The Interaction of Ribonuclease with Metal Ions

III. GEL FILTRATION STUDIES ON THE RELATIONSHIP BETWEEN CUPRIC ION AND CYTIDYLIC ACID BINDING*

(Received for publication, January 31, 1969)

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SUMMARY

The effect of 2'-cytidylic acid and 3'-cytidylic acid upon the binding of cupric ions by RNase has been studied by gel filtration, together with the effect of cupric ions upon binding of 2'- and 3'-cytidylic acids.

The results confirm that binding of 2'-CMP by RNase weakens its affinity for cupric ions; reciprocally, binding of cupric ion diminishes the affinity of RNase for 2'-CMP. The negative interactions between cupric ion and 2'-CMP upon binding to RNase have been shown to lead to distortion of the gel filtration ligand trough. At pH 5.5 these interactions are tentatively interpreted in terms of competition between 2'-CMP and Cu(II) for the strongest Cu(II)-binding site on RNase together with an acetate-dependent increase in the Cu(II) affinity of one of the weaker RNase sites in the presence of bound 2'-CMP.

Binding of 3'-CMP to RNase increases the affinity of RNase for 2 cupric ions; similarly binding of cupric ions to RNase increases its affinity for 3'-CMP. Analysis of the binding pattern of Cu(II) to the 3'-CMP-RNase complex indicates co-operative interactions between two Cu(II)-binding sites on the 3'-CMP-RNase complex; at least one of these sites differs from any on free RNase and could involve the phosphate group of 3'CMP. In further contrast to free RNase, the Cu(II)-binding sites at pH 5.5 on the 3'-CMP-RNase complex have been shown to have a diminished affinity for the cupric ion-monoacetate complex relative to free cupric ion. This suggests that, on the average, Cu(II) is coordinated with more ligands on the 3'-CMP-RNase complex than on free RNase or, alternatively, that binding of Cu(II) to the complex occurs in a more sterically limited environment than on free RNase.

The inhibition of pancreatic RNase by cupric ions (1) has prompted several investigations of the nature of Cu(II)-RNase interactions (2-7). Of these, potentiometric titrations and kinetic studies have indicated the presence of approxi-

mately four binding sites between pH 5.5 and 7 of similar affinity for Cu(II) (2). Subsequent gel filtration binding studies in the presence of acetate buffer more specifically indicated five binding sites, one of which was 12-fold stronger than each of the other four at pH 5.5 (4); binding of both free cupric ion and the cupric ion-monoacetate complex to RNase was shown (4). More recently, three Cu(II)-binding sites have been observed at pH 5 by proton relaxation rate studies; as in gel filtration studies, these sites have been divided into two classes of different affinity for Cu(II) (6).

The identity of the sites to which Cu(II) binds on RNase is not definitely known. The number of sites involved (4) and their properties (2) have led to a tentative assignment of four of the five sites as each involving one of the four RNase imidazoles together with adjacent peptide bond nitrogen atoms, and to tentative identification of the fifth site as involving the α-NH₂ terminus (4). A postulated site involving chelation between His-12 and His-119 of RNase (8) has been shown to be unlikely (9). Equilibrium dialysis studies in the presence of β-alanine led to the suggestion that the site on RNase having the greatest affinity for Cu(II) contains His-119 (3, 5). However, no allowance in these studies was made for possible ternary complex formation between RNase, Cu(II), and β-alanine, a probable factor contributing to the large discrepancy in apparent binding constants obtained (3) relative to gel filtration studies (4) in which acetate binding was taken into account. Finally, Joyce and Cohn have recently suggested that the strongest Cu(II) site at pH 5.1 contains His-12 (6).

Kinetic studies (9) have shown that RNase, Zn(II), and 3'-CMP form a ternary complex. Potentiometric and spectrophotometric studies have indicated that a similar complex is formed with Cu(II) and suggested a positive effect of 3'-CMP on the binding of 2 cupric ions to RNase (2). The same studies (2) indicated that 2'-CMP and Cu(II) did not form such a special complex with RNase; instead small effects of 2'-CMP on the potentiometric titration behavior of Cu(II)-RNase complexes were interpreted to suggest that 2'-CMP diminishes the affinity of RNase for Cu(II). These latter results are in disagreement with the conclusions of Takahashi, Irie, and Ukita (7) who interpreted their data in terms of qualitatively similar behavior of 2'-CMP and 3'-CMP in their interactions with Cu(II) and RNase.

The aim of the present study has been to determine quantitatively by gel filtration the interactions of CMP derivatives and Cu(II) on binding to RNase and to illustrate in greater
detail the application of gel filtration to Cu(Ii)-binding studies. Specifically, answers to the following questions have been sought:

1. What is the effect of 2'-CMP on the binding of Cu(II) to RNase? If binding of 2'-CMP and Cu(II) are competitive, which of the five Cu(II)-binding sites appears to be at or near the 2'-CMP-binding site?

2. What is the nature of the "ternary complex" between Cu(II), RNase, and 3'-CMP?

**EXPERIMENTAL PROCEDURE**

**Materials**

Bovine pancreatic ribonuclease A (lyophilized, phosphate free, Lots 6509, 6Ka, 7Ja) was obtained from Worthington and used without further purification. The RNase appeared homogeneous on chromatography on CM-Sephadex G-50 at pH 8.1 and on Amberlite CG-50 at pH 6. It migrated as a single component in the analytical ultracentrifuge of $s_{20, w} = 1.88$. The water content of the preparations used ranged from 5.0 to 8.5%. No significant difference among the different lots was found in Cu(II)-binding properties. N-Ethylmorpholine, obtained from Eastman Organic Chemicals, was redistilled before use and stored at 4°. Zincol (2-carboxy-2'-hydroxy-5'-sulfoformazyl-benzene) was obtained from LaMotte Chemical Products Company and from Fisher. Cytidylic acid (the mixture of 2'- and 3'-CMP) was obtained from Schwarz Bioresearch. Separation of isomers was performed as previously described (2). Purity of each isomer was checked by optical rotation (10) and by paper chromatography, with the solvent system saturated (NH4)2SO4-0.5 M sodium acetate-isopropyl alcohol, 40:9:1, v/v (10). The extinction coefficient of 2'-CMP at 270 nm was $8800 \text{ cm}^{-1} \text{ M}^{-1}$. Cytidine 3'-phosphate was also purchased from Boehringer Mannheim Corporation and found to have the same purity as the material prepared in this laboratory. All other chemicals were reagent grade, and deionized water was used throughout. Stock solutions of CuCl2 were standardized as previously described (11).

**Methods**

**Column Preparation**—Sephadex columns were prepared in glass housings measuring 0.4 to 1.3 cm (inside diameter) x 95 to 120 cm. These were equipped with Teflon stopcocks (2-mm bore) and with coarse sintered glass plugs to support the Sephadex bed. Standard taper fittings were used throughout.

Sephadex G-25 (fine) from Pharmacia was suspended in 0.2 M acetic acid and taken through six settling and decantation cycles to remove the very fine particles. Before a column was poured, the slurry was deaerated on a water pump for 1 hour with magnetic stirring. A continuous settling procedure was used, each housing being first filled with deaerated 0.2 M acetic acid, and the Sephadex slurry was introduced continually through a funnel. The bed was washed overnight with 0.2 M acetic acid and then covered with a small circular piece of nylon net (400 mesh).

**Binding Experiments and Calculations**—The column was first equilibrated with the solution of buffer containing the desired concentration of Cu(II) and cytidylic acid. At pH 5.5 the elution of two buffer systems was used: (a) 0.05 M total acetate (0.044 M acetate ion + 0.006 M acetic acid) + 0.11 M KCl, or (b) 0.2 M total acetate (0.177 M acetate ion + 0.0237 M acetic acid). At pH 7, buffers were 0.02 M in N-ethylmorpholine and contained either 0.05 M acetate ion and 0.11 M KCl or 0.2 M acetate ion. A volume of eluant equivalent to at least three Cu(II) elution volumes was passed through the column. The sample of RNase was weighed in a small beaker and dissolved in approximately 0.25 ml of eluant. Depending on the amount of protein used, and the extent of Cu(II) binding, it was often necessary at this point to adjust the pH back to that of the eluant. The adjustment was made carefully on a Radiometer pH-Meter 4, with a relatively concentrated solution of NaOH or HCl (1 to 3 N) to prevent significant volume change. The sample was then transferred to the column with a small curve-tipped pipette and, after 5 min, was allowed to run in at a slow rate. Any remaining material was washed from the beaker onto the column with two 0.1-ml aliquots of eluant. The column was then reconnected to the reservoir, and the elution rate was set at 8 to 9 ml hr⁻¹ cm⁻² for the duration of the run. All binding runs were conducted at 25 ± 1°C (ambient). Fractions of either 0.5 or 1.0 ml were collected in test tubes, 18 x 130 mm, that had been acid-washed and rinsed with deionized water. The actual volume used was determined either with a graduated "to contain" pipette or by weighing. Fractions which could not be analyzed immediately after collection were covered with Parafilm and refrigerated to minimize evaporation.

The effluent was analyzed for total copper by a modification of the spectrophotometric procedure described by Rush and Voe (12). Either whole fraction volumes or aliquots thereof in test tubes were diluted with deionized water so that the addition of 2.0 ml of 0.32 M borate buffer (pH 9.2) and 0.5 ml of Zincol, 0.2% (w/v) in 0.03 N NaOH, brought the final volume to 10.0 ml.

Absorbance was determined at 900 nm with a Beckman DU spectrophotometer and cells of 1-cm path length. Experimental analyses were restricted to the linear portion of the standard curve obtained under these conditions, i.e., from 0 to 3.8 ppm of copper. Occasionally it was necessary to increase the sensitivity of the analysis by reducing the final volume to 5.0 ml, while keeping the concentration of buffer and Zincol constant. Binding of Cu(II) was calculated both from the area of the peak in the elution profile (representing the actual Cu-RNase complex) and the area of the trough, according to the following equation:

$$f_{cp} = \frac{\sum_{n} A_{n}}{V}$$

where $f_{cp}$ represents total copper bound per mole of protein, $\sum_{n} A_{n}$ is the peak or trough area under $n$ fractions, $V$ is the fraction volume in milliliters, $f$ is the slope of the standard curve (absorbance units per pmole of copper), $p$ is RNase in micromoles. RNase is the concentration of free cupric ion and cupric ion-acetate complexes was calculated as described previously (4). Treatment of the free acetate ion concentration was assumed equal to the acetate ion present in the original buffer except in those studies in which Cu(II) concentrations were relatively high and a significant percentage of the total acetate was coordinated with cupric ion.

Elution of 2'- and 3'-CMP was monitored at 270 nm and bind-
ing was determined from the area of the trough in the elution pattern from the equation:

\[ \bar{e}_{\text{CMP}} = \frac{\sum_m \Delta A_m \ V}{\epsilon_{\text{m,M}} \ L \ P} \]  

(2)

where \( \bar{e}_{\text{CMP}} \) represents CMP bound per mole of RNase, \( \epsilon_{\text{m,M}} \) is the millimolar extinction coefficient of CMP, \( L \) is the cell path length in centimeters, and other factors are as described under Equation 1. Since the Cu(II) trough overlaps that of CMP, it was necessary in experiments conducted with both species present to correct the CMP trough for a small contribution of Cu(II) absorbance in the ultraviolet. The extinction coefficient of Cu(II) in 0.05 M acetate buffer, pH 5.5, was found to be 550 cm\(^{-1}\) M\(^{-1}\) at 270 nm, while in 0.2 M acetate buffer, pH 5.5, it was 980 cm\(^{-1}\) M\(^{-1}\).

Three factors may have contributed to the diminution of the Cu(II) and CMP concentration in the sample applied to the column, thereby leading to trough areas that are too large: (a) the water content of the protein (13), (b) the partial specific volume of the protein (13), and (c) the volume of acid or base needed for pH adjustment. The most significant of these dilution factors in these binding experiments was (c), but, even at relatively high concentrations of small molecule, the net correction in all cases was well within experimental error.

To maintain an equilibrium at a given concentration of small molecule in a gel filtration binding experiment, it is essential to use an amount of protein that will remove as little as possible of the small molecule from the “salt volume” of the column. In all binding experiments described below, unless otherwise noted, the concentration of total copper or cytidylic acid was lowered no more than 10% by the amount of RNase used; in the majority of runs the reduction was less than 5%.

The recovery of RNase was checked periodically by Folin-Lowry analysis of the pooled fractions under a copper peak (14). This was done primarily to correct for losses incurred during pH measurement. The observed recovery value was usually between 93 and 98%; calculations of \( \bar{e} \), therefore, were routinely based on RNase being 95% of the amount actually weighed.

**Precision of Data**—Within a given binding run, differences between peak and trough areas did not usually exceed 10% and were often within 5%. Agreement between average results of different runs was slightly better than between peak and trough values from a single run. The majority of experiments in which peak and trough values differed by significantly more than 10% fell into one of three categories: (a) binding was very low so that very small absolute differences were proportionately significant, (b) the column was very small (0.4 \( \times \) 100 cm), or (c) the equilibrating total Cu(II) concentration was greater than 10 mN. When runs performed on very small columns were repeated on larger columns, differences between peak and trough values were eliminated with no change in the average calculated \( \bar{e}_{\text{Cu}} \).

Three runs performed at Cu(II) concentrations of 10 mN and above gave peak values which were clearly too low (see “Results”); this is tentatively ascribed to diminished solubility of the Cu(II)-RNase complex under these conditions so that not all of the protein was eluted from the column; however, protein recoveries on these particular runs were not performed.

**RESULTS**

**Cu(II) Binding in Absence of CMP**—In Fig. 1A the type of raw data obtained in an individual chromatographic run of RNase in a Cu(II)-acetate buffer at pH 5.5 is shown. The peak contains the excluded protein-Cu(II) complex; its area is equivalent to Cu(II) bound by the protein. Similarly the trough area represents bound Cu(II); the essential equivalence of peak and trough areas confirms that the presence of protein in the peak did not interfere with Cu(II) determinations under these conditions. The plateau between peak and trough indicates that the system is at equilibrium with the Cu(II) concentration with which the column was equilibrated; such a plateau is absent if the free Cu(II) concentration is significantly depleted by binding.

Graphical analysis of Cu(II)-binding studies to RNase using gel filtration in the absence of CMP has been reported previously (4). In Fig. 2, a summary of the binding data obtained at pH 5.5 at 0.05 M and 0.2 M acetate is shown more directly as a plot of \( \bar{e}_{\text{Cu}} \) against the negative logarithm of the total equilibrating concentration of CuCl\(_2\). These studies represent a range of total Cu(II) concentrations from 0.05 to 20 mN. Higher concentrations of total Cu(II) could not be used because of the resultant precipitation of Cu(OH)\(_2\). The fact that the observed binding constants of Cu(II) to RNase (4) fall into the range in which competition with OH\(^-\) is significant, in theory, places an upper limit on attainable values of \( \bar{e}_{\text{Cu}} \). However, it is relevant to note that the binding of both Cu\(^{2+}\) (free cupric ion) and CuAc\(^{+}\) (the 1:1 complex of Cu\(^{2+}\) with acetate ion) by RNase (4) leads to higher attainable values of \( \bar{e}_{\text{Cu}} \) in the presence than in the absence of acetate.

The amount of protein used in a chromatographic run varied from 1 to 2 \( \mu \)moles for runs on the most typical column (0.6 \( \times \) 100 cm) and was proportionately increased or decreased for larger and smaller columns, respectively. The concentration of protein used in any given chromatographic run was limited on...
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FIG. 2. Binding of Cu(I1) to RNase and its complexes with 2'-CMP and 3'-CMP at pH 5.5. The abscissa refers to the total concentration of CuCl2 in the eluant. Each point represents the average of peak and trough values from a single chromatographic run; deviations between peak and trough are indicated by a line through each point. Dotted deviation lines represent studies performed on very small columns (see "Experimental Procedure").

The low side by the sensitivity of Cu(I1) determinations and on the high side by the stipulation that the protein applied to the column should not bind a significant fraction of the total column Cu(I1). The limitation in allowable total protein together with the inevitable change in protein concentration during chromatography disallowed rigorous determination of the effect of protein concentration on Cu(I1) binding. However, in several studies both in the absence and presence of CMP, a 2-fold alteration in the amount of protein applied to the same column produced no change in Cu(I1) binding that fell clearly outside the range of experimental error.

2'-CMP and 3'-CMP Binding to RNase in Absence of Cu(I1)—

The binding of 2'-CMP and 3'-CMP to RNase was studied in the absence of Cu(I1) by gel filtration with the same buffer systems as used in Cu(I1)-binding studies. Chromatographic patterns were similar to those observed by Hummel and Dreyer (15) as shown in Fig. 1B. Binding constants determined at pH 5.5 in 0.05 M acetate buffer containing 0.11 M KCl were 2.1 \times 10^4 and 2.9 \times 10^4 for 2'-CMP and 3'-CMP, respectively, and may be compared with values of 2.96 \times 10^4 and 2.7 \times 10^4 for the corresponding isomers obtained spectrophotometrically in similar buffer systems (16). At pH 7 the binding constant for 2'-CMP was determined as 1.2 \times 10^4 in 0.02 M N-ethyl morpholine buffer containing 0.2 M acetate; this value is somewhat higher than that reported at pH 7 in 0.1 M KNO3 containing 0.05 M acetate (15).

Competition between Cu(I1) and 2'-CMP—The existence of competition between Cu(I1) and 2'-CMP on binding to RNase was shown by studying binding on columns equilibrated with both components. In addition to the usual considerations, concentrations of 2'-CMP and Cu(I1) were chosen such that reduction of unbound 2'-CMP and Cu(I1) concentrations due to nucleotide-Cu(I1) interaction (17) could be assumed insignificant, as discussed in greater detail below. Actual concentrations of 2'-CMP and Cu(I1) ranged from 0.1 mM to 0.75 mM and from 0.05 to 20 mM, respectively; total protein and column dimensions were identical with those used in the absence of CMP. Acetate concentrations of 0.05 M and 0.2 M at pH 5.5 and of 0.2 M at pH 7 were used. At pH 5.5 limitations on allowable conditions, coupled with the strong binding of 2'-CMP relative to Cu(I1), permitted detailed studies only of the displacement of Cu(I1) by 2'-CMP, although some displacement of 2'-CMP by Cu(I1) was evident. Alternatively, at pH 7, binding of 2'-CMP was relatively weak compared to that of Cu(I1) and appreciable displacement of 2'-CMP by Cu(I1) was evident. Alternatively, at pH 7, binding of 2'-CMP was relatively weak compared to that of Cu(I1) and appreciable displacement of 2'-CMP by Cu(I1) was evident. Alternatively, at pH 7, binding of 2'-CMP was relatively weak compared to that of Cu(I1) and appreciable displacement of 2'-CMP by Cu(I1) was evident.

In the absence of acetate, at pH 7, ultracentrifuge studies show considerable aggregation of RNase in the presence of Cu(I1). This aggregation is considerably reduced (but not completely removed) at pH 5.5 or in the presence of acetate at pH 7. It is therefore possible that, under the conditions of these studies, protein-protein interactions play a role in binding, but this role is not yet amenable to quantitation.

Competition between 2'-CMP and Cu(I1) on binding to RNase.

The term "competition" as used here implies only a negative interaction between the two sites involved and not, specifically, a competition between two ligands for the same binding site.
RNase leads to an interesting alteration of the chromatographic patterns of 2'-CMP and Cu(II). Under conditions in which the main effect of the competition is a reduction in bound Cu(II) and in which the CMP concentration used is relatively low (pH 5.5), an additional Cu(II) peak is observed immediately before the trough in Cu(II) concentration (Fig. 3A). This peak can be shown to represent a fraction of the Cu(II) displaced by 2'-CMP. At constant levels of 2'-CMP it diminishes with increased Cu(II) ion concentration and decreased displacement of Cu(II). It does not occur in the presence of 3'-CMP and 3'-CMP does not compete with Cu(II). The presence of the peak is independent of the rate of elution and may be explained as follows. During the early stages of the chromatographic run, the protein is in equilibrium with a concentration of each ligand which is less than that with which the column was equilibrated; this follows because binding of ligand reduces the ligand concentration for a short column distance (eventually giving rise to the observed trough in ligand concentration). Because the equilibrating column concentration of 2'-CMP is low relative to the amount to be bound at equilibrium, the protein must traverse a greater column distance to reach equilibrium with the final concentration of 2'-CMP than to reach equilibrium with Cu(II). There is therefore a point early on the column when the protein is in equilibrium with the same Cu(II) concentration with which the column was equilibrated, but with a lower concentration of 2'-CMP. Since 2'-CMP and Cu(II) are competitive, more Cu(II) is bound at this point than at the higher final free CMP concentration. As the protein travels further, the free 2'-CMP concentration with which it is in equilibrium increases and bound Cu(II) is displaced as evidenced by the anomalous Cu(II) peak.

A similar phenomenon can be observed in the chromatographic

TABLE I

Representative data showing negative interactions between Cu(II) and 2'-CMP on binding to RNase

<table>
<thead>
<tr>
<th>pH</th>
<th>Eluant concentrations</th>
<th>Total</th>
<th>Cu(II)total</th>
<th>2'-CMP</th>
<th>( \bar{v}_{\text{Cu}} )</th>
<th>( \bar{v}_{\text{CMP}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>0.05</td>
<td>0.5</td>
<td>0.53 ± 0.02</td>
<td>(2)</td>
<td>0.94</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.1</td>
<td>0.23 ± 0.02</td>
<td>(1)</td>
<td>0.98 ± 0.01</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.5</td>
<td>0.79 ± 0.06</td>
<td>(1)</td>
<td>0.83 ± 0.03</td>
<td>(2)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.65</td>
<td>0.6</td>
<td>0.31 ± 0.03</td>
<td>(1)</td>
<td>1.00</td>
<td>(1)</td>
</tr>
<tr>
<td>2.5</td>
<td>0.5</td>
<td>0.12</td>
<td>0.51 ± 0.01</td>
<td>(1)</td>
<td>0.64</td>
<td>(1)</td>
</tr>
<tr>
<td>7.0</td>
<td>0.2</td>
<td>0.05</td>
<td>1.02 ± 0.01</td>
<td>(1)</td>
<td>0.47</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>0.12</td>
<td>1.95 ± 0.01</td>
<td>(1)</td>
<td>0.25</td>
<td>(1)</td>
</tr>
</tbody>
</table>

* Values of \( \bar{v} \) refer to the number of moles of Cu(II) or CMP bound per mole of total protein.
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Figure 4. Scatchard plots of binding of Cu(II) to RNase and the 2'-CMP-RNase complex at pH 5.5. Data are plotted with respect to the sum of the concentrations of Cu++ and CuAc+ in accord with the observation that both species are bound equally by RNase (4). Unless otherwise noted, data points for binding to 2'-CMP-RNase complex represent average of peak and trough determinations from a single run, corrected for binding to free RNase as in Equation 3; deviations between peak and trough are shown by a diagonal line through each point.

A, data in 0.05 M acetate buffer containing 0.11 M KCl. ●, 2'-CMP-RNase complex; ○, RNase. Line through RNase data (+-+) is theoretical curve for sum of one strong site (Kc++,KcAc+= 1.5 x 10^3) and four weak sites (Kc++,KcAc+= 1.2 x 10^2) defined in Reference 4. ---, theoretical curve for "strong" site alone; ----, theoretical curve for four weak sites alone. B, data in 0.2 M acetate. ●, 2'-CMP-RNase complex; ○, RNase. Theoretical curves are the same as described in A. Large deviations between peak and trough values at high values of eCu are explained in text; the low peak values are anomalous. Lowest point shown for the 2'-CMP-RNase complex represents a trough value only.

The presence of five (or four) sites at pH 5.5 has previously been shown to be fit with the assumption of five sites, one of which has a 12-fold greater affinity for Cu(II) than each of the other four (4); all five sites are readily shown at pH 7 (4). In view of the suggestion that the strongest Cu(II)-binding site at pH 5.1 may contain His-12 (6) and the probable involvement of His-12 in the binding of 2'-CMP (18), it is relevant to attempt to assess the effect of 2'-CMP on the strongest Cu(II)-binding site at pH 5.5. In Figure 4, Cu(II)-binding data for RNase and for the 2'-CMP-RNase complex are plotted according to the method of Scatchard (19). Shown along with the data are theoretical curves derived from the previously ascribed binding constants (4), representing binding by: all five sites (A), the strong site alone (B), and the four weaker sites in the absence of the strong site (C). At 0.05 M acetate, there is a close similarity between Cu(II) binding by the 2'-CMP-RNase complex and Curve C and a markedly weaker binding by the complex at low values of eCu than Curve B, indicating that the strongest Cu(II)-binding site on free RNase under these conditions is lost or significantly weakened when 2'-CMP is bound. The data this treatment is retained here. However, the allocation of two members to the strong class and three to the weak class, although less compatible with the data, cannot be strictly excluded. The net effect of such a treatment upon interpretation of the present data would be to change the principal site with which 2'-CMP may compete from the single "strong" site to one of the two strongest sites.

...
at 0.2 M acetate, however, although scant, deviate significantly at low values of $\vec{v}$ from that predicted for binding by the four weaker sites of RNase alone and, in fact, tend to suggest the presence of a strong site on the complex which is only slightly weaker than that on free RNase itself. A tentative interpretation of these data is given under "Discussion."

**Interactions between Cu(II) and 2'-CMP Binding at pH 7.0**—Several studies of the apparent competition between Cu(II) and 2'-CMP upon binding to RNase were also made at pH 7 in 0.2 M acetate and are shown in Table I. The observed diminution in $\vec{v}_{\text{CMP}}$ as the concentration of unbound Cu(II) is increased is too great to be due to complex formation between unbound Cu(II) and 2'-CMP, as shown by the following. The stability constant of the 1:1 complex of Cu(II) with 2'-CMP should be similar to that for Cu(II) and 5'-AMP which has been estimated as $1.1 \times 10^3$ (17). With this value, allowing for formation of 2:1 complexes of comparable stability, and neglecting competition by acetate for Cu(II), it can be calculated that, at 0.12 mM 2'-CMP, the maximum expected effect due to Cu(II)-2'-CMP complex formation should be to reduce $\vec{v}_{\text{CMP}}$ from 0.6 in the absence of Cu(II) to 0.36 in the presence of 0.125 mM Cu(II). The observed effect is a reduction in $\vec{v}_{\text{CMP}}$ to 0.25.

The effect of Cu(II) upon 2'-CMP binding at pH 7.0 can be treated by standard equations (20) in terms of direct competition between 2'-CMP and free copper ion for a single site. Assuming

**Table II**

<table>
<thead>
<tr>
<th>Eluant concentrations</th>
<th>Total acetate</th>
<th>Cu(II)total</th>
<th>$\vec{v}_{\text{Cu}}$</th>
<th>$\vec{v}_{\text{CMP}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.5</td>
<td>0.05</td>
<td>0.50 $\pm$ 0.03 (2)</td>
<td>0.53 (1)</td>
</tr>
<tr>
<td>0.05</td>
<td>1.0</td>
<td>0.05</td>
<td>0.70 $\pm$ 0.00 (1)</td>
<td>0.88 (1)</td>
</tr>
<tr>
<td>0.05</td>
<td>0.5</td>
<td>0.05</td>
<td>2.17 $\pm$ 0.00 (1)</td>
<td>0.92 (1)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.65</td>
<td>0.2</td>
<td>0.36 $\pm$ 0.01 (1)</td>
<td>0.81 (1)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.38</td>
<td>0.2</td>
<td>0.06 $\pm$ &lt;0.01 (1)</td>
<td>0.81 (1)</td>
</tr>
<tr>
<td>0.2</td>
<td>2.5</td>
<td>0.2</td>
<td>1.49 $\pm$ 0.06 (1)</td>
<td>0.92 (1)</td>
</tr>
</tbody>
</table>

$\vec{v}_{\text{Cu}}$ values refer to the number of moles of Cu(II) or CMP bound per mole of total protein.

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**Fig. 5.** Binding of Cu(II) to the 3'-CMP-RNase complex at pH 5.5. A, Scatchard plot of binding data in terms of concentration of free Cu(II) ion at two levels of acetate. Binding by RNase alone is shown for comparison. $\bullet \ldots \bullet$, 3'-CMP-RNase, 0.05 mM acetate; $\bullet \ldots \bullet$, 5'-CMP-RNase, 0.2 mM acetate. **Data points** for the complex represent average of peak and trough determinations of $\vec{v}_{\text{Cu}}$ from a single run corrected for binding to free RNase as per Equation 3. Deviations between peak and trough are shown by a diagonal line through each point; runs showing particularly large deviations are those performed on very small columns (see text). **Lines connecting data points** serve only to delineate binding pattern. $\times \ldots \times$, RNase in 0.05 mM acetate. B, Scatchard plot of binding to complex in terms of sum of concentrations of Cu(II) and CuAc+. Only average of peak and trough determinations of $\vec{v}_{\text{Cu}}$ are shown. $\bullet \ldots \bullet$, 0.05 mM acetate; $\bullet \ldots \bullet$, 0.2 mM acetate.
and /3-alanine as has been found in the presence of acetate (4). The formation of ternary complexes between Cu(II), RNase, and 3'-CMP are compared; the data confirm previous conclusions that binding of Cu(I) increases the affinity of RNase for 3'-CMP. In addition, the previously reported effect of 3'-CMP on the spectrum of the first 2 bound Cu(II) ions (2) is supported here by the magnitude of the difference in $r_{Cu}$ between free RNase and its 3'-CMP complex at the same equilibrating concentration of Cu(II). At 1 mm Cu(II), for example, 1.4 additional metal ions are bound in the presence than in the absence of 3'-CMP. Thus, as the observed binding constants will verify, the affinity of at least two Cu(II)-ion-binding sites has been increased by the presence of 3'-CMP.

The Cu(II) binding data in the presence of 3'-CMP were treated similarly to those in the presence of 2'-CMP; i.e. Cu(I) binding was formulated in terms of the number of moles of Cu(I) bound to the 3'-CMP-RNase complex according to Equation 3, although in this case data were obtained at values of $[3'\text{CMP}]$ ranging from 0.7 to 1.0. Alteration of the concentration of 3'-CMP at a given Cu(I) concentration gave a value of $[3'\text{CMP}]_{\text{Cu(II)}}$ which was independent of $[3'\text{CMP}]$, confirming the validity of the treatment.

In Fig. 2, direct plots of the binding of Cu(II) to the 3'-CMP-RNase complex at 0.05 M and 0.2 M acetate are shown as a function of the negative log of the total equilibrium Cu(II) concentration and are to be compared with binding by free RNase. In Fig. 5A, the data at both acetate concentrations are plotted according to the method of Scatchard (19) with respect to the concentration of free Cu(II) ion (Cu++) present and are compared to the binding properties of free RNase at 0.05 M total acetate. Several facts are immediately clear: (a) binding of Cu(II) by the complex is significantly stronger than by free RNase; (b) marked positive interactions among the Cu(II)-binding sites of the complex are present as evidenced by the upward trend of the data for the complex in Fig. 5 at low values of $\chi$ as well as by the steep slope at low values of $\chi$ in Fig. 2; and (c) data for the complex obtained at different acetate concentrations are very similar when plotted with respect to the concentration of Cu++. (Fig. 5A). This last effect may be shown to indicate that the complex, in contrast to free RNase, has a low affinity for the species CuAc+. Cu(II)-binding studies of free RNase are marked by a large difference between binding curves obtained at different acetate concentrations unless the data are plotted in terms of the sum of the concentrations of the species Cu++ and CuAc++; this has been interpreted as binding of both cupric ion and the cupric ion-monoacetate complex to RNase (4). In Fig. 5B, binding by the 3'-CMP-RNase complex is plotted in terms of the sum of the concentrations of Cu++ and CuAc++; it is apparent that such a treatment only increases the disparity of the data obtained at the two different acetate concentrations. Therefore, binding of 3'-CMP to RNase has altered the properties of the Cu(II)-binding sites such that accessibility to acetate ion has diminished.

Apparent binding constants governing the interaction of the first 3 Cu(II) ions to the 3'-CMP complex can be obtained from the data at 0.05 M acetate with an equation similar to that of Adair (21) where:

$$\chi = \frac{K_1(Cu^{++}) + 2K_1K_3(Cu^{++})^2 + 3K_1K_3K_5(Cu^{++})^3}{1 + K_1(Cu^{++}) + K_1K_2(Cu^{++})^2 + K_1K_3K_4(Cu^{++})^3} \quad (4)$$

Here

$$K_i = \frac{(P_{Cu})}{(P)(Cu^{++})}$$

Study of Cu(II)- and $3'$-CMP-Binding Site Interactions—The binding of Cu(II) in the presence of 3'-CMP was studied at pH 5.5 similarly to binding in the presence of 2'-CMP. Equilibrating concentrations of CuCl$_2$ ranged from 0.01 to 5 mm; 3'-CMP concentrations ranged from 0.046 to 0.65 mm. In Table II, representative binding studies in the presence and absence of 3'-CMP are compared; the data confirm previous conclusions (2) that the presence of 3'-CMP increases the binding of Cu(II) ion and that binding of Cu(II) increases the affinity of RNase

![Fig. 6. Attempts to fit binding data of Cu(II) to the 3'-CMP-RNase complex at 0.05 M acetate, pH 6.5. O represents experimental data; lines represent theoretical curves derived from Equation 4 with values of $K_1 = 12.2 \times 10^4$ and $K_2 = 2 \times 10^4$. ---, $K_1 = 7 \times 10^4$; ..., $K_1 = 2.2 \times 10^4$; --. $K_1 = 9 \times 10^4$. ---, $K_1 = 7 \times 10^4$.](http://www.jbc.org/)

That all CMP displaced in the presence of Cu(II) is displaced by direct competition with Cu++, the apparent affinity of the 2'-CMP-binding site for Cu++ calculated from our data is $9 \times 10^4 \pm 3 \times 10^4$. Although this value is in fair agreement with the value $5 \times 10^4$ obtained from direct Cu(II)-binding studies as the maximum affinity of the strongest Cu(II)-binding site for Cu++ at pH 7.0 (4), it also is a maximum value in that it does not allow for competition between 2'-CMP and more than one Cu(II)-binding site or for competition between 2'-CMP and CuAc++. As such, it is of interest that this value is significantly lower than the value $5 \times 10^4$ obtained in the presence of /3-alanine as the interaction constant of the active site with Cu(II) (3, 5). The disparity between these data parallels the disparate Cu(II)-binding constants obtained from direct binding studies in acetate (4) and in /3-alanine (5) and is a possible consequence of the formation of ternary complexes between Cu(II), RNase, and /3-alanine as has been found in the presence of acetate (4).

Study of Cu(II)- and $3'$-CMP-Binding Site Interactions—The binding of Cu(II) in the presence of 3'-CMP was studied at pH 5.5 similarly to binding in the presence of 2'-CMP. Equilibrating concentrations of CuCl$_2$ ranged from 0.01 to 5 mm; 3'-CMP concentrations ranged from 0.046 to 0.65 mm. In Table II, representative binding studies in the presence and absence of 3'-CMP are compared; the data confirm previous conclusions (2) that the presence of 3'-CMP increases the binding of Cu(II) ion and that binding of Cu(II) increases the affinity of RNase...


\[ K_3 = \frac{(PCu_3)}{(PCu_3) (Cu^{++})} \]

and

\[ K_1 = \frac{(PCu_2)}{(PCu_2) (Cu^{++})} \]

where \( P \) is the concentration of the 3'-CMP-RNase complex to which no Cu\(^{++}\) is bound, and \( PCu_2 \), \( PCu_3 \), and \( PCu_4 \) are each the sum of all species of 3'-CMP-complex carrying 1, 2, and 3 moles of Cu(II), respectively; \( (Cu^{++}) \) is the concentration of free cupric ion. If the simplifying assumption is made that the affinities of all sites on the complex for CuAc\(^{+}\) is negligible (the results indicate that the affinity of the first two sites for CuAc\(^{+}\) is less than 5% of their affinity for Cu\(^{++}\)\()), the data can be shown to lead to unique values of the product \( K_1K_3 \), which is the overall binding constant of the first 2 Cu(II) ions to the complex, and of \( K_3 \). These have been derived by successive approximations as 12.2 \( \times \) 10\(^8\) and 2 \( \times \) 10\(^8\), respectively; a variation in \( K_1K_3 \) of as little as 20% can be shown to generate results which are clearly incompatible with the data. Somewhat greater flexibility is found in the allowed values of \( K_1 \). Good fit of the data is obtained with \( K_1 = 7 \times 10^7 \) and values of \( K_1 \) greater than \( 9 \times 10^7 \) are clearly excluded. However, minimum values of \( K_1 \) cannot be defined with confidence except to exclude values lower than \( 7 \times 10^7 \). The relationship between the derived constants and the binding data can be seen in Fig. 6 where binding curves generated by Equation 4 with values of \( K_1 \), \( K_2 = 12.2 \times 10^8 \), \( K_3 = 2 \times 10^9 \), and varying values of \( K_4 \) are compared with the experimental data obtained in 0.05 M acetate.

A minimum estimate of the magnitude of positive site-site interaction occurring on binding of the first 2 Cu(II) ions to the complex can be obtained from comparison of the derived value of \( K_1K_2 \) with the maximum derived value of \( K_1 \). In the absence of any site-site interactions, statistical considerations (20) indicate that \( K_1K_2 \) cannot exceed \( K_1^2/3 \) and will be less than this value to the extent that any of the three significant binding sites of the complex are not intrinsically equivalent. The observed value of \( K_1K_2 \) is actually 45-fold greater than \( K_1^2/3 \), indicating that binding of the 1st cupric ion increases the apparent affinity of the complex for the 2nd cupric ion by a factor of at least 45. Alternatively, no positive interactions are manifest on binding of the third cupric ion, since the derived value of \( K_3 \) is significantly lower with respect to \( K_1K_2 \) than the maximum value allowed on statistical grounds for a set of three equivalent sites (20).

**Discussion**

The demonstration of strong cooperative interactions between two Cu(II)-binding sites on the 3'-CMP complex, not evident under the same conditions with free RNase, and the increase in affinity of RNase for 2 Cu(II) ions in the presence of 3'-CMP indicate that the "ternary" complex of 3'-CMP, RNase, and Cu(II) involves more than the formation of a bond between 3'-CMP and a single cupric ion on the enzyme surface. These studies are therefore in agreement with spectrophotometric and titration studies which previously indicated an alteration in the interaction of 2 cupric ions with RNase in the presence of 3'-CMP (2). The nature of the two interacting Cu(II) sites is unknown, but it seems highly plausible that the phosphate of 3'-CMP is a ligand to Cu(II) on at least one of them. In addition to arguments previously advanced (2), this is supported by the over-all binding constant \((K_1K_2)\) of the first 2 Cu(II) ions to the 3'-CMP complex which is 700-fold greater than the product of any two Cu(II) binding constants on free RNase; this in itself indicates that additional or different ligands to Cu(II) are present on the 3'-CMP complex than on free RNase. The second interacting site of the 3'-CMP-RNase complex could be similar to one in free RNase and its 2'-CMP-complex, but it may well also involve direct interaction of the second bound Cu(II), in addition to the first, with the phosphate of 3'-CMP. Such an interaction would be analogous to that of 2 cupric ions with a single carboxyl group as has been observed in several crystalline peptide-metal complexes (22).

The demonstration here by direct binding studies that 2'-CMP diminishes the affinity of RNase for Cu(II) also supports conclusions drawn from titration data (2) and further emphasizes the differences between 2'-CMP and 3'-CMP in their interactions with the Cu(II)-RNase system. The results are in disagreement with the conclusions of Takahashi et al. (7) who postulated that 2'-CMP formed a ternary complex with Cu(II) and RNase which was qualitatively similar to that formed with 3'-CMP. Their conclusions, however, may be shown to result in part from their failure to recognize that there are several Cu(II)-binding sites on RNase, and that 2'-CMP and Cu(II) can occupy the same RNase molecule even though binding to one of the Cu(II) sites is diminished by 2'-CMP. Such is the situation indicated by the present data.

The origins of the observed negative interactions between Cu(II) and 2'-CMP on binding to RNase remain uncertain, particularly in view of the stronger binding of Cu(II) by the 2'-CMP-RNase complex relative to RNase at 0.2 M and at 0.05 M acetate. However, the data are not incompatible with direct competition between 2'-CMP and Cu(II) for the strongest Cu(II)-binding site at pH 5.5 if an acetate-dependent increase in one of the weaker Cu(II) sites of the complex is also occurred. Such an assumption finds some support in the work of Joyce and Cohn (6) who showed that carboxymethylation of His-12 leads both to the apparent loss of the strongest Cu(II)-binding site at pH 5.1 and an increase in Cu(II) affinity of one of the weaker Cu(II) sites of the complex is also occurred. Such an assumption finds some support in the work of Joyce and Cohn (6) who showed that carboxymethylation of His-12 leads both to the apparent loss of the strongest Cu(II)-binding site at pH 5.1 and an increase in Cu(II) affinity of one of the weaker Cu(II) sites of the complex is also occurred. Such an assumption finds some support in the work of Joyce and Cohn (6) who showed that carboxymethylation of His-12 leads both to the apparent loss of the strongest Cu(II)-binding site at pH 5.1 and an increase in Cu(II) affinity of one of the weaker Cu(II) sites of the complex is also occurred. Such an assumption finds some support in the work of Joyce and Cohn (6) who showed that carboxymethylation of His-12 leads both to the apparent loss of the strongest Cu(II)-binding site at pH 5.1 and an increase in Cu(II) affinity of one of the weaker Cu(II) sites of the complex is also occurred.

The present studies reflect both the limitations and advantages of investigating metal ion binding by gel filtration. The dependence on pH of protein-metal ion interactions necessitates the use of \( \Pi^+ \) ion buffers in most systems. Moreover, metal ion buffers are often necessary to allow the column to hold an adequate excess of metal ion so that binding does not perturb the unbound metal ion concentration; this is particularly true when metal-protein affinity is high and values of \( \% \) below saturation are needed, or when large amounts of protein must be used because of the low sensitivity of available methods of metal ion assay. Potential complications of the presence of buffer and metal ion buffer interactions are illustrated by the binding to RNase of both free cupric ion and the cupric ion-monoacetate complex (4) and by the effect of acetate on the pattern of Cu(II) binding by the 2'-CMP-RNase complex. However, the extent

\[ ^* \text{A. Girotti and A. Breslow, unpublished observations.} \]
to which a protein can discriminate on binding between free metal ion and metal ion complexes appears to be a useful probe of the nature of the binding site. For example, the diminution in RNase affinity for the cupric ion-monooacetate complex relative to free cupric ion when 3'-CMP is bound must reflect either a greater number of nonaquo ligands to Cu(II) in the 3'-CMP-RNase complex than in RNase or a sterically hindered environment around Cu(II) in the complex. Similarly, the observed decrease in the relative affinity of the “strong” Cu(II)-binding site on free RNase for the cupric-ion monooacetate complex above pH 5.5 (4) reflects a greater degree of complexity in this site as the pH is raised. The ternary interactions between metal ion, buffer, and protein are therefore amenable to systematic investigation and can be an additional parameter by which the properties of metal-binding sites can be measured.

Acknowledgments—We are grateful to Dr. Mildred Cohn for making data available prior to publication and to Dr. Sherman Beychok for many constructive suggestions.

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