Unfolding of Ribonuclease A by Guanidinium Chloride. Protein Internal Motions Studied by Nuclear Magnetic Resonance Spin–Lattice Relaxation in an Off-Resonance Rotating Frame

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Abstract: The novel nuclear magnetic resonance (NMR) technique entailing rotating frame spin–lattice relaxation in the presence of an off-resonance radiofrequency field ($T_{1\rho}^{off}$) has advantageous features for the study of protein molecular motions. Examination of the carbon-13 resonance of the carbonyls in ribonuclease A in the presence and absence of an off-resonance rf field has enabled the overall rotational reorientation time of the enzyme to be determined as 12 ns. The technique also is used to investigate the local mobility of different moieties of ribonuclease A as it is unfolded by the sequential addition of guanidinium chloride. Specifically, the resonances of the C2 protons of the histidine residues are monitored with the experimental variable being the ratio of a peak intensity in the presence to that in the absence of an rf field applied off-resonance. The intensity ratio is related to the mobility of the residue. It is found that the mobility increases for His-12 and His-105 as guanidinium chloride is added to the ribonuclease solution (1.0 mM, pH 5.4) up to a concentration of 1.1 M, which is much below the concentration required for complete unfolding of the protein. No further significant changes in the mobility of His-12, His-105, and His-119 are detected as guanidinium chloride is added up to a concentration producing total unfolding of all the ribonuclease. At guanidinium chloride concentrations above 2 M, a histidine C2 proton resonance corresponding to the cross-linked random coil protein appears which behaves in the presence of the off-resonance rf field as originating from a residue with greater motional freedom than the histidines on the folded protein. As guanidinium chloride is added to the enzyme in concentrations greater than 3 M, the $T_{1\rho}^{off}$ experiment indicates that the mobility of His-48 is increased considerably. Use of the $T_{1\rho}^{off}$ technique, thus, has permitted the discovery of an intermediate with altered local motion in the ribonuclease unfolding process. Previous studies[2] found no change in the spin–lattice relaxation time (T_1) for any of the histidine C2 protons of ribonuclease as guanidinium chloride concentration was increased.

The means by which a protein unfolds or folds into its tertiary structure can, in principle, be elucidated by studies involving denaturation of the protein. The enzyme in the present investigation, ribonuclease A, has been the subject of several denaturation experiments, including some NMR studies. NMR has been used to examine the possible existence of intermediate states between the fully folded native state and the cross-linked, random-coil protein in experiments entailing denaturation of ribonuclease by addition of urea,[1,2] [^{2}H]formic acid,[1] potassium thiocyanate,[1] guanidinium chloride,[1-3] and heat.[3,4] The existence of intermediate states could be inferred in certain cases by noncoincident sharpening of several proton resonances upon progressive addition of denaturant. More direct evidence was obtained in some cases by the appearance of new peaks in the protein's spectrum when some denaturant was present.

NMR relaxation times are sensitive to molecular motions, overall molecular tumbling as well as motions of different molecular moieties.[5] As a protein changes states along the pathway of unfolding, NMR relaxation times may reflect alterations in the local mobilities of various protein moieties. For such an altered internal motion to be manifest in a relaxation time change, the frequencies of the motion must be in a range appropriate to produce a large spectral density for that particular relaxation process. The spin–lattice relaxation time (T_1), spin–spin relaxation time (T_2), and normal on-resonance spin–lattice relaxation time in the rotating frame ($T_{1\rho}$) are sensitive to motions in different frequency ranges. To supplement techniques measuring these relaxation times, we have been developing an off-resonance rotating frame spin–lattice relaxation ($T_{1\rho}^{off}$) technique for investigating molecular motions.[6-10]

The off-resonance $T_{1\rho}^{off}$ technique appears to have considerable potential for investigating mobility in proteins. (i) Internal motions in proteins are often in a frequency range suitable for the $T_{1\rho}^{off}$ technique. (ii) The experiment is readily performed on commercial Fourier transform NMR spectrometers with only slight modification, enabling resonances from different moieties of the protein to be examined with good signal to noise. (iii) Relaxation measurements per se need not be carried out; motional information is inherent in the ratio of the peak intensity in the presence of an off-resonance rf field to the intensity in the absence of the off-resonance rf field.

Our purpose here is to illustrate the potential of this novel technique for examining internal motions in proteins. In particular, the behavior of the C2 proton resonances of ribonuclease A in the presence and absence of an off-resonance rf field was monitored for each of the histidine residues as a function of guanidinium chloride concentration. The changes in dynamic state of different moieties of ribonuclease thus could be studied as the protein was unfolded by addition of guanidinium chloride. Utilization of the off-resonance $T_{1\rho}$ method permitted the detection of a previously undetected intermediate along the pathway of ribonuclease unfolding.

Theory

The theory for studying molecular motions via spin–lattice relaxation in the presence of an off-resonance rf field has been previously presented for homonuclear dipolar relaxation,[6] heteronuclear dipolar relaxation,[7] and scalar relaxation of the second kind.[8]

The proton spin–lattice relaxation rate in an off-resonance rotating frame for one proton relaxed by another may be expressed as[6]

$$\frac{1}{T_{1\rho}^{off}} = \frac{1}{T_{eff}} + \frac{1}{T_1} \qquad (1)$$

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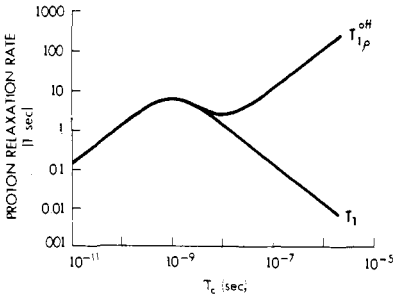

Figure 1. Dependence of the off-resonance rotating frame spin-lattice relaxation rate $1/T_{1\rho}^{off}$ and the Zeeman spin-lattice relaxation rate $1/T_1$ on correlation time. The curves were generated for illustrative purposes only by using eq 1-4 and 9 with a single isotropic correlation time, a proton-proton internuclear distance of 2 Å, a stationary magnetic field of 23 487 G, and an H_1 field of strength 0.25 G applied 6 KHz off-resonance.

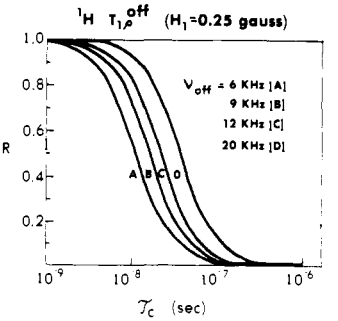

Figure 2. Theoretical dependence of the ratio of ^1H resonance peak intensities in the presence and absence of a 0.25-G rf field on the rotational correlation time according to eq 1-4, 9 by using a single isotropic correlation time for illustrative purposes. A stationary magnetic field of 23 487 G was assumed for the calculations. The curves were generated for off-resonance frequencies ν_{off} of (A) 6.0, (B) 9.0, (C) 12.0, and (D) 20.0 kHz.

where

$$\frac{1}{T_{1\rho}^{off}} = \frac{3\gamma^4h^2}{10r^6}\left(\frac{3}{2}\sin^2\theta\right)[J(\omega_e)] \quad (2)$$

$$\frac{1}{T_1} = \frac{3\gamma^4h^2}{10r^6}[J(\omega_0) + J(2\omega_0)] \quad (3)$$

γ is the proton gyromagnetic ratio, r is the proton-proton internuclear distance, ω_0 is the Larmor frequency, $J(\omega)$ is the spectral density, and T_1 is the spin-lattice relaxation time in the Zeeman frame. The other terms are

$$\theta = \tan^{-1}(\gamma H_1/2\pi\nu_{off}) \quad (4)$$

and

$$\omega_e = 2\pi\nu_{off}/\cos\theta \quad (5)$$

where ω_e is the angular frequency around the effective field vector with magnitude H_e created by application of the rf field H_1 at a frequency ν_{off} off-resonance:

$$\mathbf{H}_e = \frac{\omega_e}{\gamma} = \frac{2\pi\nu_{off}}{\gamma}\mathbf{k} + H_1\mathbf{i} \quad (6)$$

Equations 1-3 are valid if the H_1 field is applied far off-resonance. This criterion is satisfied when

$$\nu_{off} \geq 5\gamma H_1/2\pi \quad (7)$$

To gain some perspective on the motional dependence of T_1 and $T_{1\rho}^{off}$ relaxation, theoretical curves of $1/T_1$ and $1/T_{1\rho}^{off}$ are plotted in Figure 1 as a function of τ_c, the rotational correlation time for random isotropic motion. It is emphasized that random isotropic motion is undoubtedly a poor model for internal motions in biopolymers. In addition, there may be other proton-proton interactions contributing to relaxation. The point of Figure 1 is simply to illustrate that T_1 and $T_{1\rho}^{off}$ are identical for smaller correlation times, but the value of $T_{1\rho}^{off}$ is more sensitive than T_1 to slower motions, characterized by a larger correlation time, due to the $J(\omega_e)$ spectral density term.

If the magnetization is allowed to achieve a steady-state condition in the presence of an rf field applied far off-resonance, the steady-state value of the magnetization component aligned along the effective field H_e is given by

$$M_{eff} = \frac{M_0}{1 + (T_1/T_{1\rho})} \quad (8)$$

where M_0 is the thermal equilibrium value of the magnetization in the absence of the off-resonance rf field. So the ratio

$$R = \frac{M_{eff}}{M_0} = \frac{1/T_1}{1/T_{1\rho}} \quad (9)$$

The ratio $R = M_{eff}/M_0$ can be identified experimentally as the intensity of a resonance signal in the presence of the H_1 field applied off-resonance to the intensity in the absence of the H_1 field. Experimental values of the resonance intensity ratio R thus can provide motional information without recourse to direct measurement of relaxation times. That the value of the resonance intensity ratio is sensitive to motions with correlation times in a range expected for protein internal motions as well as overall protein tumbling is illustrated in Figure 2. The theoretical curves in Figure 2 were calculated for several off-resonance frequencies by using spectral densities with a single correlation time for isotropic motion for illustrative purposes only. Spectral densities appropriate for protein internal motions will require at least two correlation times. The $T_{1\rho}^{off}$ experiment can be applied more readily to carbon-13 than to protons for a quantitative study of internal motions (T. L. James, unpublished results). However, the low sensitivity of carbon-13 NMR compared with proton NMR militates against its use in many instances. Although, the intensity ratio R will not be used in the present study for a determination of correlation times, it should be evident that experimental values of R for the histine C2 protons of ribonuclease can be used in a qualitative sense to monitor motional changes as the protein is unfolded by additions of guanidinium chloride.

Experimental Section

Materials. The procedure for preparation of ribonuclease A was essentially the same as that of Benz et al.[11] Bovine pancreatic ribonuclease A, purchased from Worthington Biochemical Corp. as the phosphate- and sulfate-free protein, was dissolved in a minimum volume of 0.1 M sodium acetate, pH 5.5; any insoluble material was removed by filtration. The sample was desalted by gel filtration on a Sephadex G-25 column to remove any remaining salt contaminants. The protein-containing fractions were combined and lyophilized. About 150 mg of ribonuclease was dissolved in 15 mL of 99.8% D_2O (Bio-Rad), the pH adjusted to 7.0 with either NaOH or DCl, and the solution heated for 10 min in a water bath at 65 °C to promote proton-deuteron exchange. The sample was lyophilized, redissolved in 99.8% D_2O, and lyophilized again. The protein was then dissolved in 50 mM deuterioacetate buffer (prepared with 100% D_2O), pH 5.3, and the pH was adjusted so that the four histidine C2 resonances could be easily distinguished (pH ~5.4).

Measurements of pH were made with a Corning Model 10 pH meter equipped with an Ingold combination electrode capable of fitting into the 12-mm NMR tube (Wilmad 514A-77PP). The meter readings reported are those read directly from the pH meter without any deuterium correction. The pH meter was standardized by using standard buffers in normal isotopic water.

Perdeuterioguanidinium chloride was prepared from guanidinium chloride (Sigma) by dissolving in 99.8% D_2O, stirring for 1 day, and lyophilizing. The process was repeated a second time with 99.8% D_2O

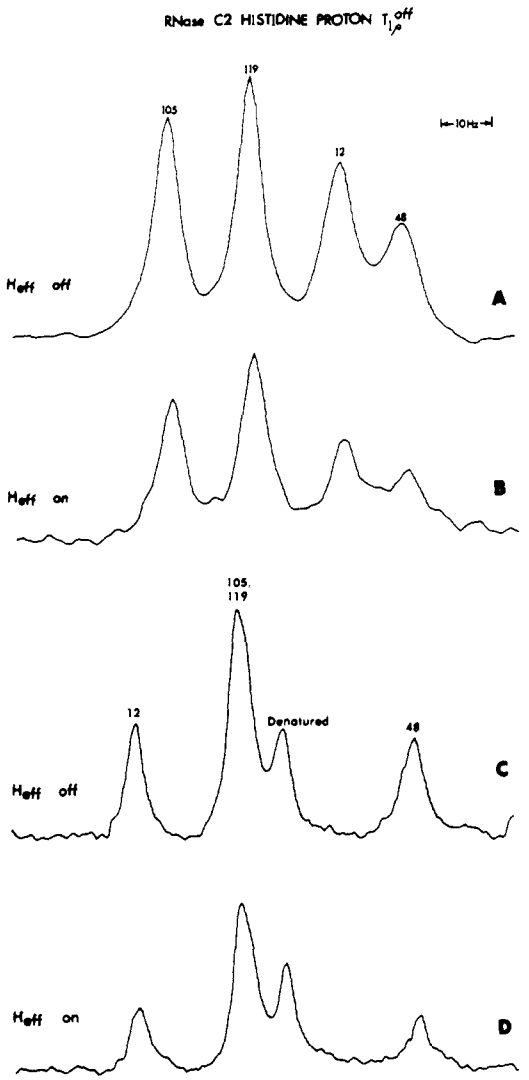

Figure 3. The 100-MHz NMR of the C2 proton resonances of the ribonuclease A histidine residues in the presence (spectra B and D) and the absence (spectra A and C) of an rf field of strength 0.25 G applied 6.0 KHz off-resonance. The ribonuclease concentration was 1.0 mM and the pH 5.4. Spectra A and B were obtained with a solution of ribonuclease in the absence of any added guanidinium chloride. Spectra C and D were obtained with a ribonuclease sample containing 2.78 M guanidinium chloride. Peaks are labeled according to their assignments to various histidine residues. Each spectrum shown is an average of 100 transients.

and a third time by using 100% D_2O, thereby replacing all exchangeable protons with deuterons. The solid perdeuterioguanidine deuteriochloride thus prepared was added directly to buffered solutions of ribonuclease A in small portions with stirring. After each addition the pH was checked and readjusted, if necessary, with dilute NaOD or DCl.

NMR Experiments. The off-resonance $T_{1\rho}^{off}$ experiments were carried out as described previously.[6,7] The proton NMR experiments were performed at 100 MHz on a Varian XL-100-15 NMR spectrometer with Nicolet Fourier transform accessories at a probe temperature of 28 ± 1 °C.

Results

Proton NMR. The resonance peak intensities for the histidine C2 protons of bovine pancreatic ribonuclease A (1 mM) were examined in the presence and the absence of an rf field applied off-resonance for solutions containing varying amounts of the denaturant guanidinium chloride. Figure 3 shows an example of the results for native ribonuclease (no guanidinium chloride (GdmCl)) in the absence (A) and presence (B) of an rf field of strength H_1 = 0.25 G applied 6.0 KHz off-resonance and for a partially denatured sample of ribonuclease (2.78 M GdmCl) in the absence (C) and presence (D) of the same off-resonance rf field. The resonance peak assignments for the native enzyme are those of Markley,[12] switching the earlier assignments of His-12 and His-119.[13] Benz and Roberts[2] followed the chemical shifts of the C2 proton resonances of ribonuclease as portions of GdmCl were sequentially added. The peak assignments for the resonances obtained in the presence of 2.78 M GdmCl are based on the GdmCl titrations of Benz and Roberts with the exception that the assignments of His-12 and His-119 resonances are reversed to be in agreement with Markley's experiments.

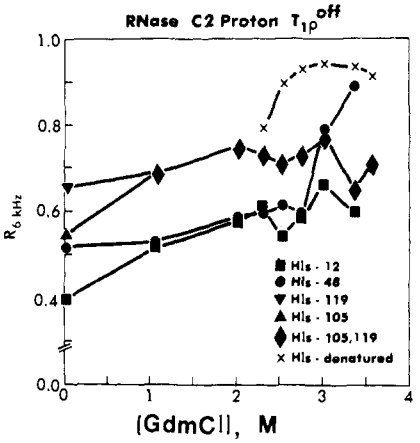

Figure 4. Experimentally determined peak intensity ratio of the histidine C2 proton resonances from ribonuclease in the presence and absence of a 0.25-G H_1 field applied 6.0 KHz off-resonance as a function of guanidinium chloride concentration. The symbols for the various histidine C2 protons of ribonuclease are shown above.

In the spectra of Figure 3, the resonance peak intensities for *folded* protein are observed to diminish in the presence of the off-resonance rf field. This is observed in both pairs of spectra (A vs. B and C vs. D). In contrast, the resonance corresponding to denatured ribonuclease shows only a slight decrease in intensity with application of the off-resonance rf field. Since the GdmCl-denatured ribonuclease is apparently a cross-linked random coil,[14] this $T_{1\rho}^{off}$ result is in accord with our intuitive notion that the histidine residues in the random coil protein exhibit a greater mobility characterized by a smaller spectral density $J(\omega_e)$.

The peak intensity ratios determined from pairs of spectra obtained in the absence and presence of off-resonance rf fields are listed in Table I for all the histidine C2 proton resonances of several ribonuclease samples prepared with varying amounts of guanidinium chloride.

It should be apparent from eq 1-3 and 9 that the value of R is independent of proton-proton internuclear distance when a proton is relaxed by a single proton or group of equivalent protons. However, this is not necessarily the case for a protein proton which may interact with more than one proton with the interactions being modulated at different rates. In this more complicated situation, the value of R will depend on the relative internuclear distances as well as the rates of the internal motions modulating the interactions.

Figure 4 shows the resonance intensity ratio of all the histidine C2 protons of ribonuclease as a function of guanidinium chloride (GdmCl) concentration; the ratio was determined for an rf field of strength H_1 = 0.25 G applied 6.0 KHz off-resonance. As implied by the discussion above, the different values of R for the protein and no GdmCl may reflect differences in number and relative distances from relaxing nuclei as well as internal motions experienced by the various histidine C2 protons. There are obvious variations in R between His-12, His-48, His-105, and His-119 in the native enzyme. But as GdmCl is

Table I. Peak Intensity Ratio of the Histidine C2 Proton Resonances of Ribonuclease A Obtained in the Presence and Absence of an Off-Resonance rf Field (0.241-0.263 G, unless Noted) as a Function of the Concentration of Guanidinium Chloride (GdmCl) in the Enzyme Solution for Several Off-Resonance Frequencies[a]

[GdmCl], M	ν_{off}, KHz	*R*				
		His-105	His-119	His-12	His-48	His-denatured
0.0	6	0.54	0.65	0.40	0.52	
	9	0.70	0.77	0.60	0.72	
	12	0.80	0.84	0.77	0.75	
	20	0.88	0.90	0.94	0.94	
	40	1.0	1.0	1.0	1.0	
1.10	6		0.70	0.51	0.52	
	9		0.80	0.65	0.73	
	12		0.87	0.80	0.85	
	20		0.96	0.95	0.96	
2.08	6		0.75	0.59	0.56	
	9		0.84	0.78	0.68	
	12		0.91	0.80	0.86	
	20		0.92	0.91	0.90	
2.30	6		0.73	0.61	0.60	0.79
	9		0.83	0.63	0.80	0.94
	12		0.86	0.78	0.79	0.94
	20		1.0	0.95	0.93	1.0
2.41	9[b]		0.85	0.75	0.77	
	12[b]		0.88	0.84	0.84	
	20[b]		1.0	0.93	1.0	
2.55	6		0.72	0.54	0.62	0.89
	9		0.81	0.73	0.78	0.98
	12		0.91	0.86	0.87	0.98
	20		0.96	0.92	0.91	1.0
2.78	6		0.73	0.58	0.58	0.93
	9		0.86	0.80	0.75	1.0
	12		0.93	0.81	0.90	1.0
	20		1.0	1.0	1.0	1.0
3.09	6		0.78	0.67	0.70	0.93
	9		0.85	0.70	0.71	0.92
	12		0.88	0.84	0.92	0.99
	20		0.90	0.88	0.92	0.99
3.37	6		0.64	0.60	0.89	0.93
	9		0.84	0.85	0.94	0.96
	20		1.0	1.0	0.98	0.99
3.61	6		0.70			0.90
	9		0.79			0.93
	20		0.90			1.0

[a] The concentration of ribonuclease A was 1.0 mM, the pH was 5.4, and the temperature was 28 °C. [b] H_1 = 0.292 G.

added, these histidine residues, as reflected in the *R* values, are altered.

Benz and Roberts[2] observed chemical shift changes going from 0 to 1.3 M GdmCl, although the enzyme is not denatured at this concentration of GdmCl. It is apparent from Figure 4 that *R* values for His-12 and His-105 change in this region as well. Benz and Roberts[2] found that there were some differences in spin-lattice relaxation time values among the four histidine C2 protons, but the T_1 value for any particular histidine did not vary with GdmCl concentration. Less extensive T_1 measurements in the present investigation corroborate those findings. Since the T_1 values do not change, any alteration in *R* value with GdmCl addition must be due to variation in $J(\omega_e)$ (see eq 1-3, 9). Since $J(\omega_e)$ contains terms of the form $\tau_c/(1 + \omega_e^2\tau_c^2)$ and the value of ω_e used for the experiments reported in Figure 4 is 3.8×10^4 rad/s, $J(\omega_e)$ will decrease as the internal mobility increases.

If the correlation time for the motion was on the order of nanoseconds, it is expected that T_1 values would reflect any change in the mobility. However, if the correlation time for the altered motion is an order of magnitude larger, the $J(\omega_e)$ spectral density term of the $1/T_{1\rho}^{off}$ expression would be sensitive to the change, but $1/T_1$ would not be. The results of Figure 4 therefore indicate that His-12 and His-105 experience greater mobility when 1.1 M GdmCl is added to the native enzyme. Several molecules of guanidinium chloride will bind to a protein.[15] This binding is apparently capable of altering the mobility of the histidine residues differentially.

The thermodynamic studies of Salahuddin and Tanford[16] indicate that ribonuclease molecules unfold to random coils cross-linked by disulfides at GdmCl concentrations above 2 M. The data recorded in Table I and Figure 4 show that the histidine residues in the random coil protein have larger *R* values and, consequently, greater mobility than histidine residues in the folded ribonuclease. Of the protein that remains folded at higher GdmCl concentrations, only the His-48 C2 proton resonance exhibits much change with a dramatically increased *R* value at GdmCl concentrations above 3 M. In contrast, the *R* values for the C2 proton resonances of His-105, His-119, and His-12 do not exhibit such a marked change.

Although the folded protein remains largely unchanged, the mobility of the His-48 residue is apparently less restricted at the higher GdmCl concentrations.

Carbon-13 NMR. The rotational correlation time for overall tumbling may be determined by monitoring the backbone carbonyl carbon-13 resonance of a protein in an off-resonance $T_{1\rho}^{off}$ experiment by using equations analogous to eq 1-3 and 9 with a single isotropic correlation time.[7] The overall reorientation time was calculated to be 12 ns for ribonuclease since the carbonyl carbon-13 resonance intensity ratio was 0.94 when ν_{off} = 0.90 KHz using an rf field H_1 of 0.135 G and an H_0 field of 23 487 G.

Discussion

The results of Table I and Figure 4 clearly illustrate that the off-resonance $T_{1\rho}^{off}$ experiment is capable of distinguishing local mobilities in different moieties of a protein and is capable of monitoring changes in these local mobilities as solution conditions are altered.

The GdmCl-induced chemical shift changes, peak area changes, and T_1 measurements for the histidine C2 proton resonances are not explicitly given here since our observations are in essential agreement with those reported by Benz and Roberts.[2] These unreported observations as well as the results in Table I and Figure 4 support the thesis of Benz and Roberts that there are intermediates along the pathway of unfolding of ribonuclease by GdmCl. This is a contradiction of the earlier conclusion of Salahuddin and Tanford[16] based on thermodynamic studies entailing optical rotation and difference spectral measurements and of Bradbury and King[1] based on NMR studies that the unfolding is a simple two-state process.

As shown in Table I and Figure 4, the addition of GdmCl up to a concentration of 1.1 M increases the R values and, consequently, the mobility of His-12 and His-105. The observed chemical shift changes of the C2 proton resonances of His-12, His-48, and His-119 could be due to binding of the denaturant molecules near the histidine residues or to local conformation changes in the vicinity of those residues.[2] Specific binding of GdmCl at the active site could result in the inhibitory effect of guanidinium chloride on ribonuclease activity[17] and provide the mechanism for the chemical shift changes of His-12 and His-119 which are in the active site cleft of ribonuclease.[18] It was not possible to unequivocally ascribe the chemical shift changes to specific denaturant binding or to conformational changes; however, Benz and Roberts did conclude, on the basis of T_1 measurements, that, if a conformational perturbation occurred due to low GdmCl concentrations, it does not represent a partial unfolding of the ribonuclease molecule.[2] T_1 measurements alone can be misleading in this regard (vide infra). In contradistinction to that conclusion, the results revealed in Figure 4 showing increased R values for the His-12 and His-105 C2 protons in the presence of 1.1 M GdmCl can indeed be interpreted by a partial unfolding of the native enzyme resulting in slightly increased mobility in the localities of His-12 and His-105. It should be pointed out, however, that the conformational changes that do occur are not large since the ultraviolet absorption spectrum and optical rotation do not change in this concentration range.[16]

The optical spectroscopic studies of Sela et al.[17] and Salahuddin and Tanford[16] indicate that ribonuclease A can unfold to a cross-linked random coil at GdmCl concentrations above 2 M. The appearance of an additional C2 proton resonance for a histidine at a frequency corresponding to a water-accessible residue provides confirmatory evidence. As shown in Figure 4, the histidine residue from the random coil protein possesses greater freedom of movement as manifested by a R value in comparison with the histidine residues of the folded ribonuclease.

The observations of Benz and Roberts[2] regarding the order of disappearance of the peaks due to histidines in the folded protein were confirmed in the present study (with a switch in assignment of His-12 and His-119). The differential disappearance of the peaks is clear evidence that intermediate species exist along the pathway of ribonuclease unfolding. The results do indicate that His-12, His-105, and His-119 do not alter their mobilities significantly (for motions in the nanosecond to millisecond time scale) as any intermediates in the unfolding process are formed at concentrations of GdmCl above 2 M. In contrast, the resonance intensity ratio R of the His-48 C2 proton in the folded protein falls sharply at concentrations of GdmCl above 3 M (cf. Table I and Figure 4). However, the T_1 and the chemical shift of the His-48 resonance do not change perceptibly. Under these solution conditions, the His-48 residue must remain buried in the interior of the protein. But the interior in the vicinity of His-48 apparently has an increased mobility in the presence of large amounts of GdmCl. The mechanism by which GdmCl can effect this change on the interior of the protein is unclear. We might conjecture, however, that the postulated hydrogen-bonded interaction of the Asp-14 residue with the His-48 imidazole at the pH used in this study[19] could provide the link between the GdmCl on the exterior of the protein and His-48 in the interior.

As noted earlier, Benz and Roberts[2] detected no significant T_1 change for any of the histidine C2 protons of ribonuclease as GdmCl was added up to a concentration of 1.3 M. The results of Figure 4 and Table I clearly show that the $T_{1\rho}^{off}$ experiment does detect mobility alterations as 1.3 M is added. These changes in dynamics occur with the apparent conformational change which results, at least to some extent, in the chemical shift changes also observed. The $T_{1\rho}^{off}$ experiment is sensitive to slower motions than T_1; apparently these slower motions are altered as the protein conformation changes with addition of 1.3 M GdmCl leading to a large R value. The carbon-13 $T_{1\rho}^{off}$ experiment indicated that the correlation time for the overall rotational reorientation of ribonuclease is 12 ns, which puts an upper limit on the correlation time of the motion which influences R.

Measurements of only proton T_1 values are not sufficient to determine the dynamics of proteins. As mentioned by Benz et al.[11] the dipolar relaxation of the histidine protons in ribonuclease entails interaction of the histidine protons with protons on other residues. Variations in internuclear distances have more influence on T_1 differences than variations in correlation times (cf. eq 3).

The present investigation has illustrated the potential of the off-resonance $T_{1\rho}$ technique for studies of motion in proteins. In particular, it has been shown to be sensitive to motions which have not previously been detected by more conventional NMR relaxation techniques. Alterations in the detected motions reveal the presence of intermediates in the process of unfolding ribonuclease.

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Synthesis and Properties of Tetraphenylporphyrin Molecules Containing Heteroatoms Other than Nitrogen. 5. High Resolution Nuclear Magnetic Resonance Studies of Inner and Outer Aromaticity

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Abstract: When the two NH groups in tetraphenylporphyrin are replaced by the group 6A heteroatoms S, Se, and Te, bonding interactions within the porphyrin core are found, as is apparent from X-ray analysis results. Because of these interactions, changes in the inner and outer aromatic pathways occur, which are expressed in the chemical shifts of the hydrogen atoms at the periphery of the molecule. The bonding interactions within the core are disrupted by either protonation or complexation, and this finds its expression in the chemical shifts in a consistent way. If two different heteroatoms are introduced, or when the *p*-phenyl hydrogens are substituted in such a way that two phenyl groups carry substituents of different electronic properties, charge transfer occurs between the heteroatoms. This charge transfer changes the inner aromatic pathway, which influences the chemical shift. These shifts relate linearly to the Hammett constant of the substituents. Starting from these results, the concept is developed that the interaction between the heteroatoms acts as an electron drain on the π electrons and especially on the pyrrolenine-N nonbonding electrons. This causes an increased contribution of the 20-membered, extended outer aromaticity Kekulé structures.

Introduction

Recently we published a new synthetic procedure by which the NH groups in the core of tetraphenylporphyrin (TPP) can be exchanged by the heteroatoms S, Se, and Te. By this method we could prepare tetraphenyl-21,23-dithiaporphyrin (S_2TPP),[2] tetraphenyl-21-selena-23-thiaporphyrin (S,SeTPP),[3] tetraphenyl-21,23-diselenaporphyrin (Se_2TPP),[3] and tetraphenyl-21-tellura-23-thiaporphyrin (S,TeTPP).[4] It appeared also to be possible to introduce substituents on the phenyl groups of S_2TPP, either symmetrically so that every phenyl group carries the same substituent, or unsymmetrically with two pairs of phenyl groups carrying different substituents.[5] Figure 1 shows the molecules which form the subject of this study.

Several of these molecules have been analyzed by X-ray structure analysis,[6] and the abnormally short distances between X and Y of Figure 1 (3.05 Å for X = Y = S; 2.89 Å for X = S, Y = Se; 2.85 Å for X = Y = Se and 2.65 Å for X = S, Y = Te) show that a chemical bonding interaction exists between X and Y in these molecules, which increases in the order S, Se, Te. This interaction influences the conjugative pathway in the molecule, which is often called inner and outer aromaticity (ring current). The degree of outer aromaticity can be expected to influence the chemical shifts of H_x and H_y of Figure 1 and to lesser extent that of H_p.

Therefore, we describe here the results of a high resolution (270-MHz) NMR study on these new porphyrins, their conjugate acids, and some of their metal complexes. These results are entirely consistent with the described X···Y interaction and also show charge transfer to occur between X and Y, when they are different or when A and B are different. Moreover, information is obtained on the binding of the proton and the metal ion in the conjugate acid and the metal complexes, respectively.

Results and Discussion

Symmetrical Heterosubstitution. A. Free Bases. In Table I the chemical shifts at room temperature of the symmetrically heterosubstituted (X = Y) porphyrins are given and compared with literature values for TPP, which were measured at −80 °C to get separate values for the pyrrole and pyrrolenine hydrogens.[7]

In order to understand the large downfield shifts of H_x and H_y of Figure 1 and the smaller ones of H_p with substitution, we must discuss the accepted Kekulé structures for porphyrins and construct a model for the core interactions, which apparently occur when the NH groups are substituted by heteroatoms of the sixth row of the Periodic Table having empty d orbitals. In Figure 2, the possible Kekulé structures for the porphyrin molecule are sketched.

The 16-membered structure corresponds to the inner aromatic pathway, which is used by Gouterman to explain the optical spectra of porphyrins.[8] The 18-membered structure corresponds to the outer aromatic pathway and, according to ring current calculations of Abraham, has a statistical weight of about 30%.[9] The 20-membered extended outer aromatic

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