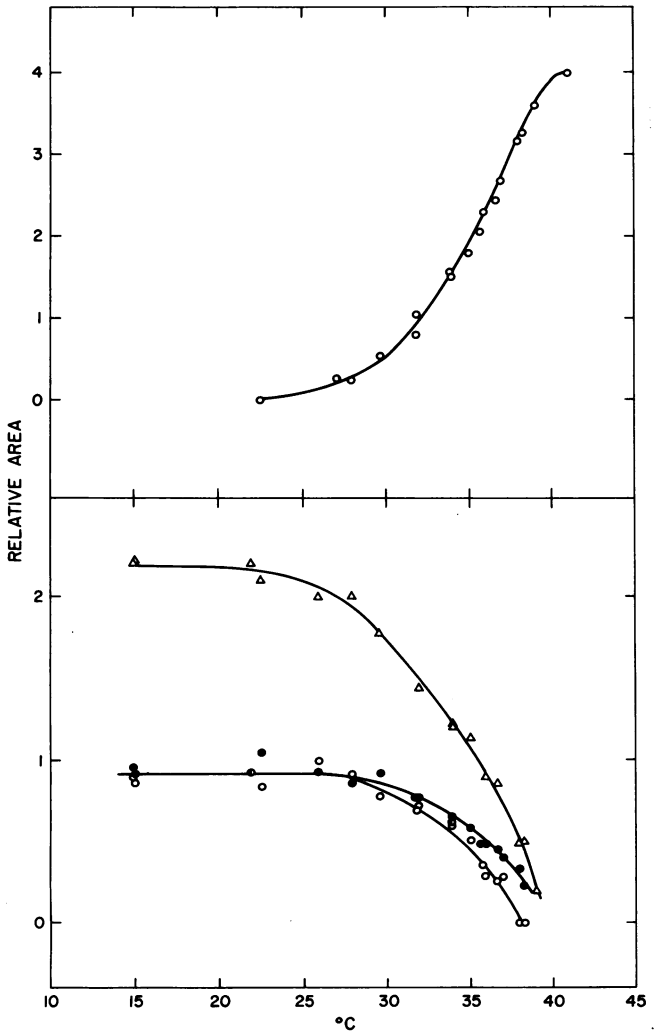

FIG. 3. Areas of the histidine imidazole C-2 proton peaks from two independent experiments as a function of temperature at pH 1.3, measured with a planimeter. The total histidine resonance area was normalized to 4.0 at each temperature. The *upper curve* represents the area of the denatured histidine resonance. The *lower curves* represent the areas of the native resonances of (△) His 12 + 105, (●) His 48, and (○) His 119. The close overlap of the His 12 and His 105 resonances prevented separation of the individual areas; the area of the composite peak is shown. *Half-filled circles* represent overlapping points for His 48 and His 119. The curves are hand-drawn approximations to the data.

in electromagnetically identical environments in the denatured conformation.

A plot of chemical shift from external $(Me_3Si)_2O$ as a function of temperature for the histidine C-2 proton peaks is shown in Fig. 4. These shifts include a small correction for the temperature-dependent bulk magnetic susceptibility of water.*

* This correction was determined by observation of the chemical shift as a function of temperature of several compounds (Me_2SO, dioxane, and *t*-butanol) whose chemical shift from the external reference was not expected to be temperature dependent. The same small downfield shift with temperature that was observed in all cases, 0.19 Hz/°C, was used as the correction.

The chemical shift of the composite C-2 proton peak in the denatured state is independent of temperature, a fact consistent with a stable denatured conformation. The chemical shift of the His 119 peak as a function of temperature demonstrates a biphasic behavior. From 10° to 30° it moves upfield; then from 30° to 40°, where the area of the native peak decreases to zero, it shifts upfield at an increased rate. The temperature dependence of the shift of the His 48 peak appears monophasic over the entire temperature range from 10° to 40°, with the peak moving downfield. The data shown in Fig. 4 for the overlapping His 12 and His 105 peaks were obtained by measurement of the shift of the composite resonance. The chemical shift displays a biphasic behavior with a break at about 30°. Close inspection of Fig. 2 shows that this peak undergoes a change in line width and line shape at higher temperatures. Initial attempts at fitting the NMR spectra with the computer program MODELAIDE suggest that the two resonances no longer overlap completely at higher temperatures.

Several studies were done to ensure that the experiments were performed under conditions where the denaturation is highly reversible. Measurements of optical absorbance at 278 nm and enzyme activity were performed on aliquots of the sample taken just before and after a series of NMR spectra were obtained. These measurements show that no appreciable loss either in concentration or activity occurred as a result of denaturation or subsequent renaturation. Complete reversibility is also observed with NMR spectroscopy. Comparison of spectra (data not shown) obtained at 35.9° during the unfolding and at 36.2° during the refolding shows no change in chemical shift, peak area, or line width for any of the imidazole C-2 proton peaks.

In order to minimize interference from the large H_2O proton signal, this study was performed in D_2O. The crystal structure of RNase A has been found to be unchanged after exchangeable protons have been replaced by deuterium (22), suggesting that the tertiary structure is the same in each case. The possibility of aggregation or other concentration-dependent effects must be considered. Under certain conditions of lyophilization, RNase A is known to aggregate; however, these aggregates can be converted to monomers by heating to 60° at neutral pH (16). Thermal denaturation of a sample of RNase A that had been previously heated at 60° for 10 min at pH 6.5 gave the same results observed when a sample was denatured immediately after lyophilization. In addition, NMR data were obtained for samples of RNase A concentrations of 10, 3, and 2 mM. Appreciable aggregation would be likely to affect the line width of the peaks, by analogy with a study of lysozyme, which shows broadening of peaks under conditions where aggregates are known to be present (23). The chemical shifts, line widths, and areas for the histidine C-2 proton resonances of native, denatured, and several partially denatured samples are the same, within experimental error, for all three RNase A concentrations. The dilution and aggregate dissociation studies both support the absence of concentration-dependent effects in these experiments.

Previous work (24) has shown that the NMR signal intensity can depend on the relative magnitudes of the time between pulses and T_1. As the T_1 values for the native and denatured resonances are less than or equal to 1.4 sec (C. R. Matthews and D. G. Westmoreland, unpublished results),

use of a 4.0-sec delay between pulses reduced this error to less than ±3%.

DISCUSSION

NMR has been applied to the study of protein denaturation in several different ways. The most powerful approach has been the preparation of selectively deuterated analogues of staphylococcal nuclease. The observation of many specific residues during the alkaline denaturation was possible and is the basis for the most detailed protein-denaturation mechanism proposed thus far (25). In another approach, study of the peak areas of individual proton resonances characterized the acid denaturation of staphylococcal nuclease as involving detectable intermediates (26).

The thermal denaturation of RNase A has been studied with NMR by following the center of gravity of a group of aromatic proton resonances (27), by observing the entire proton spectrum at 220 MHz (28), by studying the histidine C-2 proton chemical shifts at pH 5.5 (29), and by observing the carbon-13 spectrum of the native and heat-denatured protein (30). In our work the single C-2 proton resonances of the four histidines in RNase A have been used to study the thermal denaturation at pH 1.3; this study has been greatly facilitated by the use of Fourier transform NMR, which allows the rapid collection of NMR data with sufficient sensitivity to detect small changes in single proton resonances. Since the denaturation is reversible and the system is in equilibrium, the histidine residues, and thus the C-2 protons, undergo chemical exchange between sites of different electromagnetic environment corresponding to various native and denatured states.†

The chemical shift behavior of all of the histidine C-2 proton peaks is consistent with a multistate denaturation mechanism. For His 119, in the region 10°–30°, a small decrease in chemical shift with no change in peak area is the result of a fast exchange process. Since the size of this change is small compared to that occurring on complete denaturation, this shift probably represents a small, localized change in environment. In the region 30°–40°, a larger decrease in chemical shift occurs that ends about 0.40 ppm from the shift of the denatured peak, due to a decrease in peak area to zero in this region. These facts imply that in this region at least two more processes must occur: another fast exchange process accounts for the more rapid change in shift and a slow one for the decrease in area.‡ The occurrence of both a fast and a slow exchange implies that at least three protein conformations different with respect to His 119 are present in the region from 30° to 40°. The chemical shift of the His 12 + 105 peak also displays a biphasic behavior, being independent of temperature up to 30° and then decreasing from about 30° to 40°. Over the latter temperature range the area of the peak decreases to zero at a point where the chemical shift of the peak is about 0.15 ppm from the denatured peak. Although some care must be taken in interpreting the data for a composite peak, the behavior in the 30°–40° range suggests that both a fast and a slow exchange process are involved in the denaturation of these two residues. In contrast to the biphasic behavior exhibited by the shifts of His 119 and His 12 + 105, the chemical shift of His 48 appears to be a monophasic function of temperature over the range 10°–40°. It undergoes a small downfield shift that ends about 0.30 ppm from the denatured peak due to a decrease in peak area to zero in the range 30°–40°. The change in shift is due to a fast chemical exchange that may or may not be associated with the major changes that occur above 30°, since the chemical shift has a similar behavior inside and outside the temperature range of thermal denaturation. The size of the change in the shift is consistent with small, localized changes in conformation. The decreasing peak area that is also observed is due to a slow exchange process.

The fast exchange processes do not connect the native and denatured states, but rather connect the native state with

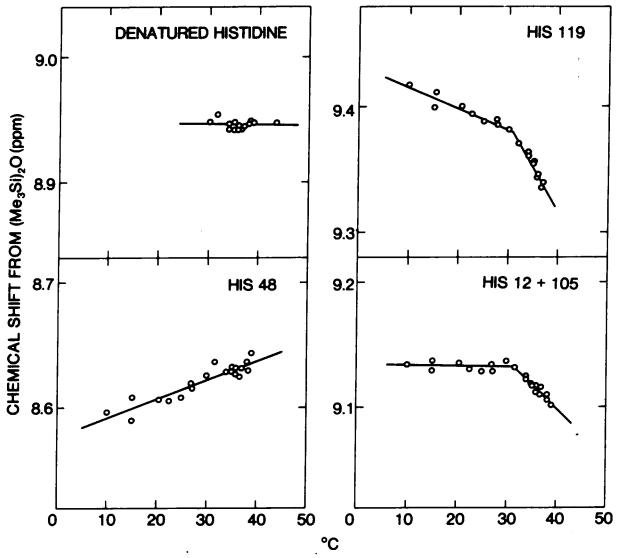

FIG. 4. Chemical shift values of the histidine-imidazole C-2 proton peaks as a function of temperature at pH 1.3 from two independent experiments. The estimated accuracy of the chemical shift values is ±0.5 Hz. The lines are only best least-squares fits to the data and are not meant to imply that the shifts must be linear as a function of temperature.

† If a proton exchanges between sites A and B then the mean lifetime τ is defined by $\tau = \tau_A\tau_B/(\tau_A + \tau_B)$ where τ_A is the lifetime in the A site and τ_B is the lifetime in the B site. A slow exchange on the NMR time scale is defined by $\tau \gg \{2\pi(\nu_A - \nu_B)\}^{-1}$ and a fast exchange is defined by $\tau \ll \{2\pi(\nu_A - \nu_B)\}^{-1}$ where ν_A is the chemical shift in site A (in Hz) and ν_B is the chemical shift in site B. For slow exchange, two peaks are observed, representing the two different electromagnetic environments. The area of each peak is proportional to the population of protons in the respective environments. The areas may change due to perturbations affecting the relative populations, but the chemical shift of each peak does not change. For fast exchange, a single peak occurs at a chemical shift intermediate to those of the two environments and varies according to the relative populations of the two environments. The area is proportional to the total concentration of protons in the two environments and therefore does not vary as the relative populations change.

‡ The requirements for observing a fast or a slow chemical exchange given in the previous footnote yield inequalities for the rate processes observed for His 119; the inequalities obtained for processes involving the other peaks are comparable. For His 119, the slow exchange has a mean lifetime of the order of 50 msec or longer and the fast exchange has a mean lifetime of the order of 3 msec or shorter.

states in which the electromagnetic environment of the histidine C-2 protons is intermediate between that of the native and denatured states. This conclusion is drawn from the fact that the extrapolation of the chemical shift behavior of the histidine peaks in the range 30°–40° intersects the shift of the denatured peak at 90° or higher. A slow exchange process that occurs along with the fast processes explains the changing peak areas and the failure of the chemical shifts of the native peaks to intersect the denatured peak at 40°.

A significant feature of the data shown in Fig. 3 is the disappearance of the areas of the His 48 and His 119 peaks at temperatures 1–2° apart, which implies that the portion of the polypeptide chain containing His 119 denatures before the portion of the chain containing His 48. The area of the composite His 12 + 105 peak maintained roughly a 2:1 ratio to the area of the His 48 resonance at temperatures near 37°, possibly but not necessarily implying a common melting temperature for all three resonances.

The plot of denatured histidine-peak area as a function of temperature correlates well with data obtained by other techniques that attempt to measure the average extent of unfolding. Measurement of the van't Hoff enthalpy at the temperature of half-conversion from the upper curve of Fig. 3 yielded a value of about 70 kcal/mol which compares closely with the value of 65 kcal/mol obtained from thermocalorimetry measurements (8).

The midpoint of the transition of the denatured peak in D_2O is about 36°, a value about 3° higher than that obtained by optical absorbance in H_2O (10). This fact is consistent with an optical rotation study of RNase A in H_2O and D_2O (31), which showed that the thermal transition in D_2O exhibited a similarly shaped curve with a transition temperature about 4° higher than that in H_2O at pH 4.3.

Studies of single-proton resonances of proteins by NMR have been shown to be a useful tool for the study of protein denaturation (23, 25, 26, 29, 32). In the case of thermal denaturation of RNase A, information is potentially available concerning the sequence of events during unfolding at different pH values. Further studies of the thermal denaturation of RNase A at other pH values are in progress.

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