Nuclear Magnetic Resonance Studies of Residual Structure in Thermally Unfolded Ribonuclease A†

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ABSTRACT: A proton nuclear magnetic resonance study of the four histidine residues of thermally unfolded ribonuclease A has provided evidence that two of the residues are in regions of residual structure, whereas the other two are freely exposed to solvent. Histidine-48 and, tentatively, histidine-105 occupy an environment at 69° characterized by residual structure and display a pK value of 5.75 and a spin–lattice relaxation time of about 0.8 sec at pH 5.5. Histidine-12 and, tentatively, histidine-119 are in an environment at 69° which is freely accessible to solvent and show a pK value of 5.96 and a spin–lattice relaxation time of about 1.1 sec at pH 5.5.

A complete understanding of the reversible thermal unfolding transition observed in many globular proteins (Tanford, 1968; Wetlaufer and Ristow, 1973; Baldwin, 1975) requires information on the structure of the unfolded form as well as the structures of intermediates. For a number of proteins, including ribonuclease A (RNase A), lysozyme, and chymotrypsinogen A, the thermally unfolded conformation has been shown to contain residual structure (Bigelow, 1964; Aune et al., 1967) as judged by further changes in uv absorbance, optical rotation, or intrinsic viscosity upon the addition of chemical denaturants such as guanidine hydrochloride. In general, these physical techniques do not permit the assignment of these changes to specific residues in the protein, making it difficult to determine whether the residual structure consists of a single or multiple forms and whether the structure is localized or widespread. Recent kinetic results (Garel and Baldwin, 1973, 1975a,b) have demonstrated that at low pH thermally unfolded RNase A is an equilibrium mixture of fast and slowly refolding forms.

Nuclear magnetic resonance (NMR) spectroscopy is an effective technique for studying specific, assignable regions of a protein, since the resonance frequency of a nucleus is sensitive to its environment. The unfolding transitions of RNase A (Westmoreland and Matthews, 1973; Roberts and Benz, 1975a,b), staphylococcal nuclease (Epstein et al., 1971; Jardetzky et al., 1971), and lysozyme (McDonald et al., 1971) have been studied by NMR by examining the resonances of individual protons in the protein. The four histidine C-2 proton resonances of native RNase A are well resolved from the remainder of the spectrum and have been assigned to the four histidine residues (Meadows et al., 1968). Of particular interest to the study of unfolded proteins is the previous observation that this same region in the spectrum of thermally unfolded RNase A shows complexity not predicted for a cross-linked random coil (Roberts and Benz, 1973; Benz and Roberts, 1975a; C. R. Matthews and D. G. Westmoreland, unpublished results).

The aims of this study were to investigate the factors that give rise to the multiple peaks in the histidine region of the NMR spectrum of thermally unfolded RNase A, to assign these peaks to specific histidine residues, and finally to obtain information on the residual structure of thermally unfolded RNase A.

Experimental Procedure

Fourier transform proton NMR spectra were collected on a Varian Associates XL-100 spectrometer operating at 100 MHz using a repetitive 90° pulse sequence. Measurements of the spin–lattice relaxation time, T_1, were made using a repetitive (180°–τ–90°–T) pulse sequence, where T is a time long compared to T_1 ($T \geq 3T_1$) and τ is a variable delay time (Vold et al., 1968). Since peak areas in spectra obtained by Fourier transform spectroscopy can depend on the relative magnitudes of the total time between pulses and the value of T_1 for the proton of interest, a 4.0-sec delay between pulses was used to reduce error in peak area determination from this source to less than 3% for the histidine C-2 protons of RNase A. Further details on experimental procedures have been published previously (Matthews and Westmoreland, 1973).

RNase A was purchased from Worthington Biochemical Corp. as a phosphate-free, lyophilized powder (grade RAF). Glycyl-L-histidylglycine (Gly-His-Gly) was purchased from Sigma Chemical Co. and guanidine hydrochloride (Gdn·HCl)[1] was purchased from Schwarz/Mann (ultra-pure). Guanidine deuteriochloride (Gdn·^{2}HCl) was prepared from Gdn·HCl by six lyophilizations from ^2H_2O.

Prior to experiments, the amide protons of RNase A were exchanged for deuterium by incubation in ^2H_2O at pH 6.5 and 60° for 10 min. This procedure decreases the background in the histidine region of the spectrum and also breaks up aggregates. The enzyme samples were then lyophilized twice from ^2H_2O and routinely made up to a 3.5% concentration in 0.2 M NaCl solution in ^2H_2O for high temperature studies. All pH values quoted are meter read-

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[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; Gdn·^{2}HCl, guanidine deuteriochloride.

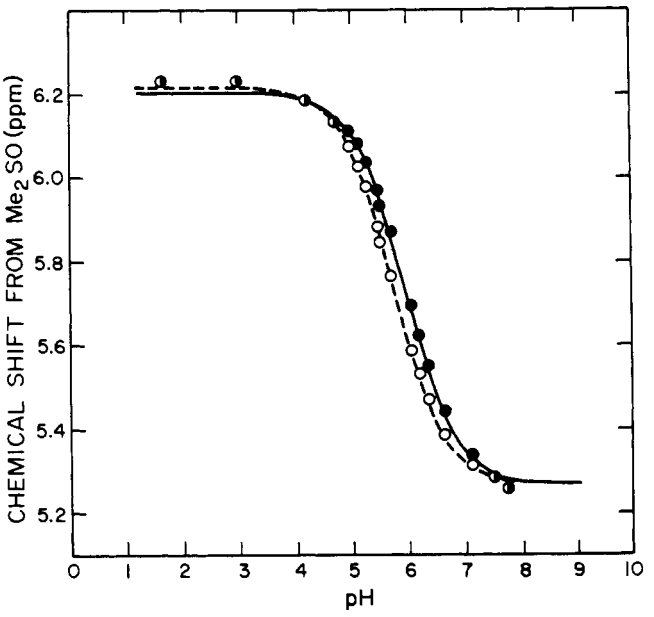

FIGURE 1: Chemical shift values of the histidine C-2 proton peaks of RNase A as a function of pH at 69°. The upfield peak (O - - - O) and the downfield peak (●—●) appear to have equal area and line width over the pH range from 4 to 7 where both are observable. (◑) Overlapping points for the two peaks. The lines represent best fits by the computer program MODELAIDE (Shrager, 1970) of a single Henderson-Hasselbalch equation to the titration curves, giving pK values of 5.96 ± 0.05 for the downfield peak and 5.75 ± 0.05 for the upfield peak. The chemical shift values were measured relative to an external capillary of dimethyl sulfoxide (Me_2SO) with an estimated accuracy of ±1 Hz.

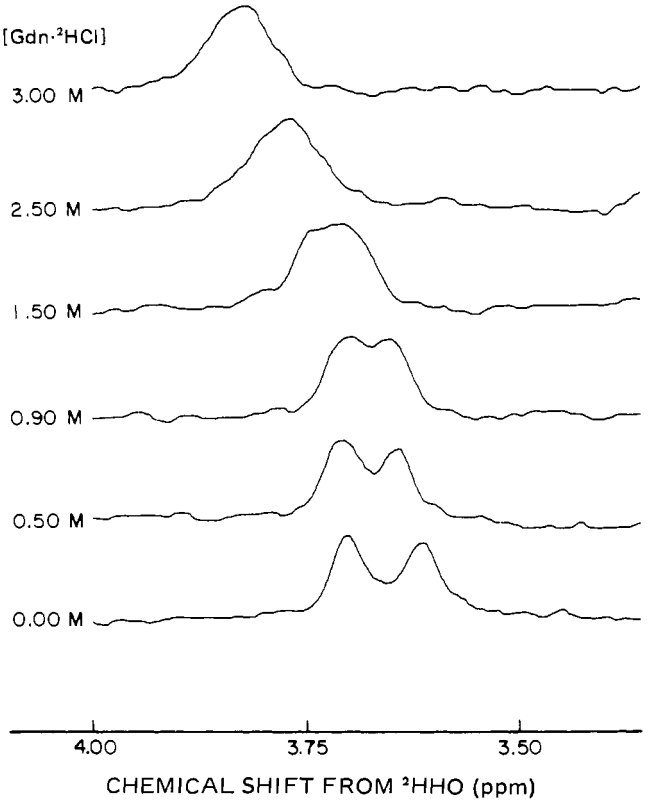

FIGURE 2: A series of Fourier transform NMR spectra of the histidine C-2 proton region of the spectrum of RNase A at 69° and pH 5.5 showing the effects of increasing concentration of Gdn·^{2}HCl. The chemical shift values were measured relative to the residual ^{2}HHO peak for convenience; the shift of this peak may be a function of Gdn·^{2}HCl concentration. The spectra shown are the results of the accumulation of between seven and ten blocks of 30 transients each.

ings uncorrected for the effect of ^2H_2O, and were obtained at the same temperature at which the NMR spectra were accumulated.

Selective exchange of the histidine C-2 protons of RNase A for deuterium was performed under two different sets of conditions. The first method has been described by Bradbury and Chapman (1972) and involves the incubation of a 1% solution of RNase A in ^2H_2O at pH 8.5 and 37° for up to 5 days (method I). The second set of conditions used has been described by Markley and Cheung (1973) and involves the incubation of a 5% solution of RNase A in 0.2 M NaCl in ^2H_2O at pH 6.5 and 40° for up to 11 days (method II). After dialysis and concentration by lyophilization, NMR spectra of partially deuterated protein samples were obtained under two different sets of conditions in order to measure the individual histidine C-2 proton peak areas. Spectra obtained at pH 5.4 and 40° of the native conformation allowed accurate measurement of the histidine-119 and histidine-105 peak areas; spectra obtained at pH 2.0 and 24°, also of the native conformation, allowed accurate measurement of the histidine-48 and histidine-12 peak areas. The number of protons in each peak was calculated by comparing the area of each peak to the total area of the peaks in the nearby aromatic region, which contains a known number of nonexchanging protons.

The conclusions drawn on the structure of the thermally unfolded protein depend upon correct assignment of these histidine resonances; therefore, it should be pointed out that the original assignment (Meadows et al., 1968) has recently come into question (Markley, 1975; Patel et al., 1975). Evidence from these reports indicates that the histidine C-2 resonances of histidines-48 and -105 were correctly assigned; however, the assignment of the resonances of the two active site histidines, 12 and 119, should be reversed. In this paper it will be assumed that the revised assignment is correct.

Results

The pH dependences of the chemical shifts of the histidine C-2 proton resonances of thermally unfolded RNase A at 69° are shown in Figure 1. At 69° RNase A is thermally unfolded between pH 1 and 8 (Hermans and Scheraga, 1961; Tsong et al., 1970). Below pH 4, where the imidazole rings are completely protonated, all four C-2 proton resonances appear in a single peak. From pH 4 to 7, where the deprotonation of the imidazole rings results in large upfield changes in the chemical shifts of the C-2 protons, the single peak separates into two peaks whose areas and line widths are equal within experimental error (±10%) over the entire range. The chemical shift change of each of the two peaks is satisfactorily explained by a single protonation-deprotonation equilibrium. Above pH 7, where the imidazole rings are unprotonated, the two peaks appear to coalesce into a single peak representing all four protons. Only a few data points could be obtained above pH 7, where unfolded RNase A undergoes irreversible aggregation.

Since peak area is directly proportional to proton concentration, the two resonances of equal area show that two of the histidine residues have a pK of 5.75 ± 0.05 at 69°, while the other two have a pK of 5.96 ± 0.05 at 69°. These two resonances can be further characterized by their spin-lattice relaxation times. At pH 5.5 and 69°, the peak of pK = 5.75 has a T_1 of 0.8 ± 0.1 sec and the peak of pK = 5.96

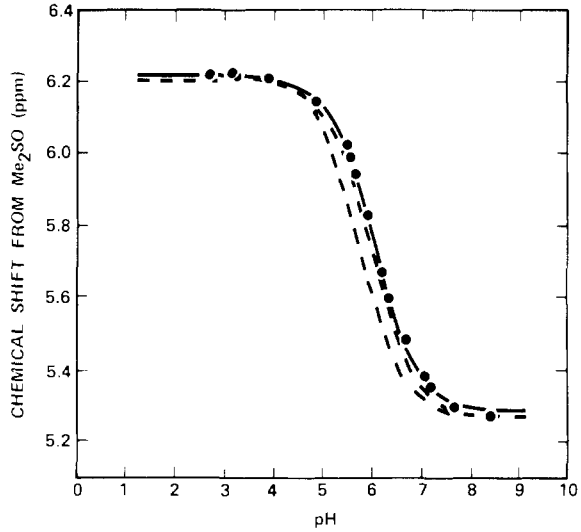

FIGURE 3: The pH dependence of the chemical shift of the histidine C-2 proton of Gly-His-Gly at 69° in 0.2 *M* NaCl in ^2H_2O. (—) Best computer fit to a single Henderson–Hasselbalch equation, giving a p*K* value of 6.03 ± 0.05. The titration curves (- - -) for the histidine C-2 protons of thermally unfolded RNase A are shown for comparison. The chemical shift values were measured relative to an external capillary of Me_2SO with an estimated accuracy of ±1 Hz.

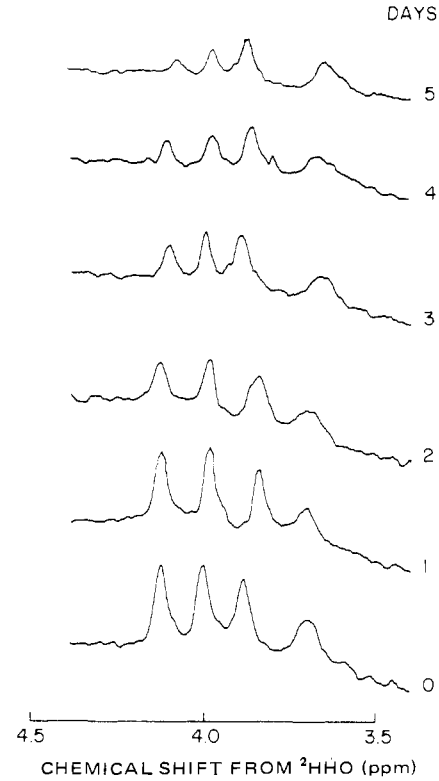

FIGURE 4: Fourier transform NMR spectra of the histidine region of RNase A showing exchange of the histidine C-2 protons for deuterium as a function of time at 37° and pH 8.5. All spectra shown were obtained at 40° and pH 5.4 and are the result of the accumulation of between 23 and 40 blocks of 30 transients each. The peaks in order of increasing shift from ^{2}HHO are the C-2 proton peaks of histidine-48, -12, -119, and -105.

has a T_1 of 1.1 ± 0.1 sec. It is difficult to determine accurate T_1 values and to make meaningful error estimates under these experimental conditions because of such possible factors as deuterium exchange, irreversible denaturation, and paramagnetic contamination (Wasylishen and Cohen, 1971). However, the data clearly show that the peak of p*K* = 5.96 has a longer T_1 than the peak of p*K* = 5.75.

Since the addition of Gdn·HCl to thermally unfolded proteins has been observed to cause further changes in optical and hydrodynamic properties of RNase A (Bigelow, 1964; Aune et al., 1967), the effect of this chemical denaturant on the NMR spectrum was studied. Figure 2 shows a series of NMR spectra of the histidine region at pH 5.5, demonstrating the effect of titrating thermally unfolded RNase A with Gdn·^{2}HCl. Increasing concentrations of Gdn·^{2}HCl change the chemical shift of one or both of the peaks until, at approximately 3.0 *M* Gdn·^{2}HCl, only one peak is observed.

The titration of a model histidine compound was performed under identical conditions to determine which, if either, of the two environments for the histidines of thermally unfolded RNase A resembles that of a completely solvated histidine. The pH dependence of the chemical shift of the histidine C-2 proton in the tripeptide Gly-His-Gly is shown in Figure 3. The pH dependence can be explained by a simple protonation–deprotonation equilibrium with a p*K* of 6.03 ± 0.05, a value within experimental error of the larger p*K* found for two of the histidines in thermally unfolded RNase A.

The environment of the histidines with the abnormal p*K* value was studied by observing the effect of NaCl concentration on the difference in resonance frequency for the two histidine peaks at 69° and pH 5.9. Near this pH value the peaks exhibit their maximum separation of 0.10 ppm and any ionic-strength dependent changes in p*K* should be evident. No change was observed in the chemical shift difference between the peaks over an eightfold range in NaCl concentration from 0.05 to 0.40 *M*, indicating that the low p*K* value for the two residues is not due to electrostatic interactions with nearby charged groups.

The two peaks in the histidine region of the spectrum of thermally unfolded RNase A may be assigned to specific histidine residues by exchanging the histidine C-2 protons for deuterium under conditions where the exchange rate is not identical for all four histidines. A series of NMR spectra demonstrating the effects of selective deuterium exchange on the histidine C-2 proton region of RNase A are shown in Figure 4. The NMR spectrum of the sample after 5 days of exchange according to method I showed peak areas corresponding to 0.57, 0.60, 0.18, and 0.32 proton at the C-2 positions of histidines-12, -48, -105, and -119, respectively. The NMR spectrum of the unfolded selectively deuterated protein at 69° and pH 5.5 showed two peaks of equal area (Figure 5). The number of protons represented by the two resonances in the unfolded, selectively deuterated protein (1.68 protons) agreed with the number of protons represented by the sum of the histidine C-2 resonances in the native conformation (1.67 protons). The NMR spectra of these samples after cooling to 45° and adjustment to pH 5.4 were found to be the same as those obtained prior to unfolding; therefore, the thermal unfolding was completely reversible and no appreciable exchange of the histidine C-2 protons for deuterium occurred during the experiment.

Table I lists the number of protons in the upfield and downfield peaks of the thermally unfolded, partially deuterated protein for all possible assignments of the histidine C-2 protons to these peaks. Comparison of the experimental results with these predicted values suggests that assignment 4 or 5 is correct, with histidine-48 and -105 in one environ-

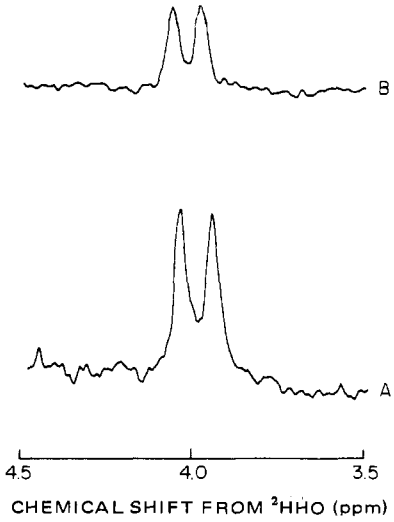

FIGURE 5: Fourier transform NMR spectra of thermally unfolded RNase A showing the histidine C-2 proton region at pH 5.5 and 69°. (A) The spectrum of unexchanged RNase A; (B) the spectrum of RNase A treated at pH 8.5 and 37° in ^2H_2O for 5 days. Each spectrum is the result of the accumulation of 10 blocks of 30 transients each.

ment and histidine-12 and -119 in the other. This partial assignment is not unique, since assignments 2 and 6, which place histidine-119 and -48 in one environment and histidines-105 and -12 in the other, also fall within the range defined by the experimental error. The assignment of histidine-48 and histidine-12 to the same peak (assignments 1 and 3) can be ruled out.

The assignment of histidine-48 to one of the two peaks was made by preparing a sample of RNase A selectively deuterated under the conditions of method II. After 11 days, it was found that histidines-12, -48, -105, and -119 were represented by peaks with areas of 0.19, 0.78, 0.09, and 0.00 proton, respectively. At 69° only a single peak was observed in the histidine region of the NMR spectrum of this selectively deuterated RNase A. The titration curve of this peak, whose area was 0.90 proton, is accurately described by a simple protonation–deprotonation equilibrium with a pK of 5.64 ± 0.06. This value is equal within experimental error to the smaller pK found for two of the histidines in unmodified thermally unfolded RNase A (Figure 6). Therefore, histidine-48 is assigned to the peak with a pK value of 5.75 and, with the results of the previous selective deuteration, histidine-12 is assigned to the peak with a pK value of 5.96.

The NMR spectrum of the thermally unfolded form of RNase A selectively deuterated by method II is predicted to contain two peaks of unequal area; however, the signal-to-noise ratio that was obtained on these samples was not sufficient to detect a peak with an area of 0.2 proton or less. Peaks of this area could be observed in the spectrum of the native conformation since, at the lower temperatures used to obtain the spectrum of the native state (temperature < 40°), a two- to threefold higher enzyme concentration could be used without aggregation. The low signal-to-noise ratio also precluded the definitive assignment of histidine-105 and -119.

Discussion

Previous studies on thermally unfolded RNase A have demonstrated the presence of residual structure. Bigelow (1964) concluded on the basis of ultraviolet absorbance

Table I: The Possible Distributions of the Histidine C-2 Protons in the NMR Spectrum of the Thermally Unfolded Form of Selectively Deuterated RNase A.[a]

	Assignment		No. of Protons[b]	
	Downfield Peak (pK = 5.96)	Upfield Peak (pK = 5.75)	Downfield Peak (pK = 5.96)	Upfield Peak (pK = 5.75)
1.	12, 48	105, 119	1.17 ± 0.17	0.50 ± 0.07
2.	12, 105	48, 119	0.75 ± 0.11	0.92 ± 0.13
3.	105, 119	12, 48	0.50 ± 0.07	1.17 ± 0.17
4.	12, 119	48, 105	0.89 ± 0.13	0.78 ± 0.11
5.	48, 105	12, 119	0.78 ± 0.11	0.89 ± 0.13
6.	48, 119	12, 105	0.92 ± 0.13	0.75 ± 0.11
Experimental observation			0.84 ± 0.08	0.84 ± 0.08

[a] Selectively deuterated under conditions of method I. [b] Calculated from data given in the text for RNase A treated for 5 days at pH 8.5 and 37°.

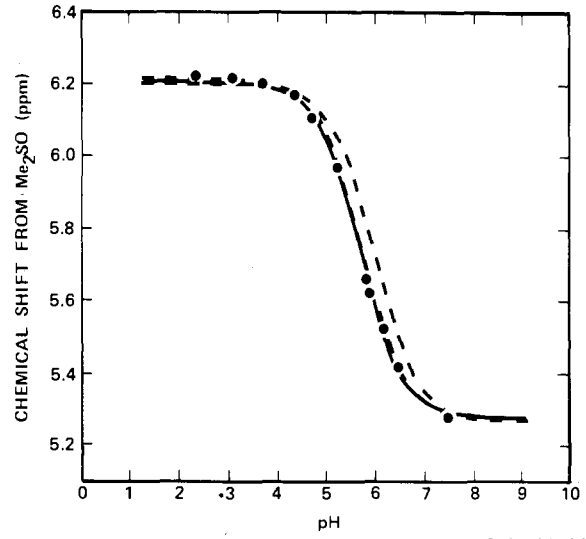

FIGURE 6: The pH dependence of the chemical shift of the histidine C-2 proton of histidine-48 in selectively deuterated RNase A at 69° in 0.2 M NaCl in ^2H_2O. The solid line represents the best computer fit to a single Henderson–Hasselbalch equation, giving a pK value of 5.64 ± 0.06. The titration curves (- - -) for thermally unfolded RNase A are shown for comparison. The chemical shift values were measured relative to an external capillary of Me_2SO with an estimated accuracy of ±1 Hz.

studies that one of the three tyrosine residues that are buried in the native state remains incompletely solvated in the thermally unfolded conformation. The results of a study by Li et al. (1966) suggest that this single remaining buried tyrosine residue is tyrosine-97. Aune et al. (1967) reported a cooperative change in the optical rotation of thermally unfolded RNase A upon the addition of Gdn·HCl, and inferred that Gdn·HCl had completed the unfolding of RNase A to a cross-linked random coil. They estimated that from one-fourth to one-third of the protein is involved in the residual structure.

The chemical shift of a histidine C-2 proton is sensitive both to the electromagnetic environment and to the state of protonation of the imidazole ring (Meadows et al., 1968). Therefore, residual structure in thermally unfolded RNase A in the vicinity of the imidazole rings might be expected to have a discernible effect on the chemical shifts by providing either different electromagnetic or different electrostatic environments for individual histidines.

The NMR studies of thermally unfolded RNase A show

that histidine-12 and, tentatively, -119 have a pK value very similar to histidine in Gly-His-Gly and appear to be as freely exposed to solvent as is possible for an amino acid in a polypeptide chain. Histidine-48, and, tentatively, -105 have a lower pK value and appear to be involved in residual structure. Although interpretation of the T_1 values must be made with caution, a shorter T_1 value for the peak containing histidine-48 and -105 is consistent with a more restricted environment for these residues.

Tanford et al. (1966, 1967) have shown that in 6 M Gdn·HCl the conformations of many proteins closely resemble a random coil or, for proteins with disulfide linkages such as RNase A, a cross-linked random coil. If the presence of two peaks in the NMR spectrum of the histidine region is indicative of residual structure, then the addition of Gdn·^{2}HCl should have a significant effect on the appearance of the spectrum. Observation of a single peak at high Gdn·^{2}HCl concentration indicates that all four histidine C-2 protons occupy a uniform electromagnetic environment and that the imidazole rings are protonated to the same extent. This finding is consistent with a cross-linked random coil configuration (McDonald and Phillips, 1969) and indicates that the residual structure in the vicinity of two of the histidines has been disrupted. Two peaks have been observed in the histidine region of the proton NMR spectrum of RNase A at pH 5.5 and 67° (Roberts and Benz, 1973; Benz and Roberts, 1975a). These workers reported that in urea only a single peak was apparent.

The titration curves shown in Figure 1 contain significant information on the local environments for the two types of histidines in thermally unfolded RNase A. The two curves have indistinguishable chemical shifts in the acidic pH region and in the basic pH region, and each is described satisfactorily by a simple protonation–deprotonation equilibrium. These observations imply that while those factors that determine the ionization constants are different for the two types of histidines, the electromagnetic environments are identical over the whole pH range from 2 to 8. The presence of two peaks between pH 5 and 7 can be attributed entirely to different states of protonation of the imidazole rings. Those factors such as charge, hydrogen bonding, or magnetic anisotropy that might have been expected to affect the chemical shift of individual histidine C-2 protons by providing different local electromagnetic environments do not appear to have a significant effect. Also, the electromagnetic environment of the two types of residues is quite similar to that observed for histidine in the Gly-His-Gly tripeptide (see Figure 3).

The low pK value (5.75) observed for two of the histidines does not appear to arise from electrostatic interactions with nearby charged groups, since the difference between the chemical shifts of the two peaks at pH 5.9 and 69° is independent of NaCl concentration over the range from 0.05 to 0.40 M. It is possible that the decrease in the pK value may be caused by the interaction of these two residues with a group of uncharged residues that stabilize the neutral form of the imidazole ring. The fact that a high concentration of Gdn·^{2}HCl is required to disrupt it demonstrates the stability of the residual structure (Figure 2).

The effect of NaCl concentration on pK values in the unfolded state observed in this work can be compared to the results of a study by Rüterjans and Witzel (1969) on the effect of NaCl concentration on the pK values of three of the histidine residues of RNase A in the native conformation. They found that the pK values of the two active site histidines, 12 and 119, increased 1.0 and 0.7 pK unit, respectively, as the salt concentration was varied from less than 0.1 to 0.4 M. This result was explained by a decreasing electrostatic effect from a nearby positively charged residue (possibly lysine-41) at higher salt concentrations. It is possible that at least a part of the change in the pK values in the native state is caused by specific binding of chloride ions to RNase A (Loeb and Saroff, 1964).

The chemical shift of the C-2 proton of protonated histidine-48 in the thermally unfolded enzyme is identical with that for the histidine proton in the Gly-His-Gly tripeptide (see Figure 3). In the native form of RNase A at low pH, the resonance from the histidine-48 C-2 proton is shifted 0.3–0.4 ppm upfield from the frequency predicted from histidine model compounds. Thus the electromagnetic environments of histidine-48 are quite different in these two conformations. It has previously been suggested (Meadows et al., 1967, 1969) that the upfield shift of the histidine-48 resonance in the native conformation may be due to the proximity of the aromatic ring of tyrosine-25, which X-ray studies (Kartha et al., 1967; Wyckoff et al., 1970; Carlisle et al., 1974) have shown is near histidine-48. The data presented above suggest that the thermal unfolding results in the disruption of the noncovalent forces that place these two residues in proximity. This conclusion is consistent with the results of previous studies (Bigelow, 1964; Li et al., 1966) which reported that thermal unfolding transfers tyrosine-25 to a more hydrophilic environment.

Other groups capable of titrating in the pH range from 2 to 8 at 69° do not appear to be in close proximity to any of the histidines, since the pH dependence of the chemical shifts of all four histidine residues is satisfactorily explained by a simple protonation–deprotonation equilibrium (Figure 1). This conclusion is based on comparison with NMR studies of histidine titrations in model compounds (Sachs et al., 1971; Shrager et al., 1972) and in RNase A (Schechter et al., 1972). Perturbations of the histidine titration curves in those instances were attributed to the titrations of nearby carboxyl and amino groups.

The influence on chemical shifts and pK values of the nearest neighbors of the histidines in the primary sequence is not significant. The negative charge on the carboxyl group of glutamic acid-49, adjacent to histidine-48, might have been expected to deshield the histidine-48 C-2 proton and result in a downfield shift. This effect would have been predicted to diminish when the glutamic acid is neutralized between pH 4 and 5. Neither of these effects on the chemical shift was observed nor was the predicted increase in pK value observed. In fact, the pK value is decreased and is also unaffected by NaCl concentration, implying that effects by nearby charged groups are not significant. Histidine-105 is adjacent to lysine-104, but the C-2 proton does not show any upfield shift due to shielding caused by this positively charged group. Also, its pK value is unaffected by NaCl concentration. Histidine-119 is adjacent to phenylalanine-120; however, no upfield or downfield shift of the C-2 proton by the anisotropic magnetic field of the aromatic ring was observed.

These conclusions concerning some of the groups involved in the residual structure in the vicinity of the two abnormal histidines would seem to preclude close proximity to amino acids that titrate in this pH region (glutamic acid and aspartic acid), charged amino acids (arginine and lysine), and amino acids possessing anisotropic magnetic fields (tyrosine and phenylalanine). The residual structure

observed would therefore appear to be composed of neutral or hydrophobic amino acids which form a stable structure that minimizes their interaction with the solvent. Benz and Roberts (1975a) observed in RNase A at 67° and pH 5.5 that the resonance from the methyl groups of valine, leucine, and isoleucine had less than the intensity found in concentrated urea or Gdn·^{2}HCl. This finding is consistent with the hypothesis that hydrophobic residues are involved in residual structure.

The change in pK of a given histidine residue in RNase A upon thermal unfolding can be estimated if the Gly-His-Gly tripeptide is assumed to be a suitable model compound for completely solvated histidines such as histidine-12 or -119. The pK value for histidine in Gly-His-Gly at 32° can be interpolated from the value at 69° (6.03 ± 0.05) and the value measured at 25° (6.75 ± 0.05) and was calculated to be 6.62 ± 0.05. In native RNase A at 32°, Westmoreland et al. (1975) reported pK values of 6.60 ± 0.02, 6.15 ± 0.05, and 6.04 ± 0.03 for histidine-105, -119, and -12, respectively. At the same temperature (32°) the maximum differences between the pK values for histidine residues in the native conformation, 0.56 pK unit between 12 and 105, is comparable to the difference between the values for histidine-12 in the native and thermally unfolded conformations, 0.58 pK unit. The similarity of the differences between pK values in the native form and between pK values for the same residue in the native and unfolded forms emphasizes the importance of the local environment in determining the pK value (Tanford and Roxby, 1972).

X-Ray crystallographic studies of the native conformations of RNase A (Kartha et al., 1967; Carlisle et al., 1974) and RNase S (Wyckoff et al., 1970) have shown that the peptide backbone nitrogen and oxygen of histidine-48 are involved in an anti-parallel β pleated sheet structure, and that the imidazole ring has a low accessibility to solvent. It is possible that the residual structure involving this residue is a portion of the original structure from the native conformation (not including tyrosine-25) that is resistant to thermal unfolding. This residual structure near histidine-48 may direct the refolding of thermally unfolded RNase A to the native conformation.

The X-ray studies have also shown that in the native conformation histidine-105 is well exposed to solvent. A similar conclusion was reached in a solution phase study of the ionization constants of the histidines in native RNase A (Westmoreland et al., 1975). In thermally unfolded RNase A, however, this residue has been tentatively identified as one of the two histidines involved in residual structure. If this tentative assignment is confirmed in subsequent studies, the refolding of RNase A to the native form may thus require the disruption of residual structure in the vicinity of histidine-105.

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Carbon-13 Nuclear Magnetic Resonance Spectroscopy of [2-^{13}C]Carboxymethylcytochrome *c*†

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ABSTRACT: Horse heart cytochrome *c* has been carboxymethylated under various reaction conditions using [2-^{13}C]bromoacetate. Direct analysis of reaction products using ^{13}C nuclear magnetic resonance spectroscopy shows that the protein can be much more extensively modified than has previously been assumed. The proximity of one carboxymethylmethionine residue to the paramagnetic center of the ferric protein allows it to be distinguished from a more constant carboxymethylmethionine residue on the basis of the chemical shift of its labeled methylene group. Refolding of cytochrome *c* after alkylation at low pH apparently gives a different configuration of modified methionine residues within the protein compared to that produced by alkylation at neutral pH in the presence of cyanide.

Cytochrome *c* is a small protein with a covalently linked heme prosthetic group which plays a major role in the electron transport mechanism associated with mitochondrial oxidation of organic acids. Since its biological function has been well defined and its structure at the amino acid sequence level determined for many widely varied animal and plant species, cytochrome *c* has become a prominent model protein system for the study of structure–function relationships. When this protein is perturbed from its native configuration the associated changes in biological activity, spectroscopic properties, and hydrodynamic properties give valuable clues in the still unsolved puzzle of how the molecular structure accommodates a functional requirement for electron transfer.

One of the first experimental techniques used for this purpose was selective chemical modification of native cytochrome *c*. Schejter and George (1965) found that carboxymethylation of cytochrome *c* in the presence of cyanide yielded a product with pH dependent spectral properties different from that of the native protein. Subsequently, carboxymethylation has become a prominent experimental tool in the study of cytochrome *c* and has greatly enhanced understanding the importance of the methionine-80 residue in the electron transfer function. Dramatic changes in electronic, ligand binding, and biological properties accompanying carboxymethylation at the sulfur of methionine-80 has made this particular residue the center of attention although other residues are also susceptible to alkylation.

The reaction product obtained by carboxymethylation under various conditions has been analyzed for carboxymethyl derivatives, either directly, or indirectly by loss of the parent amino acid. These methods require chemical degradation of the reaction product under conditions which could destroy some carboxymethyl derivatives, and has led to a variety of descriptions of reaction product from essentially equivalent preparations. Positive detection of some carboxymethyl derivatives of amino acids from carboxymethylcytochrome *c* in addition to *S*-carboxymethylmethionine has been reported by Babul and Stellwagen (1972): N^{δ}-carboxymethylhistidine, N^{ϵ}-carboxymethylhistidine, N^{δ},N^{ϵ}-dicarboxymethylhistidine, and N^{ϵ},N-dicarboxymethyllysine.

In order to determine the extent of carboxymethylation of cytochrome *c* directly without chemical degradation of the modified protein we have used [2-^{13}C]bromoacetate as the alkylating agent and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy for product analysis. As will be seen in the results presented here, the alkylated protein contains N^{ϵ}-monocarboxymethyllysine and glycolate esters in addition to those amino acid modifications previously detected.

Experimental Section

Sources of Materials. Bromoacetic acid enriched to 90+ atom % ^{13}C in the methylene carbon was a gift from Vernon Kerr and Donald Ott of The Los Alamos Scientific Laboratory. Amino acid and peptide derivatives were obtained from Foxx Chemical Company and Type VI horse heart cytochrome *c* was obtained from Sigma Chemical Company.

Carboxymethylation of Amino Acid and Peptide Derivatives. Amino acid and peptide derivatives were carboxymethylated by dissolving or suspending 100 μmol of each in separate aliquots containing 1.45 ml of 0.7 *M* sodium [2-

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