Binding of Copper(II) by Ribonuclease A and Ribonuclease S-Peptide Investigated by ^{1}H Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The binding of Cu^{2+}, in deuterium oxide solution, to ribonuclease A at pD 6.0, and its N-terminal eicosapeptide, RNase S-peptide at pD 7.9, was investigated by ^{1}H nuclear magnetic resonance spectroscopy. The interactions were followed by observing the broadening of protein and peptide resonances caused by the dipolar interaction of bound Cu^{2+} with adjacent protons. S-Peptide resonances of residues close to His-12 in the amino acid sequence were the most severely affected. Quantitation of the broadening pattern by calculation of relative Cu^{2+}–proton distances suggested that the histidine residue is involved in complexation. Ribonuclease resonances most sensitive to Cu^{2+} are those belonging to the histidine imidazole protons implying that the histidine residues provide ligands for Cu^{2+}. The rates of broadening with $[Cu^{2+}]$ ([RNase A] = 1.0×10^{-2} M) of histidine H_{C-2} resonance bands were found to be 22×10^3, 21×10^3, and 11×10^3 Hz M^{-1} for His-105, -12, and -119, respectively, at pD 6.0, and 2.8×10^3 Hz M^{-1} for His-48 at pD 5.4. It was concluded that histidine-105 and -12 provide somewhat stronger ligands for Cu^{2+} than His-119 which in turn is preferred to His-48.

The study of the binding of metal ions by amino acids, peptides and proteins is an active area of research in biochemistry (Gurd, 1970). Particular effort has been directed toward investigating the association of Cu^{2+} with peptides and proteins making extensive use of various techniques such as pH titration, spectrophotometry, equilibrium dialysis, gel filtration, and enzyme kinetics.

Increasing use is being made of magnetic resonance methods to investigate the interaction of Cu^{2+} with amino acids and peptides (Li *et al.*, 1962; Sheinblatt, 1967; Falk *et al.*, 1967; Sigel *et al.*, 1969; Ihnat and Bersohn, 1970), and proteins (Gurd *et al.*, 1967a,b; Joyce and Cohn, 1969; Roberts and Jardetzky, 1970). We describe in this report an investigation, by high-resolution ^{1}H nmr spectroscopy,[1] of the interaction of Cu^{2+} with RNase A and the N-terminal eicosapeptide, RNase S-peptide, in aqueous media.

It has been shown repeatedly that Cu^{2+} effectively inhibits the enzymatic activity of RNase A (Zittle, 1946; Davis and Allen, 1955; Takahashi *et al.*, 1967; Alger, 1970). This effect has been ascribed to the binding of Cu^{2+} to the two histidine residues at the active site, His-12 and -119. The binding of Cu^{2+} to RNase A in solution has been investigated by a variety of methods (Saundry and Stein, 1966, 1967; Breslow and Girotti, 1966; Girotti and Breslow, 1968, 1970; Takahashi *et al.*, 1967), and by the technique of nuclear magnetic resonance (Joyce and Cohn, 1969; Roberts and Jardetzky, 1970). Allewell (1969) and Allewell and Wyckoff (1971) have recently studied, by X-ray diffraction, the binding of Cu^{2+} to RNase S in the solid state. Up to five binding sites for Cu^{2+} have been reported for RNase A in solution, the histidine and the amino-terminal lysine residues providing ligands.

Seven sites have been located on RNase S in the crystal, several being intermolecular. Histidines-12, -105 and -119 but not -48 are found close to the Cu^{2+} bound to RNase S. A recent report (Levitzki and Berger, 1971) on the association of Cu^{2+} with RNase S-peptide studied by a chloroiridate oxidation technique has indicated the binding of the metal ion to the α-amino end of the molecule.

Nuclear Magnetic Resonance. The consequences of the binding of a paramagnetic metal ion by a ligand molecule are alterations of the chemical shifts and line widths of the nuclear resonances of the ligand. Transfer of some electron spin density from the metal ion to the ligand can give rise to large contact shifts which are observable with metal ions with very short relaxation times. In addition there is a dipolar interaction between the point nuclear magnetic dipole and the electronic dipole which is quantitatively understood. Both the contact and the dipolar interactions decrease the nuclear longitudinal and transverse relaxation times leading to line broadening (Carrington and McLachlan, 1967). Cu^{2+} has a long electron spin–lattice relaxation time of $\sim 10^{-8}$ sec; because of this, its dominant effect is line broadening. Protons very close to the Cu^{2+} ion have resonances broadened both by the so-called contact interaction with the spin density and by the large range dipolar interactions. A little further away from the Cu^{2+} ion the broadening is entirely due to the dipolar interaction; the line width is inversely proportional to the sixth power of the distance between the metal ion and the given proton. On the nmr time scale Cu^{2+} has a rapid exchange rate between the complex and the solution. This phenomenon gives rise to one spectrum in which the line widths are weighted averages of the two ligand species present in solution, free and complexed to Cu^{2+}, and are proportional to the

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[1] Abbreviations used are: ^{1}H nmr, ^{1}H nuclear magnetic resonance; RNase A(S), bovine pancreatic ribonuclease A(S); S-peptide, the N-terminal eicosapeptide of RNase A; CAT, computer of average transients.

total concentration of Cu^{2+}. Thus for the more distant protons

$$\Delta\nu_{1/2Cu^{2+}} = \frac{Kn[Cu^{2+}]}{\pi r^6[\text{ligand}]} \quad (1)$$

where $\Delta\nu_{1/2Cu^{2+}}$ is the observed line width increment caused by the presence of Cu^{2+}, n is the number of ligand molecules bound to Cu^{2+}, r is the distance between the proton and the metal ion, [Cu^{2+}] and [ligand] are the total concentrations of Cu^{2+} and ligand, respectively, and K is a constant. The line width is a sensitive function of r; protons close to Cu^{2+} in the complex will experience severe broadening of their resonances. Hence by observing the selective effect of Cu^{2+} on the various protons it is possible to locate the Cu^{2+} in the complex.

This technique was utilized in a previous study (Ihnat and Bersohn, 1970) determining the structure of the Cu^{2+}–carnosine complex. In the present investigation, Cu^{2+} is used to probe metal binding sites on RNase A and RNase S-peptide by following the selective broadening of the protein and peptide resonances. Besides being biochemically important (Peisach *et al.*, 1966), the relatively long electron spin–lattice relaxation time of Cu^{2+} gives the ion magnetic properties suitable to the problem at hand.

The first ^1H nmr spectrum of RNase A (40 MHz) was reported in 1957 by Saunders, Wishnia, and Kirkwood. Subsequently several other investigators have reported complete spectra or partial low-field spectra, several of the more recent ones being at 220 MHz (McDonald and Phillips, 1967a,b; Ferguson and Phillips, 1967). The low-field histidine resonances have been assigned to the corresponding residues His-12, -48, -105, and -119 (Meadows *et al.*, 1968). No complete S-peptide spectra have been reported.

Experimental Section

Materials. Ribonuclease A was obtained from several sources: Worthington Biochemical Corp. (lots RAF 7DA, 7GC, and 7JA), Mann Research Laboratories (lot S2584) and Gallard-Schlesinger Manufacturing Corp. The bulk of the work was carried out with Worthington lot no. 7JA. Ribonuclease S-peptide was Sigma Chemical Co. type XII-PE lots 117B-8000 and 117B-8090, the latter being used for Cu^{2+} binding studies. D_2O of 99.7% purity from Bio-Rad Laboratories and Columbia Organic Chemicals was used as solvent. Adjustments of pD were made with 5 M DCl and NaOD prepared from concentrated solutions obtained from Calbiochem and Stohler Isotope Chemicals.

Solutions were prepared by weight; molar concentrations were calculated from the weights of the components and measured densities of the solutions. Final concentrations were 1.0×10^{-2} and 3.4×10^{-2} M for RNase and S-peptide, respectively. Small amounts of solution of anhydrous $CuCl_2$ in D_2O were added to the RNase and S-peptide solutions to give desired concentrations of Cu^{2+}. pH was measured at *ca.* 25° (room temperature) with a Sargent combination microelectrode. The Ag–AgCl reference electrode chamber was filled with a solution of KCl in D_2O to minimize the leakage of water into the solutions. Conversion to pD units was made using the relationship pD = pH + 0.40 (Lumry *et al.*, 1951; Glasoe and Long, 1960; Mikkelsen and Nielsen, 1960). Occasionally, solutions (without Cu^{2+}) were lyophilized and redissolved in D_2O to diminish the intensity of the HOD band. To avoid the complication of Cu^{2+} binding to buffer components and the possibility of ternary complex formation, no buffers were used (Good *et al.*, 1966).

Solutions of ribonuclease were kept at pD 10 overnight at room temperature to allow the protons bonded to nitrogen and oxygen to exchange with the deuterium of the solvent. This procedure diminished the intensity of the NH absorption which obscures the histidine resonances in the low-field region of the spectrum. The pD was then adjusted as desired. (For experiments with Cu^{2+}, the metal was added prior to the adjustment of pD.) Solutions of pure RNase were generally clear and pale straw colored, whereas those containing Cu^{2+} at neutral pD had a bluish tinge. The intensity of the color depended on pD and concentration of Cu^{2+}. Alkaline solutions of RNase + Cu^{2+} were pink.

S-Peptide in D_2O gave a suspension with a very acid pD of *ca.* 1.5. Upon being made alkaline (pD 10), most of the solid dissolved to give a somewhat turbid, straw-colored solution. The solutions were left overnight at room temperature then centrifuged and/or filtered through Millipore filters to give clear, pale straw-colored solutions. The amount of insoluble material was *ca.* 5%. Cu^{2+} was then added and the pD adjusted as desired. Solutions of S-peptide with Cu^{2+} were bluish at neutral pD and pink when alkaline. Solutions of pure peptide became turbid when brought below pD *ca.* 7.0.

Methods. ^1H nmr spectra were recorded at 60, 100, and 220 MHz on Varian spectrometer Models A60A, HA 100, and HR 220, respectively, using 5-mm precision sample tubes from the Wilmad Glass Co. Preliminary experiments with RNase were carried out at 60 and 100 MHz using a computer of average transients (Varian C-1024) to enhance the signal to noise ratio. The availability of a spectrometer operating at 220 MHz enabled us to do all subsequent experiments with RNase at this higher radiofrequency, one sweep being sufficient to bring out spectral details and observe the individual histidine resonances. All S-peptide spectra were taken at 100 MHz. The field was locked either on the HOD resonance arising from the residual water in the sample, or on the resonance of H_2SO_3 contained in a capillary (Wilmad no. 520) inserted into the nmr tube. Ambient insert temperatures, as measured with methanol or ethylene glycol nmr thermometers, were 40, 31, and 17° for the A60A, HA 100, and HR 220 spectrometers, respectively. The instrumental resolution as determined by the bandwidth of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (Merck, Sharp and Dohme) was *ca.* 0.5 Hz at 60 and 100 MHz, and *ca.* 1 at 220 MHz. Chemical shifts, δ, were determined relative to the sulfonate used as an internal or external reference.[2] The internal reference was omitted from solutions containing Cu^{2+}. Addition of Cu^{2+} to RNase and S-peptide solutions did not significantly alter chemical shifts. A pD of 6.0 was chosen to study the binding of Cu^{2+} to RNase as at this pD the histidine resonances are maximally separated and the effect of Cu^{2+} on them can most conveniently be observed. A Dupont 310 curve resolver was utilized when necessary to resolve overlapping histidine resonances and for a preliminary resolution of the upfield region of RNase.

Results

Ribonuclease S-Peptide. RNase S-peptide prepared from ribonuclease A by enzymatic cleavage of the first 20 amino

[2] Chemical shifts, δ, are given in parts per million, positive when downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate: $\delta = (\nu_r - \nu_s)/\nu_0 \times 10^6$, where ν_s and ν_r are the frequencies of the sample and reference bands, respectively, and ν_0 is the operating frequency of 60, 100, or 220 MHz.

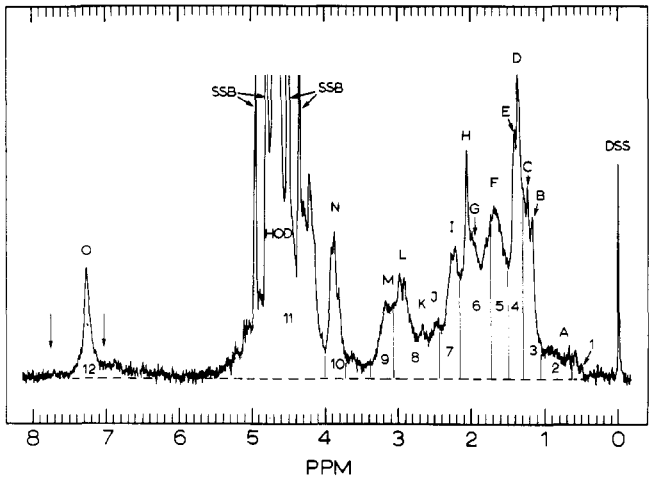

FIGURE 1: ^{1}H nuclear magnetic resonance spectrum of RNase S-peptide at 100 MHz. [S-peptide] = 3.4×10^{-2} M; pD 7.9. Arrows at 7.8 and 7.0 ppm indicate positions where the H_{C-2} and H_{C-4} imidazole protons, respectively, of the histidyl residue are expected to absorb but are not visible under the present conditions. The large resonance at 4.8 ppm, with spinning side bands (SSB), arises from residual water in the D_2O solution.

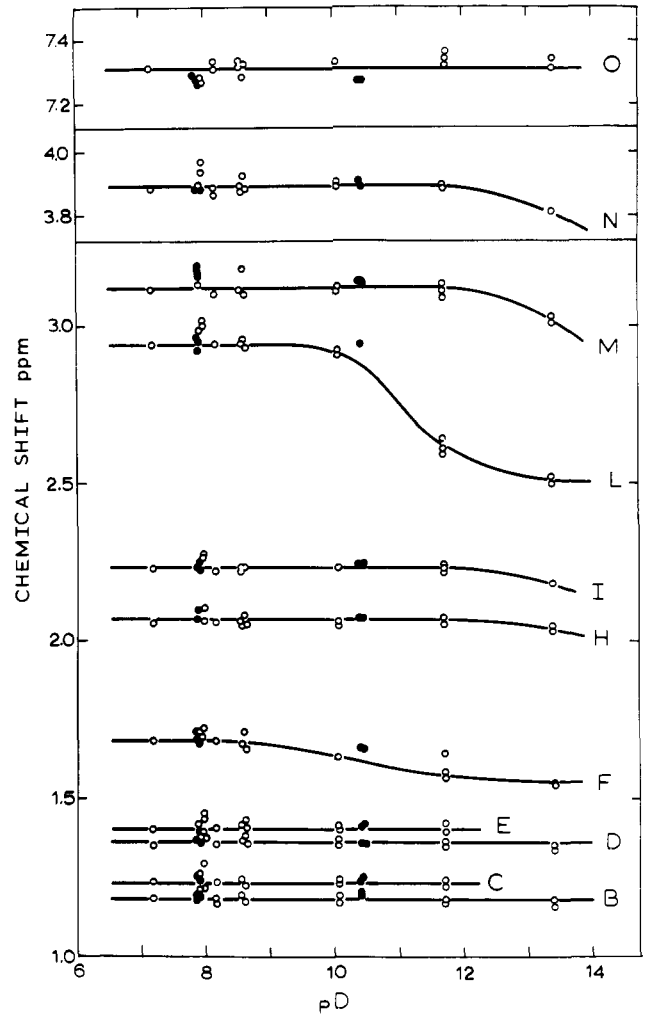

FIGURE 2: Chemical shifts of S-peptide resonances as functions of pD. (○) No Cu^{2+}, (●) $[Cu^{2+}] = 2.9 \times 10^{-4}$ to 3.7×10^{-3} M. [S-peptide] = 3.4×10^{-2} M.

acids, contains 144 protons, 41 of which are nitrogen or oxygen bonded and exchange with the deuterium of the solvent D_2O, leaving 103 carbon-bonded protons which give rise to the spectrum shown in Figure 1. Bands B–E at 1.1–1.4 ppm downfield from the sulfonate comprise the seven methyl groups of the five alanine and two threonine residues; the sharp band H, at 2.1 ppm arises from the CH_3 of Met-13. The two ϵ CH_2's of Lys-1 and Lys-7 fall at 2.9 ppm (L); peak N may be assigned to the three β CH_2's of the three serines, and the relatively sharp band O at 7.3 ppm results from the five ring protons of Phe-8. The arrows at 7.8 and 7.0 ppm indicate the positions where the imidazole protons of His-12, H_{C-2} and H_{C-4}, respectively, are expected to absorb. These protons were not visible under the present conditions (pD 7.9, 31°; Meadows *et al.*, 1968). No residual NH resonances were observed.

The assignment of the doublet L at 2.9 ppm, to the lysine ϵ methylenes was confirmed by observing the effect of pD on the chemical shift of the band. As the solution was titrated from pD 7 to 13, most bands of the peptide experienced little or no changes in chemical shift (Figure 2). Bands L and F, however, moved upfield with increasing pD by approximately 0.43 and 0.14 ppm, respectively. The pK (converted from the pD scale, Li *et al.*, 1961) of the titratable group responsible for the change in chemical shift of band L, as extracted from the point of inflection of the δ *vs.* pD plot was *ca.* 10.7. These data substantiate the assignment of L to the ϵ CH_2's of the lysine residues as the pK of an ϵ NH_2 in proteins falls in the range 9.4–10.6 (White *et al.*, 1959), and the change in shift of 0.43 ppm upon titration corresponds to the behavior of a CH_2 group adjacent to an amino group (Ihnat and Bersohn, 1970).

The chemical shift of 1.68 ppm observed for band F leads to its assignment to the β and δ methylenes of the lysine residues and the γ methylene of arginine. Furthermore, its movement with increasing pD leads to an estimated pK of *ca.* 9.9 also within the pK range of an ϵ-amino group. The two δ methylenes are each one carbon displaced from side ϵ-NH_2 groups and it is these four protons which are responsible for the change in shift of the band over the pD range 9–12. The remaining six protons are not expected to exhibit any pD dependence in that range. The alteration in chemical shift of band F of 0.14 ppm upon titration, however, is somewhat less than expected for methylenes one carbon displaced from an amino group. As the pK of the terminal α NH_2 of Lys-1 has been reported to be 7.3 in RNase A (Girotti and Breslow, 1970), some change in chemical shift of the β-CH_2 protons of Lys-1 at neutrality might be expected. The lack of any such effect on band F may be the result of these protons constituting only 20% of the band and the lack of data below pD 7.

Based on the chemical shifts expected for the protons of amino acid residues in a randomly coiled polypeptide chain in neutral solution reported by McDonald and Phillips (1969), the S-peptide spectrum was sectioned into regions 1-12, and each assigned to various protons giving rise to resonances in the corresponding range of chemical shift. The positions of the dividing lines separating adjacent regions correspond to expected absorption minima. It may be observed that for the actual spectrum, in several instances, adjacent sections do not meet at minima. This may be due to deviations of the S-peptide conformation from a random-coil conformation, the presence of secondary and tertiary structure giving rise to deviations in chemical shifts from those for a randomly coiled polypeptide. Assignments of the various regions and bands are given in Tables I and II. Although not

TABLE I: Assignment of S-Peptide Spectrum.

Region	Chemical Shift Range (ppm)	Groups Predicted for Randomly Coiled S-Peptide	No. of Protons Predicted	No. of Protons Found[a]
1 + 2	0–1.05	None	0	3.5
3	1.05–1.32	$2CH_3$(Thr-3,17)	6	7.5
4	1.32–1.52	$5CH_3$(Ala-4,5,6,19,20) $2\gamma CH_2$(Lys-1,7)	19	10.1
5	1.52–1.75	γCH_2(Arg-10) $2\beta + 2\delta CH_2$(Lys-1,7)	10	8.9
6	1.75–2.18	βCH_2(Arg-10) $2\beta CH_2$(Glu-2,9) $CH_3 + \beta CH_2$(Met-13) βCH_2(Gln-11)	13	14.5
7	2.18–2.45	$2\gamma CH_2$(Glu-2,9) γCH_2(Gln-11)	6	6.8
8	2.45–3.09	γCH_2(Met-13) βCH_2(Asp-14) $1H\beta CH_2$(Phe-8) $2\epsilon CH_2$(Lys-1,7)	9	12.0
9	3.09–3.40	βCH_2(His-12) δCH_2(Arg-10) $1H\beta CH_2$(Phe-8)	5	4.7
10	3.70–4.00	$3\beta CH_2$(Ser-15,16,18)	6	5.3
11	4.00–6.14	$20\alpha CH$ $2\beta CH$(Thr-3,17)	22	Not determined
12	6.14–7.50	5-Ring H (Phe-8) H_{C-4}(His-12)[b]	6	5.8
	7.8	H_{C-2}(His-12)[b]	1	0
	Total excluding region 11		81	79.1

[a] Calculated from areas of regions and based on the assignment of 68 protons to total of areas of regions 1–9. [b] The H_{C-4} and H_{C-2} protons are not visible under the present conditions.

TABLE II: Assignment of S-Peptide Resonances.[a]

Band	Chemical Shift at pD 7.9 (ppm)	Estd No. of Protons	Important Contributing Groups[b]	Chemical Shift in Random-Coil Polypeptide, (ppm)[c]
B	1.18	3	$1CH_3$(Thr-3,17)	1.23
C	1.23	3	$1CH_3$(Thr-3,17)	1.23
D	1.36	8	$2.5CH_3$(Ala-4,5,6,19,20)	1.41
			$2\gamma CH_2$(Lys-1,7)	1.43
F	1.68	10	γCH_2(Arg-10)	1.66
			$2\beta + 2\delta CH_2$(Lys-1,7)	1.68[d]
H	2.05	3	CH_3(Met-13)	2.06
I	2.23	6	$2\gamma CH_2$(Glu-2,9)	2.27
			γCH_2(Gln-11)	2.32
L	2.94	5	$1H\beta CH_2$(Phe-8)	2.95
			$2\epsilon CH_2$(Lys-1,7)	3.02
M	3.12	5	βCH_2(His-12)	3.18
			δCH_2(Arg-10)	3.20
			$1H\beta CH_2$(Phe-8)	3.18
N	3.89	6	βCH_2(Ser-15,16,18)	3.94[e]
O	7.33	5	Ring protons (Phe-8)	7.26

[a] Assignment is limited to those resonance bands selected for Cu^{2+}–proton distance calculations. [b] Not all groups listed are expected to contribute significantly to the band intensity (see text). [c] Chemical shifts are from McDonald and Phillips (1969). [d] The α NH_2 of Lys-1 may be partially titrated at pD 7.9. As a result, the β CH_2 of Lys-1 would have a somewhat smaller chemical shift but would still be expected to lie within the domain of band F. [e] Value for serine in zwitterionic state from Mandel (1965).

all of these assignments may be correct, they are sufficiently accurate for the present purpose of making a rough estimate of the position of binding of Cu^{2+}. The number of protons in each region was determined by cutting up the spectrum and weighing. For most regions, the agreement between the number of protons found and predicted is quite good (Table I).

S-Peptide Plus Copper. The effect of increasing concentrations of Cu^{2+} on the S-peptide spectrum is presented in Figure 3. A single broadened spectrum was observed at all concentrations of Cu^{2+} indicating that the residence times of ligand groups in the coordination sphere of Cu^{2+} are short on the nmr time scale. The most striking observation on Figure 3 is the rapid broadening, with increasing $[Cu^{2+}]$, of the phenyl protons of Phe-8 (band O), and the rapid disappearance of the resonance due to the methyl group of Met-13 (band H). Also noticeable is the perseverance, even at the highest concentration of Cu^{2+} ($[Cu^{2+}]/[S\text{-peptide}] = 0.1$), of the resonances due to the ϵ CH_2's of Lys-1 and -7 (band L), and the upfield bands B–E. Intermediate to these extremes is the behavior of the serine resonances in region 10 (band N), which do not vanish as rapidly as the phenylalanine and methionine resonances, but undergo more severe broadening than the lysine, alanine, and threonine bands. Paralleling the disappearance of the phenylalanine and methionine resonances, peak M in region 9 containing a β CH_2 of His-12, δ CH_2 of Arg-10, and 1 H of β CH_2 of Phe-8 also vanishes. Extensive overlap of most resonance bands precludes the direct determination of bandwidths as a function of $[Cu^{2+}]$. Even in the absence of Cu^{2+}, overlap of adjacent bands is sufficiently severe to require resort to extensive curve resolution techniques to extract the widths of individual bands. One resonance, however, that due to the phenyl group (O), is sufficiently well separated to permit direct width measurements. A graph of bandwidth *vs.* $[Cu^{2+}]$ shows the linearity predicted by eq 1; $\Delta\nu_{1/2}(Hz) = 3.0 \times 10^4\,[Cu^{2+}] + 7.3$, where $\Delta\nu_{1/2}$ is the observed bandwidth.

Instead of attempting resolution of the spectra to obtain bandwidths, the interaction was followed by observing the effect of Cu^{2+} on the heights of the bands and calculating the concomitant increases in widths. Consequently, plots *vs.* $[Cu^{2+}]$ of the heights, h, of bands B, C, D, F, H, I, L, M, N, and O were made, and $d\Delta\nu_{1/2}/d[Cu^{2+}]$ was computed from the slopes $dh/d[Cu^{2+}]$ from the relation

$$\frac{d\Delta\nu_{1/2}}{d[Cu^{2+}]} = \frac{-2A}{\pi h^2}\,\frac{dh}{d[Cu^{2+}]} \qquad (2)$$

in which A is the area of the band under consideration, and the factor $\pi/2$ comes from the formula $A = (\pi/2)h\Delta\nu_{1/2}$ for the area of a lorentzian curve. As the instrumental amplifica-

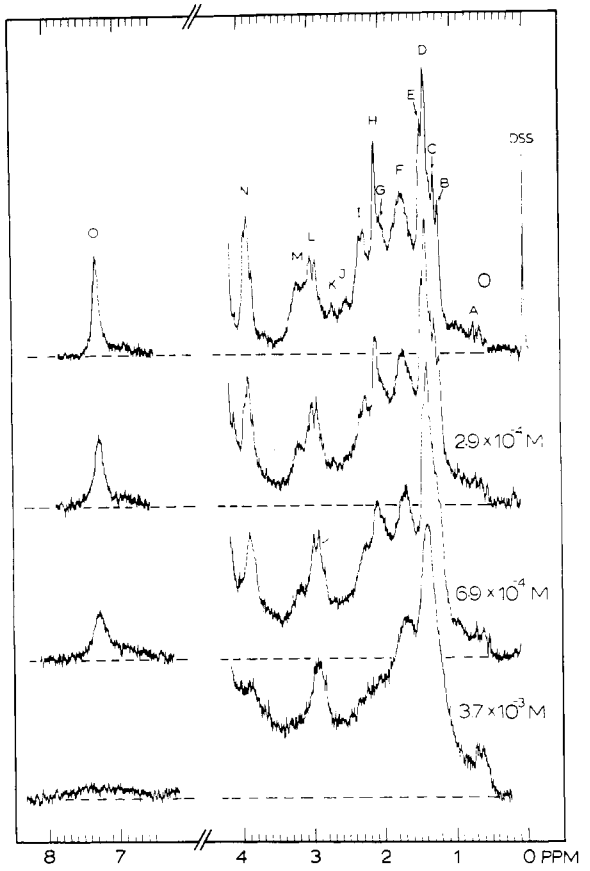

FIGURE 3: Effect of increasing concentrations of Cu^{2+} on the spectrum of S-peptide. [S-peptide] = 3.4×10^{-2} M; pD 7.9.

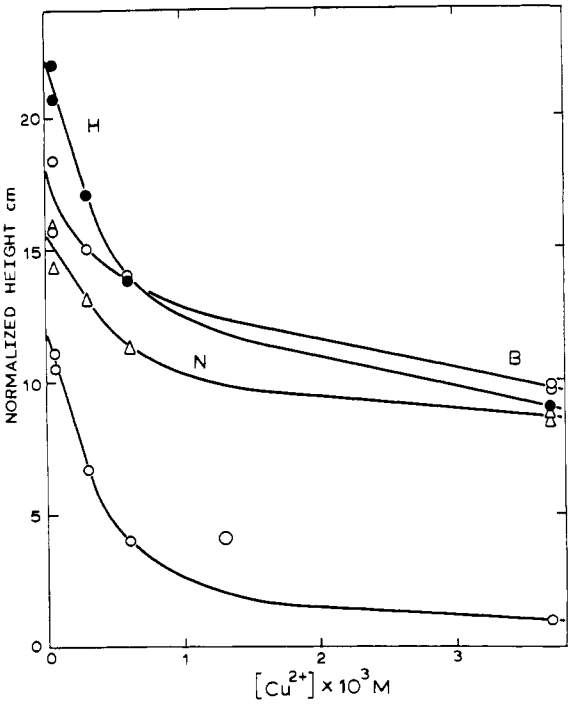

FIGURE 4: Normalized heights of several S-peptide bands as functions of $[Cu^{2+}]$ at constant S-peptide concentration of 3.4×10^{-2} M. pD 7.9.

tion varied somewhat for the different spectra, band heights were normalized prior to plotting. It was apparent that measuring heights from the base line underestimated the effect of Cu^{2+} on several of the bands. For this reason, heights of bands I, L, M, and N were also measured relative to the nearby absorption minima at 2.6 and 3.5 ppm, and average values of $d\Delta\nu_{1/2}/d[Cu^{2+}]$ were used to compute Cu^{2+}–proton distances. The number of protons contributing significantly to each band was estimated from the coincidence of the chemical shifts predicted for the amino acid residues in a random-coil conformation, and the protons' spin–spin coupling characteristics. The normalized area, A, of each band was computed from the estimated number of protons and the normalized area per proton of 38-cm Hz.

Typical plots of h *vs.* $[Cu^{2+}]$ for several bands are depicted in Figure 4. Slopes, $dh/d[Cu^{2+}]$, and values of $d\Delta\nu_{1/2}/d[Cu^{2+}]$ were calculated. The computation was also carried out for the phenylalanine ring protons; the value for $d\Delta\nu_{1/2}/d[Cu^{2+}]$ of 3.0×10^4 Hz M^{-1} is identical with that obtained directly from a plot of $\Delta\nu_{1/2}$ *vs.* $[Cu^{2+}]$.

Values of $d\Delta\nu_{1/2}/d[Cu^{2+}]$ obtained from eq 2 were used to calculate relative distances between the Cu^{2+} and protons represented by the various bands, using the relation

$$r = [K'(d[Cu^{2+}]/d\Delta\nu_{1/2})]^{1/6} \quad (3)$$

in which $K' = Kn/\pi$[ligand]. Separations, relative to the distance between Cu^{2+} and the ring protons of Phe-8 (band O) are presented in Table III. Also included, for purposes of comparison, are relative distances, computed from X-ray crystallographic coordinates of RNase S determined by Wyckoff *et al.* (1970). For this calculation it was assumed that the Cu^{2+}–S-peptide complex contained one Cu^{2+} bonded to the N-3 nitrogen of the histidine residue (refer to discussion). Distances between Cu^{2+} and the protons of the S-peptide portion of RNase S were calculated from the published coordinates. For each band a mean distance was calculated from the relation

$$\bar{r}_{rel} = \left[\frac{1}{\overline{(1/r_{rel})^6}}\right]^{1/6} = \left[\frac{1}{\frac{1}{p}\sum_{i=1}^{p}(1/r_{rel,i})^6}\right]^{1/6} \quad (4)$$

where $\bar{r}_{rel}$ is the mean distance relative to the Cu^{2+}–phenylalanine ring proton distance taken to be unity, and p is the number of protons at a distance $r_{rel,i}$ from the Cu^{2+}. This method of averaging allows comparison of the crystallographic r_{rel} with the nmr values. The number of protons, p, in the calculation is usually but not necessarily equal to the number of protons in each band listed in Table II. The reason is that although the number of protons contributing to the intensity of each band is known, it is not known which protons contribute to the band.

Upon altering the pD of a solution 3×10^{-2} M in S-peptide and 3×10^{-3} M in Cu^{2+}, both the color and nmr spectrum changed. Initially bluish at pD 7.9, the solution became pink as the pD was raised above *ca.* 9.0. The solution remained clear with no precipitate present indicating that all of the Cu^{2+} remained complexed. A concomitant sharpening of resonance bands occurred. The color change presumably results from the involvement of more nitrogen ligands. The spectral alteration may be ascribed to a decrease in the electron spin–lattice relaxation time of the Cu^{2+} due to the different ligand field.

TABLE III: Relative Distances between Cu^{2+} and S-Peptide Protons.

Band	Relative Distance: Experimental	Relative Distance: From Crystallographic Coordinates[a]	Position of Amino Acid Residue Contributing to Resonance[b]
O	1.00	1.00	8
M	0.97	1.08	8, 10, 12
L	1.59	1.24	1, 7, 8
I	1.20	1.40	2, 9, 11
F	1.50	1.52	1, 7, 10
H	1.37	1.65	13
D	1.62	1.96	1, 4, 5, 6, 7, 19, 20
B	1.44	2.89	3, 17
C	1.59	2.89	3, 17
N	1.19	3.32	15, 16, 18

[a] Crystallographic coordinates of RNase S used (Wyckoff *et al.*, 1970). Coordinates of carbon atoms were used in the calculations; it is assumed that these coordinates are fair representations of the positions of attached protons. Cu^{2+} is assumed to be bonded to His-12N-3 taken to be position XE2 in the crystallographic structure. Relative distances can be converted into absolute distances by multiplying by 5.41 Å (the distance between Cu^{2+} and the phenyl ring of Phe-8). [b] Lys-1 and -7 were omitted from crystallographic calculations for band D as their contribution to intensity of D is minimal.

Ribonuclease. A spectrum of the histidine-aromatic region of RNase A at pD 6.0 and 220 MHz is shown as the top curve in Figure 5. Even at this high frequency the multiplicity of protons with similar chemical shifts and extensive spin–spin coupling together with inherently broad bands due to a large rotational correlation time leads to extensive overlap of resonances. The 15 ring protons of phenylalanine, 24 ring protons of the tyrosines, and 4 H_{C-4} of the histidines contribute to the broad band in the region 6.0–7.5 ppm. The sharp peak labeled 4 belongs to H_{C-4} of His-105. Downfield of the aromatic region are several sharp resonances 1, 2, and 3 arising from the H_{C-2} imidazole protons of His-105, -12, and -119, respectively.

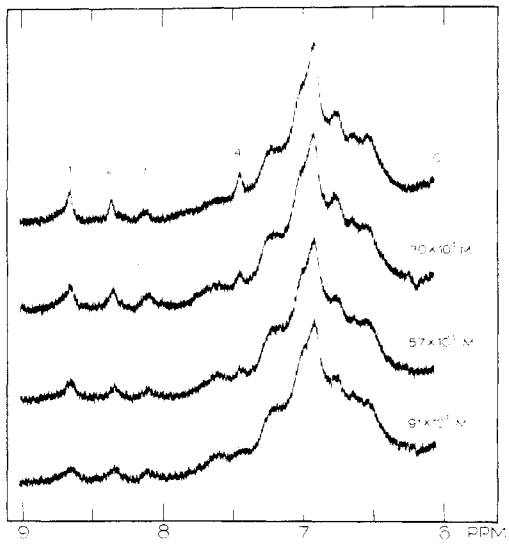

FIGURE 5: Histidine aromatic region nmr spectrum of RNase A and the effect of Cu^{2+} on the histidine aromatic resonances. [RNase] = 1.0×10^{-2} M; pD 6.0; [Cu^{2+}] is indicated. Resonance lines 1, 2, and 3 arise from the H_{C-2} protons of His-105, -12, and -119, respectively; band 4 belongs to H_{C-4} of His-105. The absorption envelope in the region 6.0–7.5 ppm comprises phenylalanyl, tyrosyl, and histidyl H_{C-4} resonances.

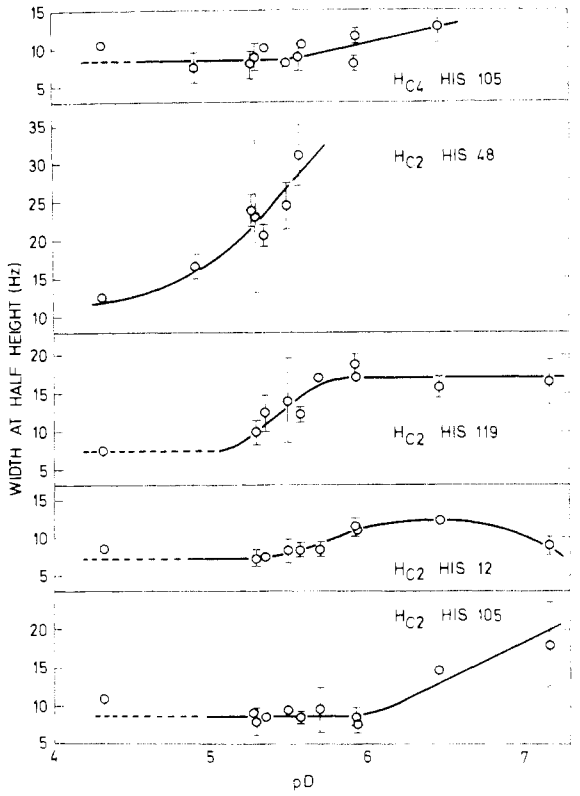

FIGURE 6: Line widths of RNase A histidine resonances *vs.* pD.

Most RNase resonances suffered rather small alterations of chemical shift with increasing pD over the range investigated (pD 4–10). The histidine resonances, however, exhibited large upfield shifts due to the titration of the imidazole groups as previously reported (Meadows *et al.*, 1967, 1968). The approximate pK's at 25° (corrected from the pK_D scale) of the imidazole NH protons were found to be: His-12, 5.6; His-105, 6.4; His-119, 5.4. These values are on the average 0.5 pH unit lower than those interpolated for 25° from the data of Roberts *et al.* (1969), and 0.1 unit lower than those reported for 32° by Bradbury and Scheraga (1966).

Besides undergoing alterations in chemical shifts as the RNase solution is titrated, the histidine imidazole resonances also suffer changes in bandwidth. The pD dependence of bandwidth is depicted in Figure 6. Whereas the widths of residues 12, 105, and 119 are in the range 8–17 Hz over the pD range 4–7, the width of H_{C-2} His-48 increases rapidly from 12 Hz at pD 4.3 to *ca.* 30 Hz at pD 5.6 making this band difficult to observe above this pD. Consequently, although a pD of 6.0 was chosen for binding studies, to observe the effect of Cu^{2+} on the His-48 resonance, resort was made to a somewhat lower pD of 5.4. The corresponding widths in a solution of pure RNase were subtracted from those in Cu^{2+}–RNase solutions to give bandwidth increments due to Cu^{2+} reported below. The broadness of the H_{C-2} His-48 resonance of RNase A in acetate buffer and NaCl solution has been re-

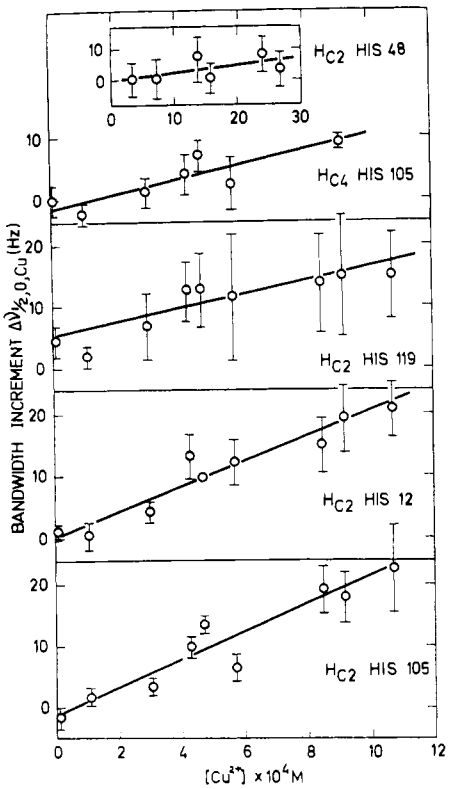

FIGURE 7: Line-width increments, due to Cu^{2+}, of histidyl imidazole bands of RNase A as functions of $[Cu^{2+}]$. [RNase] = 1.0×10^{-2} M; pD 6.0. The inset is for the H_{C-2} resonance of His-48, pD 5.4.

ported previously (Meadows *et al.*, 1968; Roberts *et al.*, 1969). Imidazole protons of histidines in trypsin, α-chymotrypsin, and their zymogens have also been reported to have broad bands of *ca.* 30–40 Hz (Bradbury and Wilairat, 1967).

Our interpretation is that the imidazole side chain reorients around the Cβ–Cα axis between two positions nonequivalent but indistinguishable by X-rays which do not distinguish between carbon and nitrogen atoms. The p*K*'s in the two positions are different thus magnifying the proton chemical shift differences. We assume that the potential barrier between the two positions is sufficiently large (12–15 kcal/mole) that we are in the slow exchange limit. The overall prediction is that the line width will be proportional to the magnetic field and will increase rapidly with temperature. Such behavior was evident when comparing spectra at 100 and 220 MHz.

Ribonuclease Plus Copper. The interaction of Cu^{2+} with RNase was studied at pD 5.4 and 6.0. Small concentrations of Cu^{2+} in the range 10^{-4} to 10^{-8} M strongly broadened the resonances of histidines-105, -12 and -119 but had little effect on the other proton resonances. The effects of increasing concentration of Cu^{2+} on the aromatic and histidine resonances of RNase at pD 6.0 are shown in Figure 5. The histidine resonances increase in width with increasing $[Cu^{2+}]$. At the highest concentration of Cu^{2+} in the figure, the band due to H_{C-4} of His-105 has become obscured in the aromatic region while the three H_{C-2} resonances of His-105, -12, and -119 are substantially broadened. In contrast, relatively little effect on the aromatic protion of the spectrum is seen. A plot of bandwidth increment as a function of $[Cu^{2+}]$ is presented in Figure 7. Higher concentrations of Cu^{2+} were required to affect seriously the aromatic and all remaining resonances of the protein. The reason for this is twofold: the Cu^{2+} is more distant

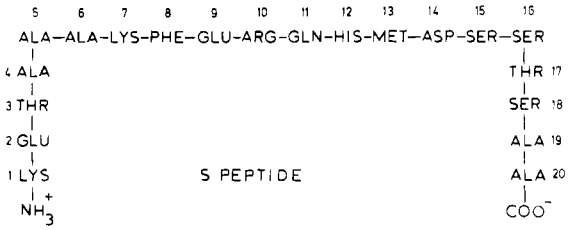

FIGURE 8: RNase S-peptide amino acid sequence.

from most of the remaining residues and the severe overlap of a multitude of resonances in all spectral regions masks any severe broadening of individual resonances which might occur.

Discussion

Site of Binding of Cu^{2+} on S-Peptide. S-Peptide contains many functional groups which are potential ligands for Cu^{2+}. These include the N-terminal α NH_2, the ϵ NH_2's of Lys-1 and -7, COO^- of Glu-2 and -9, and Asp-14, the C-terminal COO^-, imidazole nitrogens of His-12, and the amide nitrogens of the peptide groups. The carboxyls usually function as ligands in acid solution whereas the imidazole and other nitrogen ligands participate in complexation as the hydrogens are liberated in more alkaline solution.

In the present investigation, we make use of the data presented in Table III to infer from the relative distances between Cu^{2+} and peptide protons, the site at which the metal ion is attached to the S-peptide. The protons closest to Cu^{2+} constitute bands M and O, namely, β CH_2 (Phe-8), δ CH_2 (Arg-10), β CH_2(His-12) and ring protons of Phe-8 (refer to Figure 8). The protons most distant from the Cu^{2+} belong to the alanines-4, -5, -6, -19, and -20 (band D), threonines-3 and -17 (bands B and C), and lysines-1 and -7 (band L). At an intermediate distance are the three serines-15, -16, and -18 (band N), Glu-2 and -9, Gln-11 (band I), and Met-13 (band H). It thus appears that the binding site is somewhere in the region of residues 8–12 of which histidine-12 would seem to be most likely ligand. It is not possible to surmise from the nmr data the nature of the remaining ligands completing the square-planar configuration of Cu^{2+}. The most direct evidence for the participation of the histidine residue would come by observing the effect of Cu^{2+} on the imidazole H_{C-2} and H_{C-4} resonances which, unfortunately, are not visible under the conditions of the experiment. The N-terminal α-amino group of Lys-1 does not appear to participate. If this group were involved, the simultaneous proximity of Cu^{2+} to the ϵ CH_2 would be evident from the behavior of band L. In addition, the proximity of Cu^{2+} to the methyls of Ala-4, -5, and -6, and Thr-3 would be expected to cause considerable broadening of bands B, C, and D, which does not occur. Naturally, the minimal broadening of band L also preculudes the involvement in complexation of the ϵ-amino groups of Lys-1 and -7.

This result is at variance with the recent finding (Levitzki and Berger, 1971) that the preferred binding site for Cu^{2+} on S-peptide at pH 8 is the N-terminal α NH_2. Some reports concerning Cu^{2+} binding to RNase A (Girotti and Breslow, 1968, 1970) propose that the N-terminal α NH_2 group of the protein provides a site for the metal, whereas other studies (Breslow and Girotti, 1966; Joyce and Cohn, 1969) do not present clear-cut evidence for the involvement of this group. X-Ray crystallographic data on Cu^{2+}–RNase S (Allewell and Wy-

ckoff, 1971) reveals that the α NH_2 of Lys-1 is a possible intermolecular ligand. All of the RNase studies, however, have also revealed the strong affinity for Cu^{2+} of several of the histidine residues.

Several other studies on the interaction of Cu^{2+} with peptides containing multiple binding sites (Breslow, 1961; Campbell *et al.*, 1963; Shearer *et al.*, 1966; Bradshaw *et al.*, 1968) have shown that the sites of binding include the N-terminal amino groups. In peptides containing histidine residues (Bradshaw *et al.*, 1968; Shearer *et al.*, 1966), these residues are also ligands for either the first or second mole of Cu^{2+} depending on the position of the histidine in the amino acid sequence of the peptide. The present investigation presents evidence that the dominant site for Cu^{2+} on RNase S-peptide under the experimental conditions noted is the histidine residue.

Comparison of Nmr and X-Ray Results. A comparison between nmr-determined Cu^{2+}–proton distances in the S-peptide complex and these computed from crystallographic coordinates of RNase S (Table III) brings out two points. Those bands (M, L, I, F, and H) which are closest to the assumed Cu^{2+} ion site show good agreement between nmr and crystallographic distances. Bands B, C, and H which are farthest away show no agreement at all. An overall conclusion is that the local structures around histidine-12 extending perhaps from amino acid 7–13 are approximately the same in the two environments. What follows are possible reason for the discrepancies at longer distances.

The lack of complete agreement between nmr and X-ray results may be the result of several factors. Firstly, the assumption that all of the Cu^{2+} is localized on the histidine residue may not be accurate. Although the results suggest this to be essentially the case, population, by Cu^{2+}, of other binding sites would give rise to different bandwidth patterns which would modify the patterns arising from the complex of Cu^{2+} with the histidine residue. The nmr results are too crude to permit the fine distinction to be made between the case of complete localization of Cu^{2+} on His-12 and the case where a majority of the bound Cu^{2+} is on the histidine and a small proportion is on a second binding site or on several secondary sites.

Secondly, error incurred in the calculation of both the nmr and X-ray distances may have introduced further discrepancies between the two. In the nmr calculation, the variables with the largest uncertainties are A and $dh/d[Cu^{2+}]$. In the X-ray calculation, the difficulty is not knowing precisely which groups contribute resonances to some of the bands, hence being forced to consider all likely groups in arriving at a mean distance.

Thirdly, as pure S-peptide has not yet been crystallized and subjected to X-ray analysis, the X-ray coordinates of the S-peptide portion of RNase S were used in this study. There may exist a conformational difference between the S-peptide free in solution and in the solid state either as pure S-peptide or as an integral moiety of RNase S. Several studies of the circular dichroism of S-peptide in aqueous solution have been reported. Scatturin *et al.* (1967) and Tamburro *et al.* (1968) concluded that the polypeptide is essentially in a random-coil conformation, and proposed that upon binding to S-protein, the peptide undergoes a random-coil–helix transition. Similarly, Klee (1968) and Brown and Klee (1969) surmized from their CD spectra that although much of the S-peptide is in a random-coil conformation, perhaps 10–20% of the chain is in the form of an α helix most likely present in the first half of the molecule. This amount of helical structure is less than in the S-peptide portion of RNase A (Kartha *et al.*, 1967) or RNase S (Wyckoff *et al.*, 1967a,b). Other evidence exists (Epand and Scheraga, 1968) that the helix contents of free peptides are markedly lower than when incorporated into the parent protein.

Finally, complexation of the peptide with a metal ion may alter the conformation of the peptide (Shearer *et al.*, 1966; Gurd *et al.*, 1967b) so that comparison of its conformation in the complex to that of the metal-free peptide may not be strictly justifiable. Of the four possible reasons discussed to explain the discrepancies between nmr and X-ray distances, it is expected that the second and third are likely to be the ones of paramount importance.

Estimation of Distance between Cu^{2+} and Phe-8 of S-Peptide. Equation 1 may be used to extract a value for the distance between the Cu^{2+} in the Cu^{2+}–S-peptide complex and the phenyl protons of the phenylalanine residue. For this calculation the value of the constant K must be known. As defined by Ihnat and Bersohn (1970), K is given by

$$K = \frac{1}{15} S(S+1)\left(\frac{g^2\beta^2 g_N{}^2\beta_N{}^2}{\hbar^2}\right)\left(7\tau_c + \frac{13\tau_c}{1+\omega_s{}^2\tau_c{}^2}\right) \quad (5)$$

where S is the electron spin of Cu^{2+}, g and β are, respectively, the g value of the electron and Bohr magneton, g_N and β_N are, respectively, the g value of the proton and the nuclear magneton, $\hbar$ is Planck's constant, ω_s is the electron resonance frequency at the field employed for ^1H nuclear magnetic resonance, and τ_c is the correlation time for the dipolar interaction between the Cu^{2+} and ligand protons. τ_c is given by the relation

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_h} + \frac{1}{\tau_s} \quad (6)$$

in which τ_r is the rotational correlation time for Brownian motion for the complex, τ_h is the residence time of the Cu^{2+} in the complex, and τ_s is the electron spin relaxation time. K depends on the nature of the complex, and it is evident that for a given metal ion, K depends only on τ_c. Furthermore, for complexes involving Cu^{2+} we assume that $\tau_h, \tau_s \gg \tau_r$ so that $\tau_c \approx \tau_r$.

For a diffusing sphere of radius a, the rotational correlation time to be used in the magnetic resonance experiment is $\tau_c = 4\pi\eta a^3/3kT$, where η is the viscosity of water at absolute temperature T, and k is Boltzmann's constant. It is of course difficult to know what value of a to assume for the S-peptide or indeed whether to assume that its structure is effectively spherical. We can estimate the volume of the Cu^{2+}–S-peptide complex by taking its partial specific volume to be identical to that of proteins, normally 0.7 ml g^{-1} and with the molecular weight of 2230 we have a molar volume of 1560 cm^3.

The constant $(7/15)S(S+1)g^2\beta^2 g_N{}^2\beta_N{}^2/\hbar^2$ is 8.624×10^{-32} cm^6 sec^{-2}. Using the above formula for τ_c we find $\tau_c = 4.84 \times 10^{-10}$ sec at 31° and hence $K = 4.17 \times 10^{-41}$ cm^6 sec^{-1}. Using this value for K in eq 1 together with $\Delta\nu_{1/2} = 125$ Hz, $[Cu^{2+}] = 4.0 \times 10^{-3}$ M, and [S-peptide] $= 3.4 \times 10^{-2}$ M, we calculate r_{Phe} (the average distance between the Cu^{2+} ion and the five phenylalanine ring protons) to be 4.82 Å. We assume that, as in the Cu^{2+}–carnosine complex (Ihnat and Bersohn, 1970), Cu^{2+} binds to N-3 of His-12. According to the X-ray structure of ribonuclease S the N-3 atom can be at either position XE1 or XE2 of the imidazole ring. Suppose we place the Cu^{2+} in the plane of the

imidazole ring and 2.01 Å from the atom at XE1. The distance between the Cu^{2+} ion and the center of the side-chain phenyl ring is 7.96 Å. If we place the Cu^{2+} near atom XE2, we obtain 5.41 Å. There is still some disparity between this value and the experimental 4.82 Å.

Let us enumerate some possible explanations. One is that the contribution of the contact interaction to the broadening should not have been neglected. We do not favor this explanation because of the good to excellent agreement of the relative distances (calculated on the assumption that the broadening is proportional to $(1/r)^6$) in five separate bands. A second explanation is that the structure is not the same in solution as in the crystalline state. Again we point to the good agreement suggesting that the structure involving amino acids 7–13 is very similar in the two media. A third possible explanation is that the assumption of spherical structure of the S-peptide in solution implied in eq 1 is false. Woessner (1962) has investigated the effect of diffusion on dipolar broadening between two spins when they are attached to an ellipsoid. There is an enhanced rate of broadening as compared to a sphere of equal volume but the enhancement depends strongly on the axial ratio the ellipsoid (of revolution) and on the orientation of the line joining the two dipoles relative to the ellipsoidal axis. Now the actual broadening of the phenylalanine aromatic protons is $(5.41/4.82)^6 = 2.00$ times as much as predicted by eq 1. If we assume a rigid rod model for the S-peptide with a ratio of long axis to short axis of $\sim$5 and assume the line joining the dipoles is parallel to the long axis we get a factor of 3–4. Hence it appears that the discrepancy between the nmr and X-ray distances can be explained by deviation of the S-peptide conformation from spherical symmetry.

Magnetic resonance results for small molecules of mol wt $\leq$100 have shown that τ_c calculated from the diffusion model is too long by an order of magnitude. On the other hand, for a protein of mol wt 18,000 the diffusion model has been shown to work perfectly. It could be that the S-peptide which has a molecular weight of $\sim$2200 is an intermediate case. From the practical point of view it appears difficult to obtain absolute distances through the use of nuclear magnetic resonance measurements alone.

Binding Sites for Cu^{2+} on Ribonuclease A. Figure 5 indicates that among the RNase A resonances most sensitive to Cu^{2+} are those belonging to the histidine residues. The histidine bands are already quite broad at the concentration of Cu^{2+} required to affect most other protein resonances, the implication being that the histidine residues are either involved in complexation with Cu^{2+} or are in proximity to the binding sites. From the plots of Figure 7 we can calculate the rates of broadening with $[Cu^{2+}]$, $d\Delta\nu_{1/2}/d[Cu^{2+}]$, to be 22×10^3, 21×10^3, and 11×10^3 Hz M^{-1} for the $H_{C\text{-}2}$ protons of histidines-105, -12, and -119, respectively at pD 6.0, and 2.8×10^3 Hz M^{-1} for $H_{C\text{-}2}$ of histidine-48 at pD 5.4. Assuming that in all instances the mode of interaction of the Cu^{2+} with histidine ligands is identical, that is, the Cu^{2+}–$H_{C\text{-}2}$ distances are identical, we can say that residues 105 and 12 provide somewhat stronger ligands than His-119. The binding constant of the His-48 site appears to be substantially smaller than those of the other sites. As the pD, however, for observing the interaction of Cu^{2+} with His-48 was different from the pD for the other histidines, and as complexation is a function of pD, no quantitative comparison of association affinities can be made.

Our results are in good agreement with those of Roberts and Jardetzky (1970) and Joyce and Cohn (1969) derived from magnetic resonance studies, and the X-ray results for Cu^{2+} and RNase S of Allewell (1969) and Allewell and Wyckoff (1971). Roberts and Jardetzky also observed bands of histidines-105 and -12 to be broadened in preference to His-119, and concluded that Cu^{2+} binds strongly to or near His-105 and -12, and weakly to His-119. The water proton relaxation enhancement studies of Joyce and Cohn have led them to assign the strong binding site to His-12 and two weaker sites to His-105 and -119. Spectrophotometric and titration studies of Cu^{2+}–RNase A (Breslow and Girotti, 1966; Girotti and Breslow, 1968, 1970) have shown that the histidines and their adjacent peptide bond nitrogens are important binding sites. The X-ray diffraction studies of Cu^{2+}–RNase S have confirmed the solution results in finding that histidines-12, -105, and -119 are situated close to the Cu^{2+} ion. Histidine-48, however, was not observed to be involved. The involvement of the N-terminal α NH_2 in Cu^{2+} binding has also been documented (Girotti and Breslow, 1968, 1970; Allewell and Wyckoff, 1971). The present nmr study does not provide any information on the participation of the α NH_2.

Concluding Remarks

The main aim of this work has been to ascertain the position of the binding sites for Cu^{2+} on RNase A and S-peptide. Secondly, we have looked into the possibility of using the nmr technique to elucidate the secondary and tertiary structure of a moderately large polypeptide in solution. If the site (or sites and corresponding occupancies) of binding of the ion on the polypeptide is known, one can calculate from nmr relaxation, relative distances between the metal and the various amino acid residues. Making an estimate of τ_c for the polypeptide complex permits the computation of absolute distances of separation. One thus should be able to get an idea of the conformation of the polypeptide chain in the complex, and in principle can calculate the dihedral angles to define the conformation rigorously. The advantage of employing Cu^{2+} as the probe lies in the fact that Cu^{2+} predominantly broadens resonance bands rather than causing contact shifts. The broadening results from dipolar interaction which depends solely on r and not on the number and nature of intervening bonds. Although the results suggest that the binding site for Cu^{2+} on S-peptide is in the region of the histidine residue, any ideas about the conformation of the complex remain inconclusive. Increasing the external magnetic field to simplify and separate the resonances and extensive resolution of bands either by computer curve fitting or by use of a curve resolver should permit more accurate computation of metal–proton distances and a more quantitative assessment of conformation and binding sites.

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