Ribonuclease A: Carbon-13 Nuclear Magnetic Resonance Assignments, Binding Sites, and Conformational Flexibility†

Oliver W. Howarth* and L. Yun Lian‡

ABSTRACT: Assignments have been made for 11 methyl, one Gln-C$^{\gamma}$, one Thr-C$^{\beta}$, and all six Tyr-C$^{\zeta}$ carbon resonances of ribonuclease A. These partially serve to delineate the binding sites for Cu^{2+}, Mn^{2+}, phosphate, cytidine and its 2′-, 3′-, and 5′-phosphates (Cyd and Cyd-2′-P, -3′-P, and -5′-P), and one or a few urea molecules at low concentration. Evidence is presented for a conformational change, and hence flexibility, in the active site region around the optimum pD for enzymic activity and another such change at around the optimum temperature. The binding of cytidine-containing ligands is shown to have extensive conformational consequences for methyl groups but less for hydrophobic aromatic residues, implying that the former make a special contribution to molecular flexibility. The cytosine ring in Cyd-2′-P, -3′-P, and -5′-P is found to be close but far from parallel to the ring of Phe-120. In contrast to previous claims, ribonuclease A is shown not to unfold even partially before denaturation. On denaturation, it passes to a new but structured state.

Although there have been previous NMR[1] studies of the aromatic and histidine ^1H resonances of ribonuclease A (Bradbury & Scheraga, 1966; Markley, 1975a; Shindo et al., 1976; Santoro et al., 1979) and of several unprotonated aromatic and carboxyl ^{13}C resonances (Walters & Allerhand, 1980; Shindo et al., 1978; Niu et al., 1979), the aliphatic region of these spectra has never been explored at single-carbon resolution. Only four tentative ^1H assignments (Sadler et al., 1974) and one firm ^{13}C assignment (Jaenck & Benz, 1979) have been made in the native protein. Yet this region of the ^1H and ^{13}C spectra arises from inherently more flexible residues and may be expected to afford insights into their biological functions.

The present study makes many new assignments and confirms some earlier ones. It goes some way toward delineating the extent of the local protein motions associated with the binding of various ligands and connects these with the enzyme's flexibility requirements. A major aim of the study has been to attempt to understand the reported stepwise unfolding of ribonuclease A, and this is discussed along with results on lysozome in the following paper (Howarth & Lian, 1984). The detailed interpretation of the NMR spectra has depended heavily upon recent X-ray crystallographic studies (Borkakoti, 1983; Borkakoti et al., 1982, 1983).

Experimental Procedures

Materials

Bovine pancreatic ribonuclease A (Sigma, type II-A, phosphate free) was dialyzed against water at 5 °C and lyophilized before use. Reagent-grade $CuSO_4$, $MnCl_2$, and Na_2HPO_4 were used. Methyl iodide (BDH, reagent grade), urea (Sigma), cytidine 2′-phosphate (Sigma), cytidine 3′-phosphate (P-L Biochemicals, Milwaukee, WI), and cytidine 5′-phosphate (BDH) were used as supplied. D_2O (99.8%), NaOD (30% in D_2O), and DCl (20% in D_2O) were obtained from Aldrich Chemical Co.

Methods

Methylation of Met-29 in Ribonuclease A with Methyl Iodide. The reaction of RNase A with methyl iodide was carried out essentially as described by Jaenck & Benz (1979). To 100 mL of RNase A (3 mg/mL) in 0.1 M HCl 1 g of methyl iodide was added and the flask tightly capped. After being stirred for 24 h in the dark at 25 °C, the solution was lyophilized and the unreacted methyl iodide removed under vacuum. Dialysis against frequent changes of deionized water at 5 °C for 12 h removed the KNO_3 present.

NMR Samples. All the studies used 6–7 mM D_2O solutions. The different pD values were obtained by the addition of minute amounts of 0.05 M NaOD or 0.05 M DCl, followed by overnight equilibration and measurement before and after spectrum accumulation. No extra salt was added to the samples so that ionic strengths were determined by the protein and the DCl or NaOD used for the titration. Urea was added as a preweighed solid.

For each RNase A–inhibitor solution, 1.5 mL of RNase A solution in D_2O (0.05 M NaCl) was mixed with 0.8 mL of the inhibitor in D_2O (0.05 M NaCl). The final concentrations of the inhibitors in their respective solutions were as follows: Cyd-2′-P, 40 mM, pD 5.5; Cyd-2′-P, 40 mM, pD 7.0; Cyd-3′-P, 65 mM, pD 5.5; Cyd-5′-P, 80 mM, pD 5.5. In order to study the effects of Cu^{2+} and Mn^{2+} ions, small amounts of highly concentrated salt solutions in D_2O (0.5–1.0 M $CuSO_4$ or $MnCl_2$) were added to RNase A to give the desired concentration of metal ions.

The FID was processed after resolution enhancement by a Lorentz-to-Gaussian multiplication. A "pattern-recognition" method of referencing according to Walters & Allerhand (1980) was applied, with external dioxane (δ_C 67.4) as the standard. ^1H spectra used 5 mM o.d. tubes, 500 and 1000 accumulations over 16K data points, and resolution-enhancement as above. The standard was Me_4Si. NOE difference spectroscopy was carried out by using the FID subtraction method, with a presaturation pulse length of between

† From the Department of Chemistry and Molecular Sciences, University of Warwick, Coventry CV4 7AL, U.K. *Received August 15, 1983; revised manuscript received December 13, 1983.* This work was supported by SERC.

‡ Present address: Department of Chemistry, Queen Mary College, London E1 4NS, U.K.

[1] Abbreviations: ribonuclease A or RNase A, bovine pancreatic ribonuclease A; Cyd-2′-P, -3′-P, or -5′-P, cytidine 2′-, 3′-, or 5′-phosphate; NMR, nuclear magnetic resonance; COSY, *J*-correlated 2D spectroscopy; FID, free-induction decay; NOE, nuclear Overhauser enhancement; δ_H and δ_C, carbon and hydrogen NMR shifts in parts per million relative to Me_4Si; M, mol dm^{-3}; rms, root mean square.

0006-2960/84/0423-3515$01.50/0

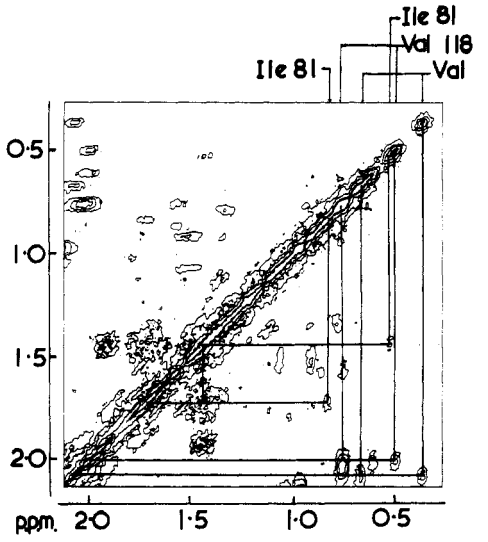

FIGURE 1: Detail of methyl region of COSY-90 2D contour spectrum of ribonuclease A, pD 3.2, 308 K. For clarity, the lowest contour levels are not shown. The main spectrum lies along the diagonal, and off-diagonal peaks with coordinates (δ_x, δ_y) arise from significant spin–spin coupling between peaks with shift δ_x and δ_y. A 1K × 1K data set, 20-h total accumulation, and double sine-bell window function were used.

0.6 and 1.0 s, and accumulation time of 1.2–1.5 s per FID, a pulse repetition time of 1.0 s, and about 3000 pulses. Calculations of the distances between the protons in the crystal structure were carried out by using refined coordinates supplied by Dr. N. Borkakoti (personal communication).

Acquisition of NMR Spectra. All NMR spectra are Fourier-transform ^1H and ^{13}C spectra, obtained with quadrature detection, and were recorded at 400.13 MHz for ^1H and 100.63 MHz for ^{13}C on a Bruker WH400 spectrometer. Sample temperatures were independently verified. ^{13}C spectra were recorded in tubes of 10 mm o.d., with approximately 7 W of gated proton-decoupling power. A 25-kHz sweep width, a pulse width of 25 μs (32 μs $\simeq$ 90°), a recycle time of 1.0 s, and 16K of data points were used for data acquisition (typically 10–12 h) and zero-filled to 32K before transformation.

The 2D COSY-90 spectra were obtained by the method of Nagayama et al. (1979) as modified by Wagner & Wütrich (1982), with a 1K × 1K data set, an interscan delay of 0.3 s, 1024 transients for each of 256 interpulse delays, double sine-bell window functions, and ca. 20 h of total measurement time.

Results and Discussion

^1H Assignment by Type. Part of a 2D COSY-90 contour spectrum (Wagner & Wütrich, 1982) of ribonuclease A at pD 3.2, 308 K, is shown in Figure 1. In the contour spectrum, the off-diagonal peaks link spin-coupled protons. Some general assignments are immediately apparent. The probable Val H^γ to H^β connectivity for two resolved resonances near δ 0.5 and δ 0.3 is marked. The peak at δ 0.5 also contains a second component coupled to a proton resonating at δ 1.45, which in turn is not coupled to any other proton resonating in the methyl region. This probably identifies it as an isoleucine H^δ. In contrast, the region from δ 1.1 to 1.4 appears to arise very largely from Ala-H^β and Thr-H^γ methyl resonances, as the peaks in this region are mostly coupled to resonances near δ 4.

^{13}C Assignment by Single-Frequency Irradiation. ^{13}C spectra were obtained whilst the proton resonance regions

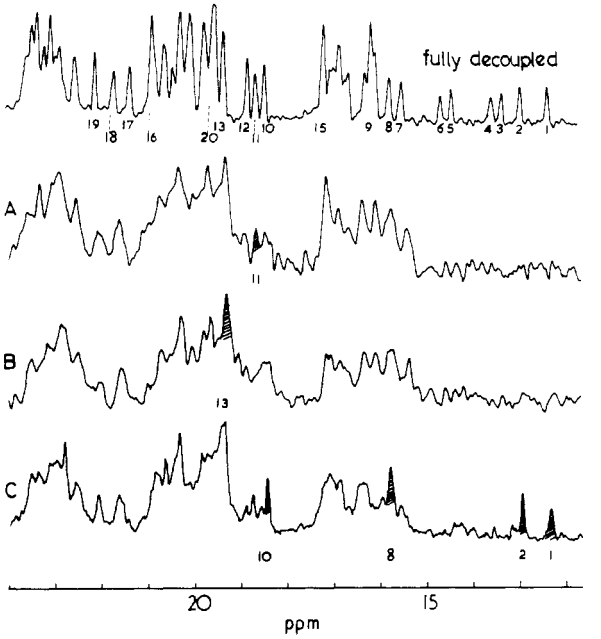

FIGURE 2: (Top trace) Spectral regions from δ 12.0 to 24.0 of the proton-decoupled ^{13}C NMR spectrum of RNase A (6 mM in D_2O) at pD 3.2, 50 °C. The numbers indicate the position of individual methyl carbon resonances. (A–C) Same as top trace, with selective ^1H irradiation at δ (A) 0.57, (B) 0.63, and (C) 0.75. The main collapsed multiplets in the individual spectra are hatched.

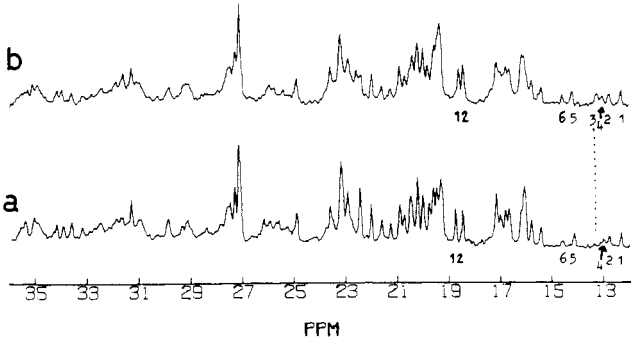

FIGURE 3: Spectral region from δ 12.0 to 36.0 of the proton-decoupled ^{13}C NMR spectrum of native and chemically modified RNase A: (a) [*S*-methyl-Met^{29}]RNase A (5.6 mM in D_2O), pD 5.5, 25 °C. (b) native RNase A (6 mM in D_2O), pD 5.5, 25 °C. The numbers indicate the positions of individual methyl carbon resonances.

mentioned in the previous section were irradiated. Figure 2 shows a few examples of the way in which this results in the selective decoupling of a few ^{13}C resonances in the region δ 12–20, which thus become assigned by type. They are discussed in more detail below. An irradiation at δ_H 2.0 was at the peak position assigned by Sadler et al. (1974) to several Met-H^ϵ resonances, and although other resonances also contribute to this region of the proton spectrum, the decoupling certainly narrows a ^{13}C resonance at δ 13.5, whose specific assignment as Met-29 C^ϵ is made below.

Chemical Modification of Met-29. Figure 3 compares the methyl regions of the ^{13}C spectra of native and [*S*-methyl-Met^{29}]ribonuclease. These were obtained at pD 5.5, where the order of peaks 3 and 4 (see Figure 2) is reversed compared with pD 3.2. The methylated protein was prepared as described above. This experiment confirms and extends previous observations (Jaenck & Benz, 1979) made with isotopic ^{13}C enrichment. Peak 3, at δ 13.42, is removed upon this conversion of Met-29 S to $-SMe_2^+$, and a new resonance appears near to the δ 26.7 position noted by the above workers. It is evident that other perturbations of the spectrum, and hence

Table I: Assigned ^{13}C Resonances of Ribonuclease A

peak no.[a]	δ_C[b]	δ_H	assignment[c]	method[d]
1	12.40	0.75	(?) Ile-107C$^{\delta}$	ligands
2	13.03	0.8	Ile-81C$^{\delta}$	Cu^{II}, ligands, COSY
3	13.42		Met-29 C$^{\epsilon}$	selective modification, T_1
4	13.65	2.0	Met-13 C$^{\epsilon}$	T_1, pK_a, urea
5	14.53	1.02	Ile-106 C$^{\delta}$	urea, ligands
6	14.76		Met-30 C$^{\epsilon}$	T_1, phosphate
7	15.60	0.63	(?) Ile-107 C$^{\gamma 2}$	ligands
11	18.78	0.55	Ile-81 C$^{\gamma 2}$	Cu^{II}, ligands, COSY
12	18.96	1.02	Val-47 C$^{\gamma}$	Cu^{II}, urea, ligands, Met-29, COSY
13	19.47	0.63	Val-47 C$^{\gamma}$	Cu^{II}, urea, ligands, COSY
19	22.6	0.5	Val-118 C$^{\gamma}$	Cu^{II}, phosphates, COSY
	27.0–27.2		Lys C$^{\delta}$	pD dependence
	35.0		(?) Glu-49 C$^{\gamma}$	pK_a
	39.9–40.1		Lys C$^{\epsilon}$	pD dependence
	68.6		(?) Thr-45 C$^{\beta}$	urea
21	154.74		Tyr-115 C$^{\zeta}$	selective decoupling
22	155.50		Tyr-92 C$^{\zeta}$	selective decoupling
23	155.80		Tyr-76 C$^{\zeta}$	selective decoupling
24	156.29		Tyr-73 C$^{\zeta}$	by elimination
25	157.16		Tyr-25 C$^{\zeta}$	pK_a, Met-29, selective decoupling
26–29	157.2–157.6		Arg C$^{\zeta}$	
30	158.26		Tyr-97 C$^{\zeta}$	pK_a

[a] Numbering for aliphatics as in Figure 2. [b] pD 3.2 except for peak 3, at pD 5.5. [c] (?) Indicates a more tentative assignment. [d] See text for details.

of the protein structure, are small, as would be expected when an exposed residue is modified. Perturbations occur only in a few peaks that are independently known to be pH dependent, and in each case, the shift is the same as would be caused by a slight lowering of pH. This suggests that the added positive charge causes the same conformational change as an added proton at a similar site. We believe that the main affected residue is His-48, which is the closest histidine to Met-29 C$^{\epsilon}$ (775 ppm; N. Borkakoti, personal communication). At least one of the perturbed peaks, 12, is assigned below to Val-47 C$^{\gamma}$, which would almost certainly be affected by any change in the pK_a of His-48. A further perturbation is analyzed below in the section concerning aromatic resonances.

Saturation. A spectrum obtained with a higher pulse repetition rate than usual showed a decrease of ca. 40% relative to neighboring peaks in the intensity of peaks 3, 4, and 6. This behavior would be expected for Met-C$^{\epsilon}$ groups, because of their relatively rapid internal motion. It supports the assignment made below.

Titration. Figure 4 shows the effect of titration (in D_2O) on the shifts of 11 residues, which may be followed with reasonable certainty from pD 3.2 to 11.2. Peak 4 was followed by titration of [*S*-methyl-Met^{29}]ribonuclease A to resolve the overlap with peak 3. The shifts were shown not to arise merely from changes in ionic strength.

Resonances 2 (Ile-81), 4 (Met-13), 5 (Ile-106), 11 (Ile-81), and 12 (Val-47) are assigned below (Table I). They are all adjacent to histidine residues (12, 48, and 105) with the exception of Ile-81. However, the measured apparent pK_a's, with no isotope correction, from Figure 4 are 5.7 (peak 2), 4.9 (8), 5.9 (11), and ca. 6 (12). These values are not highly accurate, especially for peak 8, because of a variety of minor perturbations around pD 4. But they fit broadly with the data of Walters & Allerhand (1980) from aromatic resonances. Now the pK_a's of the histidine residues measured in D_2O under comparable conditions are 5.8 (residue 12), ca. 6.3 (48), 6.7 (105), and 6.2 (119) (Jardetzsky & Roberts, 1981). Thus, most of the data are consistent with a single, substantial conformational change associated with protonation of His-12, with relatively little influence from protonation of the other three histidines.

A comparable change has been proposed for different and less direct reasons by Markley & Finkenstadt (1975) and

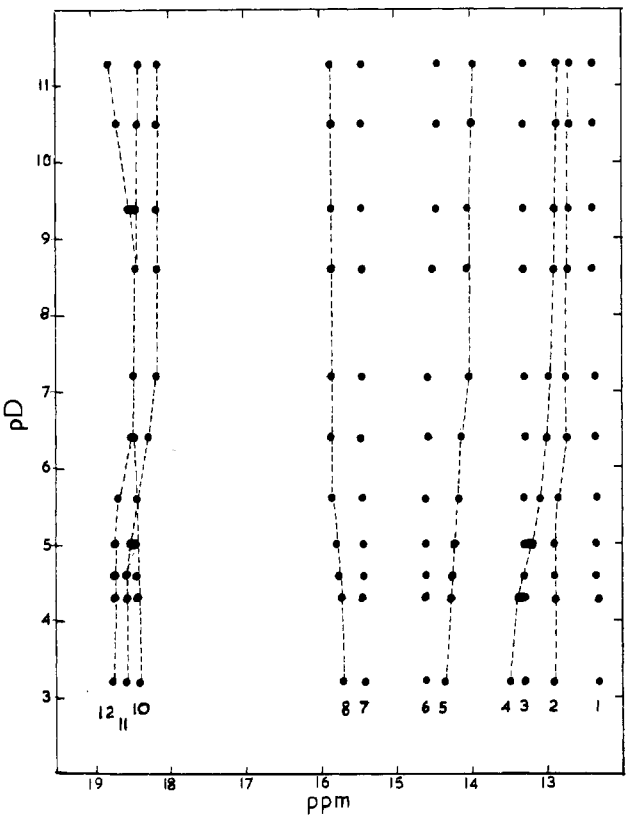

FIGURE 4: Plot of chemical shifts of the methyl carbon resonances in the proton-decoupled ^{13}C NMR spectra of RNase A as a function of pD, 25 °C.

Lenstra et al. (1979). Our proposal is entirely consistent with the known maximum in enzymic activity at pD 5.4–6.4.

The second, higher pK_a of peak 12, assigned below to Val-47 C$^{\gamma}$, is likely to arise from the deprotonation of Lys-41. A similar pD sensitivity above pD 8.4 is noted from the ^{13}C resonances lying in the ranges δ 27.0–27.2 and δ 39.9–40.1. We therefore assign peaks in these regions nonspecifically to Lys C$^{\delta}$ and C$^{\epsilon}$, respectively.

Peak 4, identified above as a Met-C$^{\epsilon}$ resonance other than Met-29, shows a particularly marked pD dependence below pD 5 as well as a histidine-related dependence. We assign this peak to Met-13 C$^{\epsilon}$, by noting its closeness to Asp-14, because

no other Ile or Met methyl groups are close to a carboxylate group in the crystalline protein. The shift of δ 13.1 (pD 5.6) is fairly close to that observed for the same residue in the reconstituted protein by Niu et al. (1979).

Urea Binding at Low Concentration. The addition of urea to aqueous ribonuclease A significantly affects some resonances in the protein's ^1H and ^{13}C NMR spectra at urea concentrations below 0.5 M, whereas the same spectra definitely exclude any denaturation below 2 M. This indicates that one or a few urea molecules bind specifically to the native protein at low urea concentration. No further changes occur from 2 to 3.5 M urea.

Benz & Roberts (1975) noted the specific perturbation at low urea concentration and pD 5.5 of a ^1H resonance subsequently reassigned to His-12 C^{ϵ_1}. We confirm a shift for this resonance of δ 0.42 between 0 and 2 M urea at pD 3.2, in contrast to near zero (δ <0.02) changes for other histidine ^1H resonances. This shift is in the opposite direction to the denatured value and, hence, is not likely to arise from local unfolding. Thus, at least one urea molecule binds near His-12 (K_M = 0.5 M). Other binding sites have also been suggested by the above authors on the basis of smaller shifts in other resonances at pD 5.5. However, these do not seem to be significant at pD 3.2 and may be related to the pH-dependent conformational change described above.

We also found a K_M = 0.5 M dependence on urea concentration for carbon resonances 4 (Met-13 C^{ϵ}), 5, 10, 12, and 13. Similar dependences were found at pD 5.5. The other resolved methyl and Tyr C^{ζ} resonances were not perturbed under these conditions, but one resolved Thr C^{β} resonance moved from δ 68.6 to 67.9. This is possibly Thr-45 C^{β}, which lies at 647 pm from His-12 C^{α}.

The perturbation of resonance 4 by urea binding near His-12 is strongly consistent with the assignment of this resonance to Met-13 C^{ϵ}, made above. The Cu^{II}-induced broadening also adds weight to the assignment made below of resonance 12 to Val-47 C^{δ} and suggests that peak 13 also arises from this residue. It also further supports the assignment of resonance 5 to the Ile-106 C^{δ}, made below.

In general, comparison of the effects of urea binding with those of Cu^{II} binding described in the next section suggests that, if one considers the active site cleft as a crude axis, the urea binds on the opposite side of His-12 in the cleft, compared with Cu^{II}, and thus lies nearer to the hydrophobic pocket noted by Carlisle et al. (1974).

Binding of Cu^{II} and Mn^{II}. Both Cu^{II} and Mn^{II} are known to bind, in a kinetically labile fashion to ribonuclease A. Walters & Allerhand (1980) have shown that Cu^{II} has at least two binding sites, one at His-105 and one at the active site (His-12 and His-119). This binding appears to involve few shift (and hence conformational) changes but several line broadenings. We confirm this.

In contrast, Fan & Bersohn (1975) have shown by ^1H NMR that at the same pD of around 5.5, Mn^{II} binds preferentially at or near to His-105, and we also confirm this observation. The broadening of His-12 and His-119 C^{δ_2} by Mn^{2+} is slight.

The corresponding ^{13}C spectra for methyl resonances are shown in Figure 5. The Mn^{II}-containing solution was shown to display severe broadening of His-105 C^{δ}, yet evidently, no methyl resonances are broadened. This is not unexpected, as His-105 is on the protein exterior.

It therefore follows that the broadening effects of Cu^{II} shown in Figure 5 arise from binding at the alternative active site location. These effects are clearly noticeable for peaks 10 or 11, 14, and 19 and region C. Peaks 11 and 14 are also perturbed by all the cytosine inhibitors (see below) and evidently arise from methyl groups in the active site. Some possibilities are Ile-81 C^{δ_2} and C^{δ} of Val-43, -108, and -118 or, for peak 14, Thr-45 C^{δ}. Peak 19 is discussed further below.

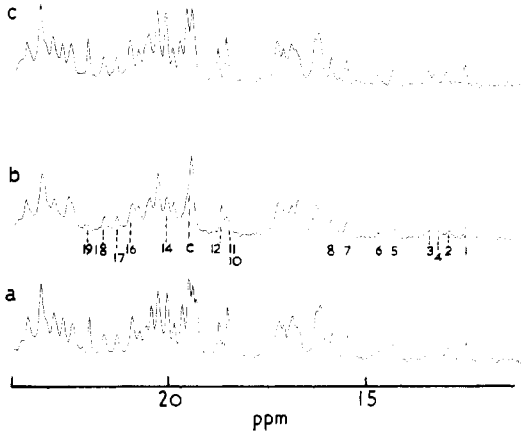

FIGURE 5: Effect of Cu^{2+} and Mn^{2+} on the resonances in the spectral region from δ 11.0 to 24.0 of the proton-decoupled ^{13}C NMR spectrum of RNase A in 0.05 M NaCl, pD 5.5, 25 °C: (a) free protein (6 mM); (b) $+Cu^{2+}$ (1.8 mM); (c) $+Mn^{2+}$ (0.8 mM). The numbers indicate the positions of individual methyl carbon resonances. The letters indicate the positions of groups of unresolved methyl carbon resonances.

Inhibitor and Phosphate Binding. Because ribonuclease A binds Cyd-2′-P, -3′-P, and -5′-P as well as cytidine and phosphate independently, it has been possible to make a comparative study of the binding of these ligands, under conditions of an approximately 10-fold ligand excess. At present, there are five relevant sets of X-ray crystallographic data for the binding of Cyd-2′-P and -3′-P to ribonuclease S (Pavlovsky et al., 1977) and for that of three inhibitors to RNase A (Borkakoti, 1983). Pavlovsky proposes that the two nucleotides are bound in the anti conformation and that the ribose ring has a 3-endo conformation. His data also show two hydrogen bonds between the ligand and Thr-45. The anti conformation of Cyd-3′-P was proposed for ribonuclease A by Karpeisky & Yakovlev (1977) using ^1H NOE measurements. Borkakoti also shows that Cyd-2′-P is anti.

For steric reasons, it is evident that at least the sugar ring must adopt different orientations for such nucleotide, and it is also likely that the protein will adapt its own conformation correspondingly. The phosphate moiety was shown by Pavlovsky et al. (1977) to bind at His-12 and Lys-41.

The present study used both ^1H and ^{13}C NMR. We have already noted (Figure 1) a composite ^1H resonance at δ 0.5 containing both Val and Ile methyl resonances. One component of the δ 0.5 resonance was shown to be perturbed by δ 0.03 upon addition of phosphate or by δ −0.03 with cytidine. Single-frequency decoupling of this component links it with carbon peak 19. This resonance must arise from a Val residue, from its ^{13}C shift and because the other component arises (see below) from Ile-81 C^{δ}. Cu^{II} and phosphate binding show it to be close to His-12 and -119. We assign it as Val-118.

Similarly, the other component at δ 0.5 shifts δ 0.04 upfield with at least cytidine and by decoupling links with carbon peak 11. In support, carbon resonance 11 shows similar shift behavior, as does carbon peak 2. This latter peak is almost certain from its chemical shift to arise from Ile C^{δ} (Howarth, 1978) because the only other reasonable possibility, Met C^{ϵ}, has been ruled out above. We may therefore reasonably assign peak 2 to Ile C^{δ} and peak 11 to Ile C^{γ_2} of the same residue. This link is also consistent with the data in Figure 1, insofar as this suggests a link between the proton isoleucine resonance at δ 0.5, via resonances at δ 1.45 and 1.7, to a resonance at

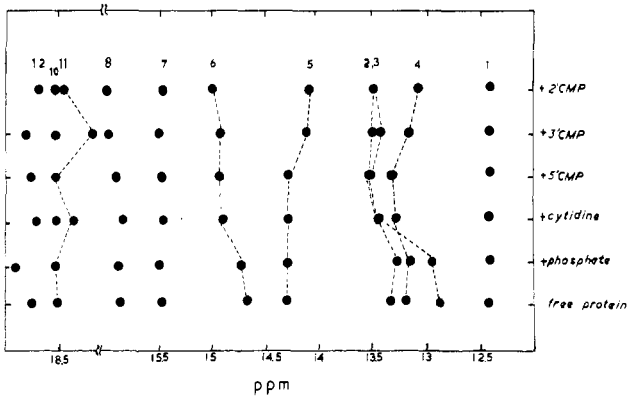

FIGURE 6: Chemical shifts of the methyl carbon resonances of RNase A (6 mM in 0.05 M NaCl), pD 5.5, 25 °C, in the absence and presence of inhibitors. The concentration of each inhibitor present is as follows: phosphate, 80 mM; cytidine, 80 mM; Cyd-5′-P, 80 mM; Cyd-3′-P, 65 mM; Cyd-2′-P, 40 mM. Peak numbers are those of Figure 5.

or near δ 0.8. Single-frequency irradiation near δ 0.8 decouples carbon resonance 2, amongst others. As peak 11 is also broadened by Cu^{II} binding at the active site (see previous section) and as the resonances of Ile-106 have already been assigned, we may now identify this Ile residue as Ile-81. The other Ile residue, 107, lies at a substantially greater distance from His-119, which is a binding site for Cu^{II} but not for phosphate.

The composite proton peak around δ 0.7 also appears from Figure 1 to contain Val and, probably, Ile methyl resonances. Decoupling linked it with carbon resonances 7 and 13. The latter resonances is assigned below to Val-47 C^{δ}, and this increases the probability that resonance 7 arises from an Ile C^{δ_2} group. Furthermore, peak 7, along with peak 1, is unusual in not being perturbed by binding any of the inhibitors. Peak 1 may therefore reasonably be assigned to Ile C^{δ} of the same residue. A likely assignment of this residue is Ile-107, because this lies further from the inhibitor-binding region than either of the other two Ile residues.

The remaining effects of inhibitor binding could only be reliably followed via ^{13}C spectra. In general, this binding produced no detectable broadening of resolved resonances but caused 90% of those that are resolvable to shift. At the inhibitor concentrations used, the proteins were in their fully complexed form.

The shifts are shown in Figure 6. It is evident from this figure that the perturbations due to Cyd-2′-P, -3′-P, and -5′-P are not identical, as anticipated above. The resolution of peaks 2 and 3 in the figure was effected via single-frequency decoupling at the quite distinct proton frequencies of Met C^{ϵ} and Ile C^{γ_2} and that of peak 4 by additionally using [*S*-methyl-Met[29]]ribonuclease, as described above, to remove peak 3.

The addition of phosphate significantly affects only peaks 2 (assigned above to Ile-81 C^{δ}), 3, 4 (assigned above to Met-13 C^{ϵ}), 6, 12 (assigned below to Val-47 C^{δ}), and 1.8. These shifts are all consistent with the binding of phosphate at His-12 and Lys-41, as proposed by previous workers, and they thus support the assignments already made.

Peak 5 is sensitive to the binding of Cyd-2′-P and -3′-P. Its shift is strongly suggestive of an Ile-C^{δ} carbon, as the three Met-C^{ϵ} resonances have already been identified with peaks 3, 4, and 6. Ile-81 and -107 C^{δ} have already been at least tentatively identified with resonances 2 and 1, respectively, and on this basis, we must assign peak 5 to Ile-106 C^{δ}. This atom lies only 386 pm from Phe-120 C^{ζ} in the crystal, and the latter residue is shown below and also by crystallography to be involved in binding of Cyd-2′-P and -3′-P. Thus, the above assignment of peak 5 is confirmed.

Peaks 3, 4, and 6 have already been assigned to Met-29, Met-13, and, by elimination, Met-30 or Met-79 C^{ϵ}, respectively. The modest effect of phosphate binding on peak 4 has already been noted. Much larger shifts are observed for each peak when cytidine inhibitors are bound. Peak 4 shifts downfield with cytidine and Cyd-5′-P and upfield with Cyd-2′-P and -3′-P. This distinction might be expected for Met-13 C^{ϵ}, as His-12 has been found to interact not only with the phosphate but also with the 2′-OH group of the ribose ring when both cytidine and Cyd-5′-P are bound.

We assign peak 6 to Met-30 C^{ϵ} because of its closeness (380 pm) to Lys-41 C^{ϵ}, in the active site. One resonance, namely, peak 12, has already been associated with Val-47 C^{γ} via urea binding. This assignment is supported by the sensitivity of this resonance to the binding of all ligands, including phosphate. Val-7 C^{γ_2} lies at 558 pm from His-12 C^{α} in the native crystal. The link between peaks 12 and 13 is also apparent from a combination of the ^1H decoupling data (Table I) and Figure 1.

One other peak, at δ 35.0 and assigned via its pD dependence to Glu C^{γ}, shows consistent perturbation with all added ligands. Glu-49 is a possible assignment, as it lies close to the active site, unlike Glu-2, -9, -87, or -110.

Protonated Aromatic Resonances. Lenstra et al. (1979) have assigned many of the aromatic resonances in the ^1H NMR spectrum of ribonuclease A. The corresponding protonated ^{13}C resonances in our spectra were too broad to be reliably identified. However, we were able to resolve one outstanding disagreement in the literature. Addition of Cyd-2′-P, -3′-P, or -5′-P to ribonuclease A leads to a new ^1H peak at δ 6.35. This has been assigned to either Phe-120 (Meadows et al., 1969) or to Tyr-25 (Markley, 1975b). We found first that when the assigned Tyr-25 H^{ϵ} at δ 7.12 is irradiated, the effects of decoupling are detectable elsewhere, namely, at δ 6.48. Secondly, an NOE difference experiment with irradiation of the new δ 6.35 peak gives an NOE at C(5)-H of Cyd-2′-P. The data of Pavlovsky et al. (1977) for Cyd-2′-P bound to ribonuclease S gives a minimum distance of 380 pm between N4 of the base and the carbon skeleton of the Phe-120 ring. Any more distant group is very unlikely to show enhancement, and therefore, both experiments confirm the δ 6.35 peak as arising from the ring of Phe-120.

Interestingly, the complexation shift of this resonance is in the opposite direction to that of the pyrimidine ring resonances. This strongly suggests that the two rings are nowhere near parallel; for if they were, they would both shift each other to lower frequency, if one above the other, or to higher frequency, if approximately coplanar.

Tyrosine C^{ζ} Resonances. The unprotonated carbon resonances appearing between δ 154.0 and 160.0 have been studied previously, as noted above. There are six resonances, 21–25 and 30, arising from Tyr C^{ζ}, as listed in Table I, together with four Arg C^{ζ} resonances, 26–29, of which three are resolved under our conditions. Peak 25 has been assigned to Tyr-25 by an argument based on its unusual pK_a of 5.9 (Santoro et al., 1979).

We confirm this assignment by an independent method, because peak 25 (alone) shifts down frequency by δ 0.45 when Met-29 is converted to methyl-Met-29 (see above). Tyr C^{ζ} lies at 434 pm from the S atom of Met-29 in the crystal. The same modification also confirms the assignment by Lenstra et al. (1979) of Tyr-25 H^{ϵ} in the ^1H spectrum.

Peaks 21–24 have not yet been specifically assigned. However, the above authors have assigned the ^1H resonances

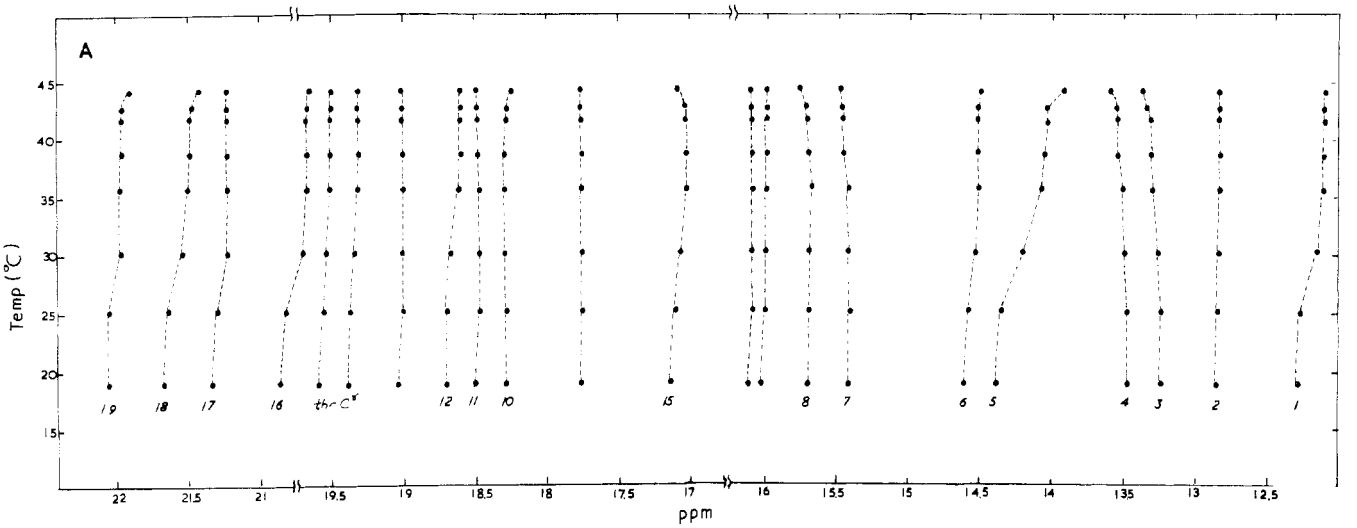

FIGURE 7: Thermal dependence of methyl ^{13}C NMR shifts in native ribonuclease A, pD 3.2. The protein denatures at 45 °C at this pD. Numbers are residue assignments as in Figure 2.

for both C^{δ} and C^{ϵ} of Tyr-25, -76, and -115 and for C^{ϵ} of Tyr-92. By using low-power single-frequency irradiation at these frequencies, we have observed narrowing of individual Tyr C^{ζ} resonances through the elimination of two- and three-bond $^{13}C-^{1}H$ couplings. The results are reported in Table I.

Peak 22 is unusual in that it shifts upfield slightly between 18.0 and 25.0 °C at pD 3.2, to a position very close to that found in the denatured protein, and thereafter remains constant. Such normalization behavior has already been proposed by Burgess & Scheraga (1975) for Tyr-92, and thus, its assignment is supported.

The remaining peaks, 24 and 30, may be assigned to Tyr-73 and -97, respectively, by accepting the assignment of Tyr-97 made by Egan et al. (1978) via its high pK_a value.

We note that none of these resonances are measurably affected by the binding of Cu^{II}, urea, or, excepting Tyr-97, inhibitors (Walters & Allerhand, 1980). Nor are they sensitive to temperature, saving the exception noted above. This contrasts with the methyl resonances, whose rms shift under the same perturbations is twice as great. Presumably, the methyl groups contribute substantially to local flexibility, whereas the tyrosines have a more structural role.

Reversible Thermal Denaturation of Ribonuclease A. In contrast to lysozyme (see following paper), ribonuclease A shows clear evidence of undergoing conformational change well below the temperature of the reversible denaturation transition even when a substrate is bound.

Burgess & Scheraga (1975) have proposed that the protein unfolds in stages, giving first free loops and eventually a random coil. However, our measurements point to quite different conclusions. Figure 7 shows the temperature dependence of the methyl resonances at pD 3.2. The first major transition starts at 25 °C (but see Tyr-92, above) and is complete by 35 °C. It involves at least resonances 1, 3–6, 12, and 16–19 and also two Thr resonances. Some thermal variation is also evident in several of the Tyr-C^{ζ} resonances, although in this case the transition is not distinct. Resonances 1, 3–6, 12, and 19 are assigned above the Ile-107 C^{δ}, Met-29 C^{ϵ}; Met-30 C^{ϵ}, Val-47 C^{γ}, and Val-118 C^{γ}, respectively, and one Thr resonance has been assigned to Thr-45 C^{β}. In all cases, the shift with increasing temperature is in the direction of the solvent-exposed "peptide" shift. This 25–35 °C transition explains the observation by Matheson & Scheraga (1979a) of a discontinuity in the gradient of the Arrhenius plot for enzymic activity at 32 °C.

Essentially, the same residues are involved in a second conformational change above 42 °C, i.e., just below the denaturation temperature at this pD. In this case, the change is not complete before denaturation begins. The direction of change is once again toward increased solvent exposure where the assignment is known, but no resonance normalizes, even approximately. Only peak 15 reverses direction, and only peaks 1 (Ile-107 C^{δ}), 12 (Val-47), and 17 do not apparently undergo the second transition although they do the first. These trends are not altered significantly when cytidine 2′-phosphate is bound, and the only marked difference at pD 5.0 is that the shift of peak 19 (Val-118) becomes completely independent of temperature from 25 to 45 °C.

Because no resonance normalizes and many (e.g., Phe) are thermally invariant, we conclude that no portion of the main peptide chain actually unfolds below the denaturation temperature, and indeed that with the exception of Tyr-92, no side chain becomes fully exposed to solvent. Instead, all the observed shifts are consistent with a modest expansion of the protein involving motion of both the N-terminal helix and C-terminal residues en bloc so as to permit limited penetration of solvent. In the case of the N-terminus, the partly exposed portion (Chothia et al., 1983) encloses the active site. This explains the shifts of Met-13, -29, and -30, Thr-45, and Val-47 and is not inconsistent with Tyr-25 remaining unaffected (Lenstra et al., 1979) in what may be a "hinge" region. The movement of the C-terminal section will affect Ile-106 and -107 and, at pD 3.2 only, Val-118.

Our observations are largely compatible with earlier ones, although they point to different conclusions. Burgess & Scheraga (1975) and later Matheson & Scheraga (1979b) have combined many observations in support of increased but partial exposure of the N-terminal section (1–24) well below the temperature for denaturation, with at least residues 1–12 remaining structured. These worker's observation of regions resistant to proteolysis even at higher temperatures is also consistent with our observations on structuring in the denatured state described below. The same considerations account for the observations made at lower magnetic field in this laboratory (Howarth, 1979).

In the main thermal denaturation transition of ribonuclease A, there is no significant difference between the spectrum of the part-denatured protein and a computer superimposition with appropriate weighting of the fully native and the fully

denatured spectra. This confirms that the main transition is strictly two state. The same observation holds for lysozyme and for urea denaturation of either protein, within the ±1 °C resolution of the method. There is also no evidence under our conditions for stepwise unfolding by urea.

The Denatured State. As reported in a preliminary paper (Howarth & Lian, 1981), the spectrum of the denatured state of ribonuclease (and lysozyme; see following paper) clearly shows that many residues remain inequivalent. This holds true for both thermal- and urea-induced denaturation and also even when the S–S bonds of ribonuclease A are cleaved, either by oxidation or reduction. We propose that this is due to substantial clustering of hydrophobic side chains, because the most marked shift heterogeneity is for hydrophobic residues (e.g., Ile, Met, and Phe) and also because the heterogeneity diminishes when the solvent is changed from water to less polar mixtures. The heterogeneity is 1 order of magnitude too large to be explained simply by known sequence effects. Some implications of these observations are discussed in the following paper.

Acknowledgments

We thank Dr. N. Borkakoti, Department of Crystallography, Birbeck College, London WC1E 7HX, U.K., for access to crystal coordinates for ribonuclease A.

Registry No. RNase A, 9001-99-4; Ile, 73-32-5; Met, 63-68-3; Val, 72-18-4; Lys, 56-87-1; Glu, 56-86-0; Thr, 72-19-5; Tyr, 60-18-4; Arg, 74-79-3; Cyd, 65-46-3; Cyd-2′-P, 85-94-9; Cyd-3′-P, 84-52-6; Cyd-5′-P, 63-37-6; Cu, 7440-50-8; Mn, 7439-96-5; phosphate, 14265-44-2; urea, 57-13-6.

References

Benz, F. W., & Roberts, G. C. K. (1975) *J. Mol. Biol. 91*, 367–387.

Borkakoti, N. (1983) *Eur. J. Biochem. 132*, 89–94.

Borkakoti, N., Moss, D. S., & Palmer, R. A. (1982) *Acta. Crystallogr., Sect. B B38*, 2210–2217.

Borkakoti, N., Palmer, R. A., Haneef, I., & Moss, D. S. (1983) *J. Mol. Biol. 169*, 743–755.

Bradbury, J. H., & Scheraga, H. A. (1966) *J. Am. Chem. Soc. 88*, 4240–4246.

Burgess, A. W., & Scheraga, H. A. (1975) *J. Theor. Biol. 53*, 403–420.

Carlisle, C. H., Palmer, R. A., Mazumdar, S. K., Gorinsky, B. A., & Yeates, D. G. R. (1974) *J. Mol. Biol. 85*, 1–18.

Chothia, C., Lesk, A. M., Dodson, G. G., & Hodgkin, D. C. (1983) *Nature (London) 302*, 500–505.

Egan, W., Shindo, H., & Cohen, J. S. (1978) *J. Biol. Chem. 253*, 16–17.

Fan, S., & Bersohn, R. (1975) *Biochim. Biophys. Acta 397*, 405–411.

Howarth, O. W. (1978) *Prog. Nucl. Magn. Reson. Spectrosc. 12*, 1–40.

Howarth, O. W. (1979) *Biochim. Biophys. Acta 576*, 163–175.

Howarth, O. W., & Lian, L. Y. (1981) *J. Chem. Soc., Chem. Commun.*, 258–259.

Howarth, O. W., & Lian, L. Y. (1984) *Biochemistry* (following paper in this issue).

Jaenck, G., & Benz, F. W. (1979) *Biochem. Biophys. Res. Commun. 86*, 885–892.

Jardetzsky, O., & Roberts, G. C. K. (1981) *NMR in Molecular Biology*, Academic Press, New York.

Karpeisky, M. Y., & Yakovlev, G. I. (1977) *FEBS Lett. 75*, 70–72.

Lenstra, J. A., Bolscher, B. G. J., Stob, S., Beintema, J. J., & Kaptein, R. (1979) *Eur. J. Biochem. 98*, 385–397.

Markley, J. L. (1975a) *Acc. Chem. Res. 8*, 70–80.

Markley, J. L. (1975b) *Biochemistry 14*, 3456–3554.

Markley, J. L., & Finkenstadt, W. R. (1975) *Biochemistry 14*, 3562–3566.

Matheson, R. R., & Scheraga, H. A. (1979a) *Biochemistry 18*, 2446–2450.

Matheson, R. R., & Scheraga, H. A. (1979b) *Biochemistry 18*, 2437–2445.

Meadows, D. H., Roberts, G. C. K., & Jardetzky, O. (1969) *J. Mol. Biol. 45*, 491–511.

Nagayama, K., Wütrich, K., & Ernst, R. R. (1979) *Biochem. Biophys. Res. Commun. 90*, 305–311.

Niu, C., Matsuura, S., Shindo, H., & Cohen, J. S. (1979) *J. Biol. Chem. 254*, 3788–3796.

Pavlovsky, A. G., Borisova, S. N., Vagin, A. A., & Karpeisky, M. Y. (1977) *Sov. J. Bioorg. Chem. (Engl. Transl.) 3*, 1378–1386.

Sadler, P. J., Benz, F. W., & Roberts, G. C. K. (1974) *Biochim. Biophys. Acta 359*, 13–21.

Santoro, J., Juretschke, H. P., & Ruterjans, H. (1979) *Biochim. Biophys. Acta 578*, 346–356.

Shindo, H., Hayes, M. B., & Cohen, J. S. (1976) *J. Biol. Chem. 251*, 2644–2647.

Shindo, H., Egan, W., & Cohen, J. S. (1978) *J. Biol. Chem. 253*, 6751–6755.

Wagner, G., & Wütrich, K. (1982) *J. Mol. Biol. 155*, 347–366.

Walters, D. E., & Allerhand, A. (1980) *J. Biol. Chem. 255*, 6200–6204.