Magnetic Resonance Studies of the Interaction of Cupric Ion with Native and Modified Forms of Ribonuclease*

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SUMMARY

The binding of copper to bovine pancreatic ribonuclease A, its carboxymethylated histidine-119 and histidine-12 derivatives, ribonuclease S and S-protein, has been investigated at pH 5 and 6 by measuring the enhancement of the magnetic proton relaxation rate (PRR) of water due to bound copper. The magnitude of the PRR enhancement factor is characteristic for the binding site and reflects its protein environment. Titration of copper and protein yield values of the binding constants and the number of sites and reveal interaction between binding sites. At pH 5, RNase A and RNase S exhibit three independent binding sites, one strong binding site with a dissociation constant, $K_D$, of 6 to 7 x $10^{-4}$ M and two weaker ones with $K_D$ of approximately 8 x $10^{-3}$ M. The 1-carboxymethylhistidine-119-RNase and S-protein have only two binding sites, one strong and one weak, and 3-carboxymethyl histidine-12-RNase has one strong site. The enhancement of the strong site, $e_b$, is 6.4 for RNase A, almost the same for the 1-carboxymethylhistidine-119-RNase, 5.9, but significantly lower for RNase S, 5.1, suggesting a more flexible structure for RNase S at the first Cu(II)-binding site. Modification of histidine-12 by carboxymethylation results in a drastic change in the environment of this Cu(II)-binding site, $e_b$ equals 30. To a lesser extent, removal of S-peptide also results in an increased value of $e_b$ at the tight binding site, $e_b = 9.0$ in S-protein. At pH 6.0, the binding patterns become much more complex; all species except the histidine-12 derivative exhibit cooperativity in Cu(II) binding and some association, rendering it impossible to extract the number of sites or values of $K_D$ and $e_b$ for individual sites. 3-Carboxymethylhistidine-12-RNase is exceptional in that it reveals two equivalent noninteracting binding sites at pH 6, $K_D$ equals 4 x $10^{-4}$ M, and $e_b$ equals 24.3. Measurements of the enhancement as a function of temperature indicated that the chemical exchange of water ligands in the Cu(II) ribonuclease complexes is so fast that the observed relaxation rates of all of the complexes are determined by the dipolar correlation time in the first hydration sphere of Cu(II). The magnitude of the energies of activation of 3 to 5 kcal per mole indicates that rotational motion determines the relaxation rate.

The inhibitor constants for Cu(II) were determined at pH 5 for RNase A and RNase S, respectively, with cytidine-2',3'-cyclic phosphate as substrate and agreed within a factor of 2 or less with the $K_D$ of the tight binding site determined from PRR enhancement data. In electron paramagnetic resonance spectra at pH 5 of solutions of Cu(II) and RNase A, two peaks were observed corresponding to free copper and bound copper. Titration of RNase A with Cu(II) by electron paramagnetic resonance yielded the same values for free Cu(II) as those calculated from the PRR enhancement data, indicating that all of the Cu(II)-binding sites were enhanced and that the PRR data accounted for the total Cu(II) bound.

On the basis of the enhancement data and other known properties of RNase A, the strong Cu(II)-binding site is tentatively assigned to histidine-12 and the two weaker sites to histidine-105 and histidine-119.

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The inhibition of bovine pancreatic ribonuclease by cupric ions is well established (1-3) and has been ascribed to the binding of copper to the essential histidine residues, -12 and -119. The original suggestion by Crestfield, Stein, and Moore (4) that a single Cu(II) chelates between these two histidines was based on their observation of an inhibition of the rate of the reaction of histidine-12 and -119 with iodoacetic acid at pH 5.5. Subsequently, the binding of cupric ions to RNase A has been investigated by spectrophotometric and potentiometric methods (5), by equilibrium dialysis and enzyme kinetics (3, 6), and by gel filtration (7). Although the likelihood of a Cu(II) chelate between histidine-12 and -119 has been ruled out, the results from
different laboratories are not in agreement on the number of binding sites or on the binding constants.

Since the cupric ion is paramagnetic, it seemed possible to study the proton relaxation rate of solvent water in solutions of the bound form and to determine the binding constants and number of binding sites as had been done previously for the binding of manganese ions to proteins (8, 9). The enhancement of the PRR is a function of the environment of the cupric ion; consequently, another parameter in addition to the binding constant would be available to characterize the site. In order to assess the involvement of the essential histidine residues -12 and -119 in the binding, the investigations of Cu(II) binding to RNase A were extended to CMHis-12-RNase, CMHis-119-RNase, RNase S, and S-protein. The PRR enhancement also indicates differences in conformation at the Cu(II)-binding sites among the various modifications of RNase A, thus giving some insight on structural changes induced by chemical modification of the protein.

**MATERIALS AND METHODS**

Cu(II) solutions were prepared from CuCl₂ of Johnson, Matthey, and Company, Ltd., London. The CuCl₂ was standardized by a titration of the CuCl₂ in a solution of 0.5 M NH₄Cl at pH 7.9 with a solution of disodium ethylenedinitrilotetraacetate. Murexide was the indicator used and cupric oxide of Jarrell-Ash Company was the primary standard. Cytidine-2',3'-cyclic monophosphoric acid was obtained from Sigma. All other materials were reagent grade.

**Preparation of Enzymes**

Ribonuclease (type II-A) was obtained from Sigma, in a few experiments RNase from Worthington was used. Ribonuclease A free of phosphate and other anions was prepared from the commercial crystalline protein by chromatography on the Amberlite resin IRC-50 (XE-64) as described by Hirs, Moore, and Stein (10) and Richards and Vithayathil (11). Ribonuclease S and S-protein were prepared by the method of Richards and Vithayathil (11) with subtilisin (Nagarse Company, Tokyo). 3-CMHis-12-RNase and 1-CMHis-119-RNase were prepared according to the method of Crestfield, Stein, and Moore as modified by Yang and Hummel (13). All enzyme preparations were passed through mixed bed columns of the Amberlite resins 1K-120 (hydrogen cycle) and 1R-400 (hydroxide cycle), 20 to 50 mesh. When it was necessary to use a pump for chromatography, a Holter micro bilateral roller pump (model RD 145) was used.

The protein concentrations were determined spectrophotometrically. The molar extinction coefficient of RNase A is 9.8 × 10⁶ at 278 mA at pH 0.5 (14), and this value was assumed for CMHis-119-RNase and CMHis-12-RNase and RNase S (12, 15). A solution of S-protein (1 mg per ml) has an extinction value of 0.781 at 280 mA (15).

**Enzyme Assay**

Ribonuclease activity was assayed by the spectrophotometric assay of Crook, Mathis, and Rabin (16) at 286 mA in a Cary model 15 recording spectrophotometer with cytidine-2',3'-cyclic phosphate as substrate. In the kinetic investigation of copper inhibition, the same procedure was used except for the omission of buffer; NaOH or HCl was added for adjustment to the proper pH. To determine initial velocities in the inhibition studies the extent of the reaction did not exceed 1.5% of the total since 3'-CMP is a potent product inhibitor at pH 5 and pH 6 (17).

**Magnetic Resonance Measurements**

The proton relaxation rate of water was measured by the pulsed nuclear magnetic resonance method of Carr and Purcell (18) at 24.3 Mc per sec at 22°C unless indicated otherwise. Enhancement (e*) has been defined as the ratio of the PRR due to a paramagnetic metal ion in the presence and absence of a complexing agent and is calculated from Equation 1 (19)

\[
\frac{1/T_{1p}}{1/T_{1}} = \left( \frac{1/T_{1p}^*}{1/T_{1p}^*} \right) - \left( \frac{1/T_{1p}^*}{1/T_{1p}^*} \right)
\]

1/T₁p is the paramagnetic contribution to the relaxation rate which equals the difference between 1/T₁, the observed relaxation rate in the presence of copper and 1/T₁, the observed relaxation rate in the absence of copper. The terms with asterisks refer to the same parameters in the presence of a complexing agent, which in this study is RNase A or one of its derivatives. The enhancement of free Cu(II) equals 1 by definition.

Two types of titrations of copper and protein were performed with enhancement as the measured parameter. In the type I titration, the concentration of Cu(II) was held constant and the protein concentration was varied; the titration was carried out by titrating 100 μl of a Cu(II) solution with small aliquots of a solution of the same Cu(II) concentration which also contained protein, usually 10 times the Cu(II) concentration. In the type II titration, protein concentration was held constant and the concentration of Cu(II) was varied. In these titrations 100 μl of protein solution were titrated with small aliquots of a solution of the same protein concentration containing Cu(II) at 4 or 6 times the protein concentration.

**Control of pH**

The pH of each solution was previously adjusted to the desired pH with HCl or freshly prepared NaOH prior to the titration, at the end of the titration the pH had changed no more than 0.1 pH unit. All titrations were performed in 160 mM NaCl, so that ionic strength effects would be negligible. Measurements of pH were made on a Radiometer pH meter, type PHM 26, with a Metrohm combination electrode EA 125 U.

**EPR Spectra**

The spectra of Cu(II) and of Cu-RNase A complexes were recorded with a Varian E-3 spectrometer at a frequency of 9.2 GHz with the aid of a Varian C-1024 computer of average transients. The latter made it possible to subtract the background spectrum.

**Analysis of Data**—From type I titrations, one obtains a value of eₐ₀, enhancement of the binary copper complex at the site or sites of the strongest binding, by extrapolation of e*, the observed enhancement to infinite protein concentration as illustrated in Fig. 1. The observed value of enhancement, e*, at any particular concentration of the components of the system, is a linear
combination of the contributions of each species in the system. As discussed in detail previously (8), the concentrations of free and bound Cu(II) may be calculated for any value of $e^*$ if the enhancement of the bound form, $e_b$, is known. The binding constant may then be determined analytically or graphically if $n$, the number of binding sites, is known from a type II titration as described in detail previously (8). From a type II titration, $1/e^*$ may be plotted as a function of $Cu(II)$, as illustrated in Fig. A4. The extrapolated value of $e^*$ at zero $Cu(II)$ concentration is a lower limit of $e_b$ since not all of the $Cu(II)$ is bound at the protein concentrations used (8). To determine the number of binding sites of each class and $K_a$, the binding constant of the weaker sites, secondary plots of the type II titration data were made in the form of either a Scatchard (20) or a Hughes-Klotz (21) plot. The reported values are based on the assumption that $e_b$ is constant.

If there is more than one class of sites, the value of $K_a$, the average value of the association constants for the class of weaker sites, was determined from the Scatchard plot. $K_a$ may be evaluated from the intercept on the ordinate (20); when $P = 0$, then:

$$e_b/(Cu) = n_1K_{A1} + n_2K_{A2}$$

where $n_1$ and $n_2$ are obtained from the intercepts on the abscissa and the value of $K_{A1}$ is known from a type I titration.

RESULTS

Effect of pH on PRR Enhancement in Binary Complexes of Cu(II)

The effect of acetate buffer on the enhancement parameter was investigated in type 1 titrations. As shown in Fig. 1, in a double reciprocal plot of $e^*$ against RNase A concentration, at 0.05 M acetate ion, there is no significant effect of acetate on the titration curve (Curve 1, no acetate; Curve 2, 0.05 M acetate) at 0.2 mM CuCl$_2$ and pH 6. When the acetate ion concentration is increased to 0.2 M (Curve 5), both $e_b$ and the slope of the titration curve are affected. The decrease in the apparent $e_b$ is due to ternary complex formation (7) and the shift in the titration curve may be ascribed to both binary and ternary complex formation of Cu(II) with acetate. To avoid the complication of Cu(II) binding to other reagents and particularly the possibility of ternary complex formation, no buffer was used in subsequent experiments. The pH was adjusted either with HCl or freshly prepared NaOH to the desired pH in solutions containing 0.16 M NaCl.

As shown in Fig. 2, over a pH range of 3 to 8.5, the observed enhancements of solutions containing Cu(II):RNase A ratios of 1, 2, 3 and 4, respectively, with an RNase A concentration of 1 mM exhibit a maximum in the range of pH between 5.5 and 6.5. At RNase A:Cu(II) ratio of 1, the optimal pH, was approximately 5.8 to 6.5. As the Cu(II):protein ratios increased, the maximum enhancement shifted to a more acid pH, so that when the ratio is 4, the pH optimum is lowest.

A similar pattern, with a maximum in $e^*$ in the range of pH 5 to 6, was observed with various modified forms of RNase A at a Cu(II):protein ratio of 0.1. For CMHis-119-RNase and CMHis-12-RNase, the pH for maximum observed enhancement is approximately pH 6, as found for RNase A. For the Cu(II)-binary complexes of RNase S and S-protein, the pH optimum was found to be somewhat lower, approximately 5.

Fig. 1. Type I titrations of RNase A in the presence and absence of acetate buffer, pH 6.0. 1/$e^*$ is plotted against 1/(M[RNase]). The concentration of CuCl$_2$ was 0.21 mM and the other components were: Curve 1, 250 mM NaCl; Curve 2, 50 mM sodium acetate and 200 mM NaCl; Curve 3, 200 mM sodium acetate and 50 mM NaCl.

Fig. 2. Variation of $e^*$ with pH at 1 mM RNase A and different Cu(II):RNase A ratios, CuCl$_2$ concentrations were: ■ — ■, 1 mM; ⌉ — ⌉, 2 mM; ● — ●, 5 mM; and ▲ — ▲, 4 mM.
Habitually large for calculations of binding constants and number of sites. The PRR measurements were limited to few moles of protein. Curve 1 (O—O), CMHis-119-RNase; Curve 2 (Δ—Δ), RNase A, 0.105 mM CuCl₂ (●—●), CMHis-12-RNase, 0.094 mM CuCl₂.

Since it has been shown that the binding constants increase as the pH increases (3, 6, 7) and therefore the fraction of copper bound is increasing beyond the observed pH maximum for e*, it must be concluded that the average enhancement factor of the very low observed enhancement at pH 6.

For RNase S, the measurements were done only at pH 5 because of binding sites with the low values of e* at the higher pH values.

The variation of 1/e* as a function of total copper concentration is shown in Fig. 4.1 in a type II titration. The variation of 1/e* at the pH range above pH 6. The PRR enhancement observed for the Cu(II) complex of RNase A with Copper at ~pH 5—The variation of 1/e* as a function of total copper concentration is shown in Fig. 4.1 in a type II titration in which the RNase A concentration was maintained constant at 2.05 mM and the CuCl₂ was varied from 0.10 to 12.5 mM at pH 4.8. The biphasic linear curve revealed the existence of at least two classes of binding sites. An extrapolation to zero Cu(II) from the low range of Cu(II) concentration yields a lower limit of e₀ of 5.2 for the strong binding sites. The secondary Scatchard plot of the type II titration (Fig. 4B) indicates one strong binding site and two weaker sites. With the value of K₀ of 7.3 × 10⁻⁴ M obtained for the tight binding site in a type I titration on the assumption that n equals 1, an average K₀ for the two weaker sites is calculated from the intercept on the ordinate axis to be 8 × 10⁻¹ M. The two binding constants differ by approximately one order of magnitude. The values of all of the parameters are summarized in Table I.

**Derivatives of RNase A**—For CMHis-119-RNase, type I titrations were carried out at 0.1 and 0.2 mM Cu(II) concentrations, respectively, and the same e₀ value of 5.0 was obtained at both Cu(II) concentrations, a value very close to the native protein, e₀ = 6.1 (see Table I); the curves were similar to Fig. 3. A biphasic curve was obtained in a type II titration (Fig. 4A). As shown in Table I, CMHis-119-RNase exhibited a similar pattern of Cu(II) binding as the nonalkylated protein with the notable exception that only one, rather than two, weak binding site was found. The Scatchard plots in Fig. 4B show that both proteins have one tight binding site but the total number of binding sites changes from three in RNase A to two in CMHis-119-RNase. The dissociation constants for the tight binding site were calculated from a type I titration and for the weak binding site from a type II titration as for the native protein and the values are listed in Table I.

The PRR enhancement observed for the Cu(II) complex of RNase A and CMHis-12-RNase as shown in a type I titration in Fig. 3 differed radically from those of RNase A and CMHis-119-RNase, since the e₀ value rose to 30. Although the type II titration (Fig. 5A) was monophasic, the secondary Scatchard plot in Fig. 5B with n₁ about 0.8 revealed a break in the curve at approximately 0.5 mole of Cu(II) bound per mole of protein. A possible explanation of this phenomenon, that a dimer was formed with a Cu(II) bridge between 2 protein molecules, was ruled out by determination of the sedimentation velocity by ultracentrifugation which yielded a value consistent with the monomer and no evidence for dimer formation. The apparent biphasic nature of the Scatchard plot is unexplained and the assignment of only one Cu(II) binding site in this modified form of RNase A must be made with some reservation.

**RNase S and S-Protein**—As summarized in Table I, the value of e₀, for RNase S, 5.1, was somewhat lower than for RNase A, 6.1. The binding constants and number of binding sites were the same for RNase A and RNase S within experimental error (cf. Table I). In the S-protein, which lacks histidine-12, the number of weak binding sites decreased from two to one as shown in the Scatchard plots in Fig. 6 derived from data of type II titrations. The enhancement value, e₀, of 9.0 for the first Cu(II) binding site was higher for S-protein (see Table I) than for RNase S.
TABLE I
Enhancement values, dissociation constants, and number of binding sites for Cu(II) complexes of RNase A, CMHis-119- and CMHis-12-RNase, RNase S, and S-protein at pH 5

The solutions contained 0.16 M NaCl and the pH was adjusted to the value indicated with HCl or NaOH. The temperature was 22°. The parameters were calculated as described in the text; the values of n have been rounded off to the nearest integer.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>pH</th>
<th>Titration</th>
<th>Protein concentration</th>
<th>Cu(II) concentration</th>
<th>( \varepsilon_1 )</th>
<th>( K_D )</th>
<th>( n_1 )</th>
<th>( K_{D2} )</th>
<th>( n_2 )</th>
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<td>0.39-2.05</td>
<td>0.105</td>
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<td>7.3</td>
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<td>8</td>
<td>2</td>
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<td></td>
<td>4.8</td>
<td>II</td>
<td>0.05</td>
<td>0.10-15.2</td>
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<td>I</td>
<td>0.23-2.0</td>
<td>0.105</td>
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<td>8.6</td>
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<tr>
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<td>5.0</td>
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<td>II</td>
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<td>5.0</td>
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* The values of \( \varepsilon_1 \) for RNase S and S-protein varied more from preparation to preparation than for other proteins, but \( \varepsilon_1 \) was always significantly higher for S-protein than for RNase S.

Effect of Cu(II) on Activity of RNase A and RNase S—An investigation of the inhibition of the enzymic activity of RNase A and of RNase S by Cu(II) was made under conditions similar to those used in the studies of the enhancement of the PRR. Analysis of the kinetic data showed that Cu(II) is a noncompetitive inhibitor with respect to cytidine-2',3'-cyclic phosphate for both RNase A and RNase S. The average \( K_i \) values, 3.6 X 10^-4 M and 6.0 X 10^-4 M, respectively, were calculated from the kinetic data with the relationship (22)

\[
K_i = \frac{I}{V/V_p - 1}
\]

where \( I \) is the concentration of inhibitor and \( V \) and \( V_p \) are the maximum velocities in the absence and presence of inhibitor, respectively. As shown in Table II, the value of \( K_i \) for Cu(II) determined kinetically at pH 5.0 for RNase S is in good agreement with the value found for \( K_D \) of the tight binding site by PRR measurements determined under the same conditions; the two constants agree within a factor of 2 for RNase A. For comparison, the values obtained by other investigators under conditions most similar to those of the present investigation are also listed.

Cu(II) Complexes at pH 6

The behavior of the Cu(II) binding to RNase A, CMHis-119-RNase, and S-protein was very complex at pH 6; the binding...
TABLE II

Comparison of Cu(II) inhibition constants and dissociation constants at pH 5

The values of \( K_i \) were obtained with 2',3'-cyclic-CMP as substrate at 22° in solutions containing 0.16 M NaCl; \( K_D \) values are for the strong binding site listed in Table I.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>( K_i ) ( \times 10^4 ) M</th>
<th>( K_D ) ( \times 10^4 ) M</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase S</td>
<td>6.0</td>
<td>6.0</td>
<td>2</td>
</tr>
<tr>
<td>RNase A</td>
<td>3.6</td>
<td>7.3</td>
<td>3</td>
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<tr>
<td></td>
<td>6.7b</td>
<td>0.8b</td>
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<tr>
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<td></td>
<td>6.7c</td>
<td>7</td>
</tr>
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</table>

a Acetate buffer, 0.01 M, and cytidine 3'-phosphate benzyl ester as substrate; ionic strength, 0.1; 37°.
b Acetate buffer and &-alanine; ionic strength, 0.1; equilibrium dialysis; pH 5.2; 25°.
c Acetate buffer, 0.05 M; Sephadex gel filtration; pH 5.5; ionic strength, 0.16; 25°.

Fig. 7. Type I titrations of RNase A, S-protein, and CMHis-12-RNase at pH 6. In addition to 160 mM NaCl, the concentration of the components and the pH were: Curve 1, 0.2 mM CuCl2, RNase A, pH 5.9; Curve 2, 0.21 mM CuCl2, S-protein, pH 5.8; Curve 3, 0.11 mM CuCl2, CMHis-12-RNase, pH 5.9.

Fig. 8. Hughes-Klotz plot of type II titration of RNase A, S-protein, and CMHis-12-RNase at pH 5.9. The solution contained 0.17 mM CMHis-12-RNase and 160 mM NaCl.

Fig. 9. Type II titration of RNase A, S-protein, and CMHis-119-RNase at pH 6. \( 1/\varepsilon^2 \) is plotted as a function of \( Cu \). In addition to 100 mM NaCl, the concentration of the components and the pH were: Curve 1, 2 mM RNase A, 0.07 to 6 mM CuCl2, pH 5.8; Curve 2, 2 mM S-protein, 0.09 to 8.4 mM CuCl2, pH 6.1; Curve 3, 2 mM CMHis-119-RNase, 0.09 to 12.6 mM CuCl2, pH 5.9.
5.9. A secondary Hughes-Klotz plot of this type II titration in Fig. 8 yields two equivalent binding sites with $K_D$ equal to $3.6 \times 10^{-4}$ M. An analytic determination of $K_D$ and $n$ with simultaneous equations (Equation 6 of Reference 8) for pairs of experimentally observed $e^*$ values between Cu(II) concentration of 0.06 mM and 0.4 mM gave average values of $n$ equal to 1.7 $\pm$ 0.5 and $K_D$ equal to 2.9 $\pm$ $1 \times 10^{-4}$ M. The corrected value of $e_b$ of 25.3 is calculated (8) from the lower limit value in a type II titration of 12.3 and is in excellent agreement with the value of 24.3 obtained in a type I titration. The pattern of binding is consistent with two noninteracting binding sites with the same value of $e_b$ and $K_D$.

In Fig. 9, titration curves of $1/e^*$ with respect to Cu(II) concentration are presented for RNase A (2 mM, pH 5.8), CMHis-119-RNase A (2 mM, pH 5.85), and S-protein (2 mM, pH 6.15). The values of $1/e^*$, instead of increasing monotonically with [Cu(II)], as observed at pH 5 (cf. Fig. 4A), exhibit three regions: $1/e^*$ initially decreases, then reaches a plateau, followed by an increase. This complex multiple site binding pattern could not be fitted to an assumption of $e_bZ$ greater than $e_bI$; the first two classes of sites could not be separated. Thus in the initial portion of the binding curve, a progressively greater fraction of copper appears to be bound as the copper concentration increases, contrary to the situation encountered with noninteracting sites where the fraction bound always decreases with increasing metal concentration. It must be concluded that there are interacting sites such that either the binding or the enhancement or both exhibit cooperative interaction. That a curve of this general shape may be expected for multiple interacting sites has been shown mathematically (23) but it is not possible to extract any exact values of the various enhancement and binding constant parameters. Some qualitative conclusions may be drawn, however. The maximum value of $e^*$ observed in Fig. 9 is about the same for CMHis-119-RNase and RNase A (Curves 1 and 3), but is significantly lower for S-protein; at pH 5, $e_b$ is higher for S-protein. As shown in Fig. 10 for RNase A, if copper titrations are done at increasing protein concentrations, the maximum value of $e^*$ in the plateau region, which represents a species with copper bound at a minimum of two sites, appears to be approaching a limiting value of approximately 5.4. Similarly for CMHis-119-RNase, the value of $e^*$ for the complex with at least two Cu(II) sites occupied, reflected by a similar plateau region, is approximately 6.0.

**EPR Spectra of RNase A**—It seemed possible that the PRR enhancement data at pH 6 might be amenable to analysis if an independent method were used to determine free and bound copper. Furthermore, an independent determination of free and bound copper would indicate whether some bound copper was undetected by the PRR method; i.e., the binding could be of such a nature that the enhancement was very close to 1 and such bound copper would be indistinguishable from free copper. In previous studies of manganese binding to proteins (8, 9), free manganese had been determined from EPR spectra at room
bound Cu(I) as illustrated in Fig. 11, the free Cu(I) in the spectrum of free Cu(I) remains undistorted in the presence of parison of Curves A and B that only the ascending limb of the used for quantitative determinations. It is obvious from a com-

The free Cu(I) concentration was determined by EPR in a titration of 2 mM RNase A with CuCl₂ varying from 0.2 mM to 21 mM. The peak to peak distance in the EPR spectrum is not a linear function of concentration and therefore standards very close to the peak height of the free Cu(I) found in the solutions of RNase A:Cu(II) were always used for quantitative determinations. It is obvious from a comparison of Curves A and B that only the ascending limb of the spectrum of free Cu(I) remains undistorted in the presence of the complex; therefore, the concentration of free Cu(I) was measured by the height of the peak from the base line. From Curve B with 2 mM RNase A, the free Cu(II) was calculated to be 0.71 mM of the total 1.68 mM. The concentrations of free Cu(II) in the titration calculated in this way from the EPR spectra agreed well with those calculated from measurements of the PRR enhancements of the same samples. It may therefore be concluded that all of the bound copper is accounted for in PRR measurements of the complexes at pH 5.

At pH 6, from the EPR spectra in the region of low Cu(II) concentration (CuCl₂, 0.4 mM; RNase A, 1 mM) shown in Curve C, the free Cu(II) was less than 2%. Thus in the region of the tight binding sites, the binding constant is too high to be determined by EPR. When the CuCl₂ concentration was increased to 3 mM (Curve D), only 20% was free, indicating that at least the first 2 moles of Cu(II) are very tightly bound. The observed PRR enhancement for this sample was 2.5, so that the average enhancement for all of the bound Cu(II) is 2.9. This value is obviously lower than the εₚ value of 5.4 found when 1 or 2 moles of Cu(II) are bound per molecule of RNase A. Thus, at pH 6 as well as at pH 5, the more weakly bound Cu(II) also has a lower PRR enhancement.

The spectra of the complexes at pH 5 and 6 were very similar. Furthermore, the EPR spectra yielded no information which could be used to elucidate the different complexes at pH 6 since the spectra of the complexes at a Cu(II)-RNase A ratio of 0.4 (Curve C) and of 3.0 (Curve D) were identical. EPR spectra of frozen aqueous solutions might lead to differentiation of the various complexes.

Dependence of Enhancement on Temperature—in order to interpret the observed proton relaxation rates of water and their enhancement factors in terms of the relaxation rate in the coordination sphere of the bound Cu(II) and consequently as a function of the rotational motion at the site of binding, it is necessary to establish that the rate of chemical exchange of the water ligands between the coordination sphere and the solvent is not the rate-limiting process in the observed rate. The observed paramagnetic contribution to the relaxation rate when exchange is occurring is (24)

\[ \frac{1}{T_{1p}} = \frac{p}{T_{1M} + \tau_M} \] (4)

where \( p \) is the ratio of the number of protons in the coordination sphere exchangeable with water to the total number of water protons in the protein; \( T_{1M} \) is the relaxation time of the water protons in the coordination sphere and is inversely proportional to \( \tau_M \), the dipolar correlation time; \( \tau_M \) is the residence time of a water ligand in the coordination sphere. If the rate of chemical exchange is rapid, i.e. \( \tau_M \) is small compared to \( T_{1M} \), then the observed relaxation rate is a function of \( T_{1M} \) (24). As pointed out previously (24), \( T_{1M} \) increases with increasing temperature and \( \tau_M \) decreases with increasing temperature; consequently, a determination of \( 1/T_{1p} \) as a function of temperature can distinguish whether \( \tau_M \) or \( T_{1M} \) predominates the observed relaxation rate in metal-protein complexes (25).

In Fig. 12 the results of such an experiment plotted as log 1/\( T_{1p} \) against the reciprocal of absolute temperature are presented for the Cu(II) complexes of RNase A, CMHis-119-RNase, and for the Cu(II) aquocation. Each point on the curve was obtained from the extrapolated value of 1/\( T_{1p} \) at infinite protein concentration in a type I titration at the indicated temperature by normalization to a Cu(II)
The magnitude of the energies of activation is in the range of 3 to 5 kcal per mole; these values are consistent with the dissociation constant. The apparent break in the curve between 33° and 39° for RNase S, if not due to experimental error, indicates that the rate of chemical exchange is rapid relative to the relaxation rate in the temperature range investigated, thus establishing that the rate of chemical exchange is rapid relative to the relaxation rate in the temperature range investigated.

**Discussion**

The complexity of Cu(II) binding to RNase with its multiple binding sites was indicated in previous investigations, particularly those of Breslow and Girotti with spectrophotometric and spectrofluorometric procedures (5) as well as gel filtration (7). The present investigation shows that considerable simplification may be achieved by studying the binding at pH 5 in the absence of buffers. For both RNase A and RNase S at pH 5, three non-interacting binding sites were found by the PRR method, one strong site with a dissociation constant of $7 \times 10^{-4}$ M and two weaker sites with dissociation constants of about $8 \times 10^{-4}$ M. The strong binding site thus determined would appear to be the site of inhibition of the enzyme by Cu(II) since the value of the inhibitory constant for Cu(II) determined kinetically with 2',3'-cyclic-CMP as substrate under the same conditions as the equilibrium experiments was found to be $3.0 \times 10^{-4}$ M and $6.0 \times 10^{-4}$ M for RNase A and S, respectively, in good agreement with the $K_d$ values for the strong binding site in the two proteins (cf. Table II). It is difficult to make direct comparisons with values determined by other investigators because the conditions such as ionic strength, temperature, and buffer were not identical. However, at somewhat comparable conditions, i.e. pH 5, acetate buffer, 37°, Takahashi (3) observed a $K_d$ of $6.7 \times 10^{-4}$ M with cytidine 3'-phosphate benzyl ester as substrate; at pH 5.5, 25°, in acetate buffer Girotti and Breslow (7) found that $K_d$ equals $6.7 \times 10^{-4}$ M. Both of these values are in the same range as those found in the present investigation, but the value of $K_d$ found by Saundry and Stein (6), $0.8 \times 10^{-4}$ M in the presence of β-alanine and acetate buffer at pH 5, is about one order of magnitude smaller. The effect of acetate interpreted by Girotti and Breslow (7) to be due to the formation of a ternary Cu(II)-acetate-RNase A complex has been confirmed in this investigation since it was found that acetate at 0.2 M lowers the value of $e^*$ as anticipated in the formation of a ternary complex.

Since it is well established that histidine-12 and histidine-119 are essential for the activity of RNase, the site of binding of Cu(II) which results in inhibition is assumed to involve one of these histidines as a ligand. The lack of evidence for a complex in which Cu(II) is coordinated with two imidazoles has been pointed out by Breslow and Girotti (5). It is not possible on the basis of the data which are now available to make an unequivocal choice between histidine-12 and -119 as the ligand involved in the inhibitor site but the assignment of histidine-12 is plausible on the basis of the following arguments. Of the three Cu(II)-binding sites observed at pH 5 for RNase A and S, only two remain after carboxymethylation of histidine-119 in RNase A; two remain in S-protein after the removal of S-peptide from RNase S, and only one remains after carboxymethylation of histidine-12 in RNase A. The simplest interpretation of these observations is that the histidine-12 site is no longer available in S-protein, the histidine-119 is no longer available in CMHis-119-RNase, and neither the histidine-12 nor the histidine-119 site is available in CMHis-12-RNase. To identify these sites in the active proteins RNase A and S, there are two criteria available, the dissociation constants and the enhancement data. Unfortunately, the modification of one site on the protein may affect these parameters at another site as has been shown to be the case for the pK values of the histidines of RNase A (26). In fact, the magnitude of the Cu(II) dissociation constant is unsatisfactory as a criterion for identification of a particular histidine ligand since all five of the proteins investigated, whether active or inactive forms and whether they have a total of three, two, or one binding sites, retain one strong binding site, i.e. a site with $K_d$ about $7 \times 10^{-4}$ M. The slightly higher value for CMHis-119-RNase may be due to the higher pK of histidine-12 in CMHis-119-RNase (20). The explanation may well be that such a value of $K_d$ is characteristic of the first Cu(II) that binds to RNase regardless of which histidine is involved.

The value of the enhancement factor, $e^*$, would appear to be a more satisfactory criterion for differentiating the binding sites. Thus the strong binding sites of RNase A, CMHis-119-RNase, and RNase S have very similar values of $e^*$ namely, 6.1, 5.9, and 5.1, respectively, while for CMHis-12-RNase $e^*$ has a value of 30 and for S-protein $e^*$ is 9.0. The last two proteins do not have histidine-12 available for binding; therefore the strong binding site with $e^*$ equal to 5 to 6 is assigned to histidine-12. This assignment is reinforced by the high resolution NMR studies of Roberts and Jardetzky (2) which showed that, at pH 5.5 in D$_2$O, Cu(II) first binds to histidine-12 and histidine-105 at all higher concentrations of Cu(II) also binds to histidine-119.

The assignment of one of the weaker binding sites in RNase A and RNase S to histidine-119 based on the loss of one binding site in CMHis-119-RNase and of two sites in CMHis-12-RNase is supported not only by the NMR data quoted above but also by data obtained by many different techniques. The compatibility of the pK values with imidazole ligands has been shown (5). The accessibility of both histidine-12 and histidine-119 has been shown by x-ray diffraction studies (27) and photo-oxidation studies (28, 29) of RNase A and S.

The possibility exists that additional Cu(II)-binding sites in RNase would remain undetected by the PRR enhancement criterion if the enhancement at such sites were close to 1; i.e. the PRR is not very different for free and bound forms of Cu(II) as in the case of the Cu(II):triglycylglycine complex for which the $e^*$ value is 1.1 (30). This possibility has been eliminated for RNase A at pH 5 since a titration of RNase A with Cu(II) with EPR spectra to determine free Cu(II) concentrations directly gave the same results as those calculated from the PRR measurements over a similar range of Cu(II) concentrations.

However, the limitation of the PRR method when values of the enhancement factor approach 1 does become apparent for all forms of RNase above pH 6 and for RNase S at pH 6, as shown in Fig. 2. The ascending limb of the pH curve in Fig. 2 is readily explicable on the basis of an increase in binding of Cu(II) to all forms of RNase as the pH is increased with a consequent increase in the fraction of Cu(II) in the enhanced bound form. The decrease in the fraction of Cu(II) in the enhanced bound form.
crease in observed enhancement above pH 5 to 6 must be ascribed to a decrease in enhancement of the bound forms since it has been shown that the binding constants for the Cu(II):RNase A complex become greater with increasing pH (3, 6, 7). The lowered \( \epsilon_o \) values at the higher pH values may be due to several causes: (a) a smaller number of water ligands in the coordination sphere of the Cu(II) resulting from displacement by other ligands (i.e., \( p \) becomes smaller, cf. Equation 4); (b) change in the structure at the binding site which decreases the rotational correlation time, thereby increasing \( T_{1M} \) (cf. Equation 4); (c) the rate of exchange of the water ligands in the coordination sphere of Cu(II) with the solvent water becomes slow (i.e., \( \tau_M \) becomes large); and (d) aggregation of RNase occurs with concomitant reduction of available binding sites in the presence of Cu(II) under these experimental conditions above pH 6.3. The phenomenon of aggregation for S-protein even at pH 6 is probably responsible for decreasing \( \epsilon_o \) at high protein concentrations as shown in Fig. 7.

The decrease in enhancement above pH 6 was not investigated because of the complexity of the Cu(II)-binding phenomenon encountered at pH 6. The change in the structure of the complexes between pH 5 and pH 6 as evidenced by earlier observations (5) on the visible spectrum is also manifested in the PRR pattern. The most striking change between pH 5 and pH 6 is from two classes of noninteracting binding sites in RNase A, CMHis-119-RNase, and S-protein to cooperative interaction as shown in Fig. 9. On the basis of potentiometric titrations, Breslow and Girootti (5) also suggested cooperative interactions between the first and second Cu(II) bound. Both S-protein which lacks a histidine-12-binding site and CMHis-119-RNase which presumably lacks a histidine-119-binding site nevertheless exhibit cooperativity among their remaining binding sites. The unique behavior of CMHis 12 RNase at pH 6 with two equivalent noninteracting binding sites may be ascribed to the very different structure of this derivative in the vicinity of the binding site as indicated by the unusually high value of \( \epsilon_o \) at pH 6, 24.3, which probably prevents cooperative interaction between binding sites. When the pH is raised from 5 to 6, the number of binding sites in this derivative increases from one to two. Perhaps Cu(II) does not bind significantly to histidine-119 of CMHis-12-RNase at pH 5 but does at pH 6 because the pK of histidine-119 in this derivative has been shifted from 5.8 in the unmodified form to 7.6 (26).

The introduction of the spectroscopic parameter, the magnetic relaxation rate of the protons of water, can serve to give some indication of changes in the flexibility of the protein (\( \tau_M \)) at the binding sites of Cu(II) upon modification of the protein molecule. The validity of comparing the enhancement factors of the strong binding site of various species of ribonuclease in terms of rotational motion was established by showing in the temperature studies that the relaxation mechanism is the same for all five proteins and that it is dominated by a rotational correlation time. It must be assumed in addition that the number of water ligands on Cu(II) is the same in all cases (\( p \) in Equation 4). It should be emphasized that the enhancement is a measure of a change in structure involving the rotational motion of a highly localized portion of the molecule, namely, the binding site of Cu(II) and need not have a 1:1 correspondence with changes in over-all tertiary structure measured by other criteria.

At pH 5, the \( \epsilon_o \) values of the strong binding site for RNase A and CMHis-119-RNase are quite similar while that of RNase S is somewhat lower (cf. Table I). Yang and Hummel (13) have shown that at pH 5.5 or below CMHis-119-RNase has retained most of its native tertiary structure by such criteria as transition temperature, denaturation in urea, and absorbance. Thus, it might be expected that the environment around the copper-binding sites of RNase A and CMHis-119-RNase would be similar. The slightly looser structure of RNase S (15, 31, 32) correlates well with a greater rotational motion reflected at the Cu(II)-binding sites as measured by the correspondingly lower \( \epsilon_o \) value. The increasing enhancement above 35° would be consistent with dissociation of RNase S to S-protein and S-peptide. Binding of Cu(II) may facilitate this dissociation as does extensive iodination (33).

The \( \epsilon_o \) of CMHis-12-RNase at both pH 5 and pH 6 is too high relative to the unmodified protein to be explained solely by an increased number of water ligands to the copper in the derivative. The high \( \epsilon_o \) value involves an increase in \( \tau_M \), which is interpreted as a change in structure around the Cu(II)-binding sites such that the rotational motion is more hindered. The noncooperativity of the two binding sites at pH 6 is unique for this protein and further supports the conclusion that a profound change in structure in the vicinity of the binding sites has occurred in this chemical modification of RNase A. The work of Yang and Hummel (13) indicates that some conformational changes occur in this derivative; at pH 7.5 or above, it was denatured in urea much more rapidly than were RNase A and CMHis-119-RNase and, at pH 8, the transition temperature was lowered about 10°. These properties, unlike the PRR of water, would suggest a less rigid structure. However, any changes in structure are necessarily localized since CMHis-12-RNase cannot be distinguished immunologically from RNase A and CMHis-119-RNase (34). From the large change in the enhancement factor, it is apparent that the regions of the protein which bind Cu(II) are among those which do change in the direction of lowered flexibility. Thus it is apparent that the measurement of the proton relaxation rate, \( 1/T_1 \), may serve as a probe which reflects changes in mobilities at the binding sites of paramagnetic ions in a protein not detectable by other methods. Information concerning the nature of the ligands to the Cu(II) at these sites might be obtained by an extension of the EPR investigations to frozen solutions.

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