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Copper(II) and Manganese(II) Effects on Ribonuclease A Activity*

Terry D. Alger

ABSTRACT: This work attempts to delineate mechanisms for the metal activation and inhibition observed in enzyme–substrate hydrolysis by two approaches: (1) direct investigation of the effects of metal ions on the ribonuclease A activity utilizing the substrates, ribonucleic acid and cyclic 2′–3′-cytidine monophosphate; and (2) binding studies of both metal–enzyme and metal–substrate interactions. The assay indicates that metal activation is substrate dependent (activation with RNA, none with cyclic 2′–3′-CMP) while the binding studies show the strongest metal binding sites are the phosphate groups on tRNA. Such data indicate that metal activation of the hydrolysis is a substrate phenomenon occurring only when binding of the metal ion to the phosphate groups on the substrate is pronounced. In contrast, metal inhibition of the reaction appears to be an enzyme phenomenon occurring when metal concentrations are sufficient to bind to the active sites of the enzyme. Thus, in the case of the hydrolysis of ribonucleic acid by ribonuclease, metal ions first activate by binding to the phosphates on RNA; and then with increasing metal concentration, inhibit as the active sites of RNase A are blocked. With cyclic 2′–3′-CMP, no activation is observed since the metal binds weakly with the single phosphate; but inhibition is observed for both Cu^{2+} and Mn^{2+} ions. Manganese(II) concentrations necessary for inhibition are about 100 times larger than copper(II) concentrations as a result of similar variations in their binding constants to ribonuclease A.

The general inhibition of ribonuclease A by divalent metal ions is well established (Anfinsen and White, 1961; Crestfield *et al.*, 1963; Ukita *et al.*, 1964; Glitz and Dekker, 1964; Takahashi *et al.*, 1967; Eichhorn *et al.*, 1969) and has been ascribed to the binding of metal ions to the essential histidine-12 and -119. However, there are contradictions in the literature regarding which divalent metal ions inhibit and whether or not activation also occurs. The resolution of such contradictions is difficult because the varying substrates, metal ions, experimental conditions, and reactions used by the various authors make cross-correlation of their work impossible. To demonstrate this, we will abstract the results of two recent papers. Takahashi *et al.* (1967) demonstrated that Cu^{2+}, Zn^{2+}, and Hg^{2+} ions had an inhibiting effect on the ribonuclease A hydrolysis while Mg^{2+}, Ca^{2+}, and Mn^{2+} were found to have little effect. No activation was observed. The substrates used were cyclic 2′–3′-cytidine monophosphate (cyclic 2′–3′-CMP) and benzylcytidine 3′-phosphate, while the experimental conditions were pH 7.0, $\mu = 0.1$, $T = 37°$, and metal concentrations from 10^{-4} to 10^{-3} M. On the other hand, when RNA was used as the substrate, Eichhorn *et al.* (1969) demonstrated that metal concentrations of about 10^{-4} M activated the hydrolysis at pH 5. The metal ions used were Cu^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+},

* From the Department of Chemistry, Utah State University, Logan, Utah 84321. *Received February 17, 1970.* This work was supported by a National Science Foundation Grant (GP8255).

Co^{2+}, and Ni^{2+}. When the metal concentrations were increased, inhibiting effects were observed for all metals. Eichhorn *et al.* (1969) also found that whereas the metal concentration necessary to activate the reaction appeared independent of the ion used (about 10^{-4} M for all metal ions), the metal concentration necessary to inhibit hydrolysis was extremely metal dependent (varying from about 10^{-3} M for Cu^{2+} to 10^{-1} M for Mn^{2+}). Although the two papers appear to be in disagreement, it is difficult to compare when the substrates, metal concentrations, and experimental conditions differ.

Such distinctions in experimental results may also cause variations in proposed models of inhibition and activation. Takahashi *et al.* (1967) concluded that their results were in agreement with previous work ascribing the inhibition to metal binding at the essential histidine residues of the enzyme. Eichhorn *et al.* (1969) indicated that the latter was a possibility, but also indicated that inhibition could result from binding of the metal ions to the substrate. This is possible since binding of divalent metal ions to both DNA and RNA *bases* is known to result in a disruption of interbase hydrogen bonding and an uncoiling of the molecules (Eichhorn and Shin, 1968; Hiai, 1965). Eichhorn *et al.* (1969) also proposed that the activation of this enzyme–substrate reaction was most likely a result of metal interactions with the substrate since it is known that the binding of divalent metal ions to the *phosphate* regions can catalyze RNA hydrolysis even in the absence of RNase A (Eichhorn and Shin, 1968; Huff *et al.*, 1964).

We propose to delineate mechanisms for the metal inhibition and activation of this enzyme hydrolysis by a twofold approach. (1) We propose extending the work of Takahashi *et al.* (1967) and Eichhorn *et al.* (1969) so that the direct effect of the metal ion upon the activity of the enzyme can be determined for two different substrates (RNA and cyclic 2′–3′-CMP) with two different metal ions (Cu^{2+} and Mn^{2+}) under the same experimental conditions. (2) We propose studying the metal–enzyme and metal–substrate binding by pulsed nuclear magnetic resonance techniques to provide quantitative agreement for our models. Since various workers (Cohn *et al.*, 1969; Eichhorn and Shin, 1968; Eisenger *et al.*, 1965; Shulman *et al.*, 1965) have shown that the binding sites on ribonucleic acids have relatively the same affinity for metal ions, we will study the metal–tRNA interaction since tRNA is a more well-defined molecule.

Experimental Procedure

Materials. RNase A (type XII-A), tRNA (type III), and cyclic 2′–3′-CMP were purchased from Sigma Chemical Co. and used without further purification. All other chemicals were of Reagent grade quality. The water employed was glass distilled after passing through an ion-exchange column. Stock solutions of materials were prepared daily as needed. The ionic strength was maintained at $\mu = 0.16$ by addition of KCl, and the pH of the solutions was adjusted just prior to any measurements. Buffers were not added since the author was concerned that they could bind to the enzyme, substrate, or metal ion and thereby confuse the study.

Measurement of Hydrolysis Rates of Cyclic 2′–3′-CMP. A spectrophotometric method based on those described by Takahashi *et al.* (1967) and Eichhorn *et al.* (1969) was used.

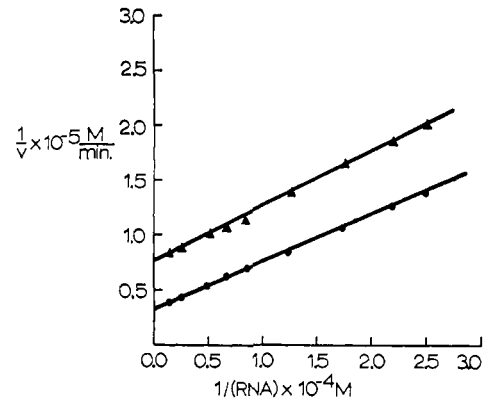

FIGURE 1: A plot of reciprocal rate ($1/v$) *vs.* reciprocal substrate concentration is shown for the metal inhibition of the RNase A–cyclic 2′,3′-CMP hydrolysis. The inhibition appears to be noncompetitive. (●) RNase A (2 μM)–substrate reaction; (▲) the same reaction with 3.0×10^{-4} M Cu^{2+} added.

The reaction was studied in a Beckman Model DU spectrophotometer thermostated at 25°. Silica cells of 1-cm light path were used. The assay solution in the cell consisted of substrate solution (final concentrations 0.15 and 1.5 M in nucleotide units), enzyme solution (final concentration 2 μM), metal solution (varying from 10^{-6} to 10^{-2} M Cu^{2+} and from 10^{-5} to 10^{-1} M Mn^{2+}), and sufficient KCl solution to make the final ionic strength, $\mu = 0.16$ (pH 5). Ribonuclease activity was determined by measuring the increase in optical density at 286 mμ after 10 min. Activity in the absence of metal was taken as 100%, and the activity in the presence of metal determined by the ratio of the increases in optical density for the metal and metal-free solutions. An analysis of the linearity of enzymatic activity with time indicated that the hydrolysis was not quite zeroth order when the concentration of substrate was 0.15 M (for higher concentrations the reaction was zeroth order). However, since the inhibition of the hydrolysis appeared to be by a noncompetitive mechanism (Figure 1), the variation in per cent inhibition with time was minimal. For 0.15 M RNA the per cent inhibition increased from 60 to 70%, respectively, for times increasing from 1 to 10 min.

Measurement of Hydrolysis Rates of RNA. The spectrophotometric method described above was used except that an equal volume of MacFadyen's reagent (2.5% trichloroacetic acid and 0.25% uranyl acetate) was added to the solution after 10-min incubation, and the absorbance of the soluble material was determined after 30 min at 25° and 260 mμ on the Beckman DU.

Magnetic Resonance Measurements. The proton relaxation rate of water was measured by the Carr and Purcell (1954) null method at 40 MHz and 24°. An NMR Specialties Model PS-60 pulsed nuclear magnetic resonance spectrometer was used in conjunction with a Varian magnet system (a V-3402 9″ magnet, a V-2608 power supply, and a V-3506 magnet flux stabilizer). The enhancement, ϵ^*, has been previously defined (Eisenger *et al.*, 1962; Joyce and Cohn, 1969) and is reproduced in eq 1, where the presence of the

$$\epsilon^* = \frac{(1/T_1^*) - (1/T_1^*_{(0)})}{(1/T_1) - (1/T_{1(0)})} \quad (1)$$

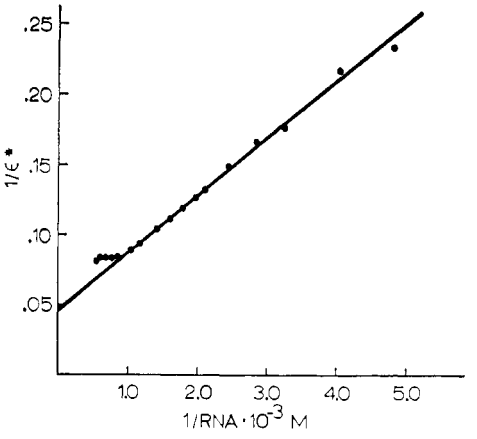

FIGURE 2: Type I titration plot of the Cu^{2+}–tRNA system, $1/\epsilon^*$ *vs.* 1/RNA is displayed. All solutions contained 9×10^{-5} M Cu^{2+}–0.16 M KCl, and were at pH 5.0. The variation in the line between 1/RNA = 0 and 1 ($\times 10^{-3}$ M) is most likely due to aggregation of the RNA at higher concentrations.

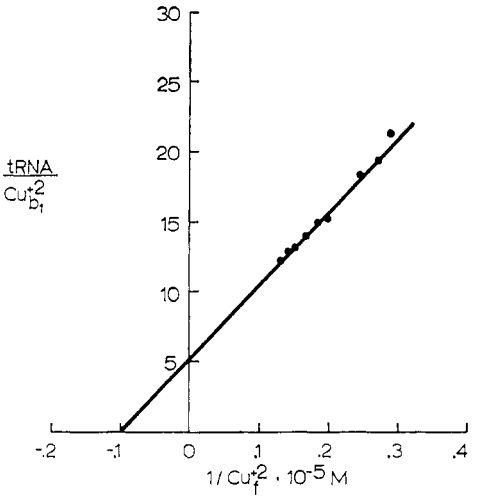

FIGURE 3: Hughes–Klotz (1956) plot of the data in Figure 2 is shown. A value of $\epsilon_{b1} = 19.5$ was used for this analysis. The graph predicts an average binding constant of $K_{A1} = 1.10^4$ M^{-1} with 16 sites total. The total number of sites for the tRNA molecule are determined by taking the reciprocal of ordinate intercept ($n = 0.20$) and multiplying by the number of nucleotide units in tRNA (about 80). (This is necessary since the tRNA concentration is expressed in nucleotide units.)

macromolecular ligand is indicated by an * and the absence of metal ion is indicated by (0). Enhancement data were obtained for two types of metal and ligand systems. In the type I titration (Joyce and Cohn, 1969), the metal concentration was held constant and the ligand concentration varied; in the type II titration, the ligand concentration was held constant and the metal concentration varied.

Analysis of Data. Since complete details for the analysis of enhancement data are reported in papers by Cohn and coworkers (Mildvan and Cohn, 1963; Joyce and Cohn, 1969), we will only outline the method used. The enhancement, ϵ^*, observed at any particular concentration of the system components is a linear combination of contributions from each species in the system as indicated in eq 2 (Mildvan and Cohn, 1963), where Me_f, Me_t, Me_{b1}, and Me_{b2}, respec-

$$\epsilon^* = \frac{(Me_f)}{(Me_t)}\epsilon_f + \frac{(Me_{b1})}{(Me_t)}\epsilon_{b1} + \frac{(Me_{b2})}{(Me_t)}\epsilon_{b2} \qquad (2)$$

tively, represent the free metal, total metal, bound metal in the primary site, and bound metal in the secondary site ($\epsilon_f \equiv 1$). From type I titrations, graphs of 1/[ligand] *vs.* $1/\epsilon^*$ can be prepared and a value of ϵ_{b1} can be obtained from an extrapolation to infinite ligand concentration (Figure 2). Next, limiting values of ϵ_{b1} and ϵ_{b2} can be obtained from extrapolation of type II graphs (Me_t *vs.* $1/\epsilon^*$) to zero metal concentrations.

Utilizing the value of ϵ_{b1} from the type I titration in conjunction with the data from the type II titration, a Hughes–Klotz graph (Hughes and Klotz, 1956) of ligand/(Me_{b1}) *vs.* $1/(Me_f)$ can be prepared (Figure 3). Extrapolations of the above graph to abscissa and ordinate intercepts produce values of, respectively, the negative of the binding constant for the primary site, $-K_{A1}$, and the reciprocal of the number of primary binding sites, $1/n_1$. If the system possesses secondary binding sites, one can also obtain the weight-average binding constant for the primary and secondary sites, K_{AVE}, as well as the total number of primary and secondary binding sites, n_t. Refinement of these data can be performed to obtain the binding constant for the secondary binding site, K_{A2}, as well as the number of secondary binding sites, n_2.

Experimental Results

Measurement of Hydrolysis Rates of Cyclic 2′-3′-CMP. Figure 4 displays the results of activity measurements for the RNase A hydrolysis of cyclic 2′-3′-CMP in the presence of Cu^{2+} and Mn^{2+}. There is apparently no activation of this reaction for metal concentrations from 10^{-6} to 10^{-1} M. On the other hand, inhibition is observed for both Cu^{2+} and Mn^{2+}; Cu^{2+} inhibition varies from 0 to 100% between 10^{-5} and 10^{-3} M Cu^{2+}, while Mn^{2+} inhibition varies from 0 to 100% between 10^{-3} and 10^{-1} M Mn^{2+}. Nevertheless, there is no disagreement between these results and those of Takahashi *et al.* (1967). For Cu^{2+} and Mn^{2+} concentrations which varied from 10^{-4} to 10^{-3} M, Takahashi *et al.* (1967) observed no activation for either metal ion, inhibition for only Cu^{2+}, and very little effect for Mn^{2+}. Between these concentration ranges, the results are in agreement with the present work. Note again that Mn^{2+} inhibition is only observed at Mn^{2+} concentrations greater than 10^{-3} M (Figure 4).

Measurement of Hydrolysis Rates of RNA. Figure 5 reproduces the results of activity measurements for the RNase A hydrolysis of RNA in the presence of Cu^{2+} and Mn^{2+}. The results are duplications of the work by Eichhorn *et al.* (1969). Figure 5 has been included, therefore, only for reference purposes. As in the work by Eichhorn *et al.* (1969), the activation by both Cu^{2+} and Mn^{2+} is initiated at the same concentration level (10^{-4} M) with the same initial slopes for both metal ions. Again, however, the concentration levels of the two ions producing inhibition vary markedly ($\approx 10^{-3}$ M for Cu^{2+} compared with $\approx 10^{-1}$ M for Mn^{2+}). It is these differences in metal concentration levels producing inhibition which account for Mn^{2+} activation reaching

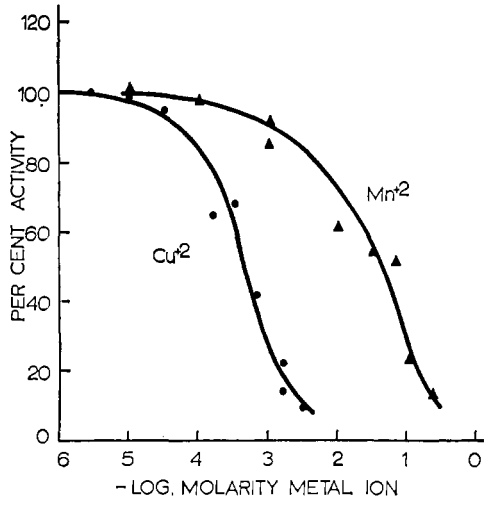

FIGURE 4: The effect of metal ions on the activity of RNase A (2 μM) is shown. Cyclic 2′–3′-CMP (0.15 mM) was treated with chlorides of Cu^{2+}, ●; and Mn^{2+}, ▲. After treatment with the enzyme at pH 5.0 (μ = 0.16), activities were measured as ratios of increases in absorbance (with and without metal ions present, respectively). Studies at 1.5 mM substrate did not differ from this graph.

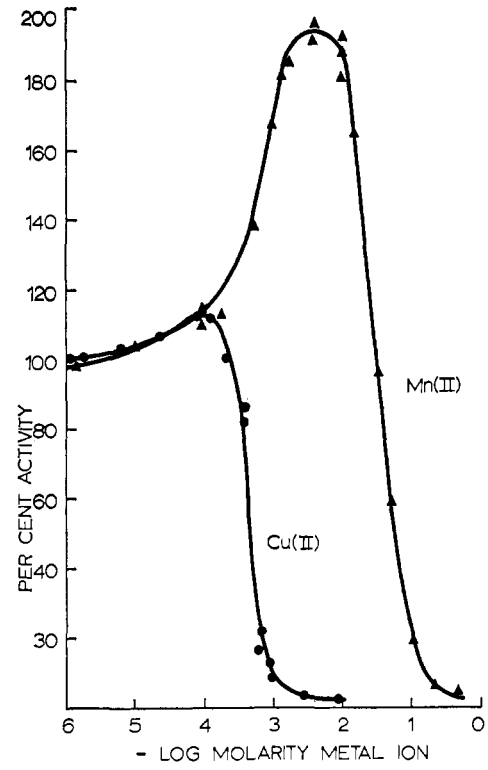

FIGURE 5: The effect of metal ions on the activity of RNase A (2 μM) is depicted. Ribonucleic acid (1.5 mM) was treated with chlorides of Cu^{2+}, ●; and Mn^{2+}, ▲. After treatment with the enzyme at pH 5.0 (μ = 0.16), activities were measured by the absorbance ratios of the solution left after precipitation by uranyl trichloroacetate (with and without metal ions present, respectively). The concentration of RNA is expressed in nucleotide units.

190% while Cu^{2+} activation reaches only 115%. In terms of the double-site analysis of Eichhorn *et al.* (1969), the Cu^{2+} inhibiting site inactivates the reaction before activation can increase. Mn^{2+}, on the other hand, does not inhibit until concentration levels at which activation is pronounced. It can also be observed that the metal concentrations causing total inhibition are almost identical for the two substrates in Figures 4 and 5.

Measurements of Metal–Ligand Binding Interactions. Table I contains the results of pulsed nuclear magnetic resonance measurements of interactions between the metal ions, Mn^{2+} and Cu^{2+}, and the ligands, RNase A, and tRNA. It was impossible, by pulsed nuclear magnetic resonance techniques, to study the metal interactions with cyclic 2′–3′-CMP. Either the interaction is too weak, or the value of ϵ_{b1} is too small. It can be observed in Table I that the binding constants of Cu^{2+} and Mn^{2+} to tRNA are of comparable magnitudes and are larger than those of Cu^{2+} and Mn^{2+} to RNase A. In the case of Cu^{2+}, the binding constant for the tRNA complex is seven times larger than that for the RNase A complex (K_{A1} = 1500 for Cu^{2+}–RNase A compared to K_{A1} = 10,000 for Cu^{2+}–tRNA at pH 5); while in the case of Mn^{2+}, the binding constants for the two complexes differ by a factor of 150 (K_{A1} = 60 for Mn^{2+}–RNase A compared to K_{A1} = 9000 for Mn^{2+}–tRNA at pH 5). Note that all equilibrium constants increase with increasing pH.

Of lesser interest is the fact that there is only one primary site for the Mn^{2+} and Cu^{2+} interaction with RNase A, while there are, respectively, 6 and 16 primary sites for the Mn^{2+}– and Cu^{2+}–tRNA interactions (Table I). The latter data agree with qualitative work which demonstrated that Mn^{2+} binds predominately to nucleic acid phosphate groups while Cu^{2+} binds about *equally* well to both the nucleic acid phosphate and base groups (Cohn and Hughes, 1962; Shulman *et al.*, 1965; Eichhorn *et al.*, 1966; Eichhorn and Shin, 1968). The upward variation in the graph of $1/\epsilon^*$ *vs.* 1/tRNA (Figure 2) for the Cu^{2+}–tRNA system is most likely due to aggregation of the tRNA at higher substrate concentrations. It has been assumed that the 16 binding sites for the Cu^{2+}–tRNA system incorporate both the phosphate and base binding sites for Cu^{2+}, and that the binding constants are too similar to allow separate analysis. The secondary binding sites for the Mn^{2+}–RNase A and Cu^{2+}–tRNA systems could not be studied because, respectively, the binding was too weak and the higher Cu^{2+} concentrations aggregated the tRNA.

The final point of interest is the enhancement values. The values of ϵ_{b1} for the Cu^{2+} and Mn^{2+} interactions with tRNA are of similar magnitudes and are larger than the comparable ϵ_{b1} values for the Cu^{2+} and Mn^{2+} interactions with RNase A. The increased magnitude of ϵ_{b1} for the tRNA ligand most likely results from binding in the more restrictive double-stranded regions of the molecule (Cohn *et al.*, 1969). Finally, it is of interest to note the extreme change in the ϵ_{b1} value of the Mn^{2+}–RNase A system with pH. Breslow and Girotti (1966) observed that with increasing pH, Cu^{2+} binds to two, three, and finally four ligands (at pH 5–6, two ligands; at pH >6.3, three ligands; and at pH >8.0, four ligands). Eisenger *et al.* (1962) stated that the value of ϵ was a product of the number of exchanging water ligands in the metal coordination sphere and the rotational correlation time of the complex. Assuming that Mn^{2+} bonds as Cu^{2+} does and further assuming that the rotational correlation time remains relatively constant, the ratios of ϵ_{b1} for

TABLE I: Binding Interaction Studies of Metal–Ligand Systems.

System	Probable Site	pH	Titration	Ligand[a] Concn (mM)	Metal Concn (mM)	ϵ_{b1}	K_{A1} (M^{-1})	n_1[b]	K_{A2} (M^{-1})	n_2[b]
Cu^{2+}–RNase A[c]	(His, His, His, His)[d]	5.1	I	0.39–2.05	0.105	6.1	1,500			
		4.8	II	2.05	0.10–15.2			1	125	2
		5.8	I	0.20–1.25	0.06	5.8	17,000			
		5.7	II	1.25	0.06–8.0			1	1600	2
Mn^{2+}–RNase A	(His, His, His, His)[e]	5.0	I	0.8–5.0	0.025	14.1	60	1		
		6.0	I	0.8–5.0	0.025	14.5	120	1		
		7.0	I	0.8–5.0	0.025	9.9	170	1		
		8.0	I	0.8–5.0	0.025	5.6	230	1		
Cu^{2+}–tRNA	(P and base)[f]	5.0	I	0.20–2.5	0.09	19.5	10,000	16		
Mn^{2+}–tRNA	(P, P)[h]	5.0	I	0.20–2.5	0.025	18.2	9,000	4		
		5.0	II	0.50	0.025–1.0					
Mn^{2+}–tRNA[g]	(P, P)[h]	7.2	I	0.1–10.0	0.1	14.5	29,000			
		7.2	II	3.7	0.1–10.0			11	1500	6
Mn^{2+}–RNA[i]	(P, P)	7.2	I	0.1–10.0	0.1	14.3	25,000			
		7.2	II	0.58	0.05–2.0				1000	
Mn^{2+}–tRNA[j]	(P, P)	7.5	I	0.03–3.0	0.1	19.2	30,000			
		7.5	II	2.6	0.05–3.0			6		13

[a] The tRNA concentrations are in nucleotide units. [b] The number of sites is the total for the molecule (*i.e.* for tRNA, not per nucleotide unit). [c] Work on this system was done by Joyce and Cohn (1969) at pH 5.0, and by the author (1969) at pH 5.8. [d] The binding sites for the Cu^{2+}–RNase A system have been suggested to be the histidine residues (Breslow and Girotti, 1966). The primary site was tentatively identified as the active site residue, histidine-12 (Joyce and Cohn, 1969). [e] We have assumed the primary site for all metal ions–RNase A systems to be the active site histidine residue. [f] The binding sites of RNA have been identified as the phosphate and base groups, Cu^{2+} binding about *equally* well to both (Eichhorn and Shin, 1968). [g] Work done by Eisinger *et al.* (1965). [h] The strongest binding sites for Mn^{2+} to RNA are the phosphate groups (Eichhorn and Shin, 1968; Shulman *et al.*, 1965). [i] Work done by Eisenger *et al.* (1965). This is the same work as in footnote *g*. It has been included only to indicate that the binding constants etc. are the same for binding to tRNA as to RNA. [j] Work done by Cohn *et al.* (1969).

the Mn^{2+}–RNase A system at pH's of 6, 7, and 8 would be respectively, 1:0.75:0.5, corresponding to 4, 3, and 2 water ligands remaining bound. Thus, considering only changes in the number of bound water ligands, we predict the values of ϵ_{b1} at pH's 7 and 8 to be, respectively, 10.6 and 7.1 (based on ϵ_{b1} = 14.2 at pH 6). Since the predicted values agree reasonably well with the experimental values (Table I), it would appear that the rotational correlation time is relatively pH invariant for this complex.

Discussion

The bivalent action of metal ions on the RNase A–RNA hydrolysis (Figure 5) is indicative of a multisite binding phenomenon (Eichhorn *et al.*, 1969). For this reason, we will separate our discussion of proposed models into subsections on activation and inhibition.

Activation. The data in Figures 4 and 5 and in Table I demonstrate that the metal activation of the RNase A–RNA hydrolysis results from the binding of metal ions to the phosphate regions of the substrate, RNA. To begin the defense of this proposed model, it is noted that metal activation of the reaction is substrate dependent. Thus, whereas hydrolysis of RNA is enhanced by the presence of Mn^{2+} and Cu^{2+} at low concentrations (10^{-4} M), the hydrolysi of cyclic 2′-3′-CMP is unaffected at similar Cu^{2+} and Mn^{2+} concentrations (Figures 4 and 5). Such evidence indicates that activation results from metal–RNA interactions. Binding studies (Table I) further suggest that the metal–RNA complex is responsible for metal activation. The binding constants (Table I) for the primary metal–tRNA interactions (K_{A1} = 10,000 for Cu^{2+}–tRNA at pH 5, K_{A1} = 9000 for Mn^{2+}–tRNA at pH 5) are larger than the binding constants for the corresponding primary metal–RNase A interactions (K_{A1} = 1500 for Cu^{2+}–RNase A at pH 5, K_{A1} = 60 for Mn^{2+}–RNase A at pH 5). Since metal activation occurs before inhibition (Figure 5) the stronger metal–RNA sites would appear to be responsible for activation (see Appendix for quantitative comparison). When it is noted that both Mn^{2+} and Cu^{2+} can catalyze hydrolysis of RNA without the presence of RNase A (Huff *et al.*, 1964; Eichhorn, 1966; Eichhorn and Shin, 1968), the evidence seems conclusive that the metal–RNA complex is the origin of metal activation. However, one more point in proof can be added. The binding constants for the primary metal–tRNA interactions (Table I) are of comparable magnitudes (K_{A1} = 10,000 for Cu^{2+}–tRNA at pH 5, K_{A1} = 9000 for Mn^{2+}–tRNA at pH 5), whereas the binding constants for the primary metal–RNase A interactions differ

TABLE II: Predicted Metal Ion Effects Compared to Observed.

System	Metal Concn (M)	% RNA[a] Bound	% RNase A[b] Bound	Total %[c] Effect Predicted	Actual %[d] Effect Obsd
Cu^{2+}–RNase A–cyclic 2′–3′-CMP	10^{-6}		0.15	100	100
	10^{-5}		1.3	98.7	98
	10^{-4}		13	87	84
	10^{-3}		60	40	26
	10^{-2}		94	6	5
Mn^{2+}–RNase A–cyclic 2′–3′-CMP	10^{-5}		0.06	100	102
	10^{-4}		0.6	99.4	98
	10^{-3}		6.0	94	92
	10^{-2}		37	63	70
	10^{-1}		86	14	22
Cu^{2+}–RNase A–RNA	10^{-6}	0.4	0.06	101	100
	10^{-5}	4.0	0.6	103	104
	10^{-4}	33	7.5	123	115
	10^{-3}	90	58.0	79	20
	10^{-2}	99	94	12	3
Mn^{2+}–RNase A–RNA	10^{-5}	4	0.02	104	104
	10^{-4}	33	0.3	133	116
	10^{-3}	90	5.2	182	170
	10^{-2}	99	37	131	178
	10^{-1}	100	86	28	22

[a] In determining the per cent RNA bound, the total metal ion concentration was used. The amount used in binding to RNase A (2 μM) was negligible compared to the amount binding to RNA (1.5 mM). [b] In determining the per cent RNase A bound, the metal ion concentration bound to RNA was subtracted from the total metal ion concentration to give a new total metal ion concentration available for RNase A binding. [c] Calculated as outlined in Appendix text. [d] From Figures 4 and 5.

by factors of about 25 (K_{A1} = 1500 for Cu^{2+}–RNase A at pH 5, K_{A1} = 60 for Mn^{2+}–RNase A at pH 5). From Figure 5, it can be noted that the initial slopes of activation for Mn^{2+} and Cu^{2+} are essentially identical (see also Eichhorn *et al.*, 1969), indicating that the binding constants for the metal ligand sites responsible for activation should be of comparable magnitude (as they are for the primary metal–tRNA binding sites).

The identification of the metal–RNA binding sites depends upon previous work. Shulman *et al.* (1965) demonstrated that Mn^{2+} binds 100% of the time to the phosphate sites (see also Eichhorn and Shin, 1968). Eichhorn and Shin (1968) qualitatively showed that Cu^{2+} binds *equally* well to both the phosphate and base sites on RNA. Work by Eichhorn and Clark (1965), however, stated that low concentrations of Cu^{2+} stabilized RNA and DNA (phosphate binding), while higher Cu^{2+} concentrations destabilized and unwound the compounds (base binding). If anything, the previous data seem to indicate that the Cu^{2+} phosphate sites of RNA are stronger than the base sites, thereby strongly suggesting the metal–RNA activating sites as the phosphate groups (as in Table I). The lack of metal activation when the substrate cyclic 2′–3′-CMP is used can then be attributed to a much reduced binding constant between the metal ions and the single phosphate of the cyclic nucleotide. Such a decrease in K_A is expected since divalent metal ions normally bind to two phosphate groups on nucleic acids and nucleotides (Cohn and Hughes, 1962; Eisenger *et al.*, 1961, 1962; Shulman *et al.*, 1965).

Inhibition. The data in Figures 4 and 5 and Table I indicate that the general metal inhibition of RNase A–substrate hydrolysis results from the binding of metal ions to the active-site hystidine residues of the enzyme. To initiate defense of this model, we point out that the metal inhibition of the reactions is substrate independent (Figures 4 and 5; Takahashi *et al.*, 1967; Eichhorn *et al.*, 1969). Thus, RNase A hydrolysis is inhibited by metal concentrations above 10^{-3} M whether the substrate is a general mixture of RNA (Eichhorn *et al.*, 1969; Figure 5), cyclic 2′–3′-CMP (Figure 4; Takahashi *et al.*, 1969), or benzylcytidine 3′-phosphate (Takahashi *et al.*, 1969). Such evidence seems to indicate that inhibition arises from metal–enzyme interactions. Binding studies (Table I) also indicate that the metal–RNase A complex is the origin of metal inhibition. Since the histidine residues at the active site are only one of several possible weaker binding sites in this metal–substrate–enzyme system, one has no *qualitative* reason for selecting it. However, there is *quantitative* agreement (Table II in Appendix) between metal concentrations necessary for inhibition as predicted by the binding constants for the histidine sites (Table I) and those concentrations as observed in Figures 4 and 5. Such agreement provides strong evidence that the active-site histidine residues are the inhibiting sites. Of special interest is the way in which the variation in binding constants (K_{A1} = 1500 for Cu^{2+}–RNase A at pH 5, K_{A1} = 60 for Mn^{2+}–RNase at pH 5) explain the 100-fold variation between Cu^{2+} (10^{-1} M) and Mn^{2+} (10^{-3} M)

concentration levels producing inhibition (Figures 4 and 5).

Note that the work of Breslow and Girotti (1966), Joyce and Cohn (1969), and Alger (1969) strongly suggest that the primary metal–enzyme binding sites are the active-site histidine residues.

Although the above arguments demonstrate that the general metal inhibition of RNase A hydrolysis is substrate independent, it does not preclude the fact that additional inhibition can occur from metal–substrate binding (additional to the general metal–enzyme inhibition). Such inhibition could result from the uncoiling of substrate bipolymers (RNA, tRNA) by metal binding which disrupts the normal interbase hydrogen bonding of the nucleic acids. Eichhorn and Clark (1965), and Eichhorn and Shin (1968) have shown that denaturation occurs in metal–DNA solutions, while Hiai (1965) demonstrated that hyperchromicity is displayed at room temperature for Cu^{2+}–tRNA solution. If such unwinding results in inhibition, one would again expect a 100-fold variation between Cu^{2+} and Mn^{2+} effects, since uncoiling results from metal–base binding on the substrates (a 100-fold variation in the Cu^{2+}- and Mn^{2+}-base binding constants is expected). Such inhibition should not occur, however, for metal ions binding to single nucleotides such as cyclic 2′–3′-CMP because its tertiary structure in solution is not dependent upon hydrogen bonding between bases in neighboring nucleotides. Nevertheless, the observation of such effects is extremely difficult due to the necessity of separating them from the general metal–enzyme inhibition, and we have chosen not to attempt to assign quantitative values to their magnitudes. However, the *predicted* data in Table II (Appendix) demonstrate that metal–RNase A–RNA systems should not be inhibited as much as are metal–RNase A–cyclic 2′–3′-CMP systems (for comparable metal ion concentrations), unless an additional inhibition mechanism is proposed. Since the *observed* data in Figures 4 and 5 show comparable levels of inhibition for both systems (at comparable high metal ion concentrations), this could be interpreted as evidence of an additional inhibition mechanism for the RNA system. The inhibition, of course, results from disruption of the RNA structure by metal ions.

Appendix

In order not to disrupt the text, this section contains the quantitative agreement between the metal concentration levels necessary for activation and inhibition as computed from the binding constants of Table I and the data from Figures 4 and 5. Activation only occurs for RNA, and thus only the Cu^{2+}- and Mn^{2+}–tRNA binding interactions are included in Table II. A value of $K_{A1} = 1 \times 10^4$ was used for both interactions at pH 5. Inhibition is enzyme dependent; therefore, only the Cu^{2+}- and Mn^{2+}–RNase A interactions are included in Table II. Values of $K_{A1} = 1.5 \times 10^3$ and $K_{A1} = 6.0 \times 10^1$ were used for the Cu^{2+}–RNase A and Mn^{2+}–RNase A interactions, respectively, at pH 5. The general formula used to determine the amounts of free and bound metal in the metal–ligand systems was

$$K_{A1} = \frac{(Me_{b1})}{(Me_f)(nL - (Me_{b1}))}$$

where Me_{b1}, Me_f, n, and L are, respectively, the amount of metal bound in the primary sites, the amount of free metal, the number of primary binding sites, and the total ligand concentration. The per cent of RNA bound and RNase A bound calculated in Table II was then based on the ratios of the metal–ligand complex to the total amount of ligand available. The calculated values of free and bound ligand are based upon concentration levels in Figures 4 and 5 (RNase A, 2 μM; RNA, 1.5 mM in nucleotide units, and metal ion concentrations varying as in Figures 4 and 5). The total per cent effect predicted was calculated by the following procedure. (1) The number of available RNase A molecules (per cent unbound) was determined from the per cent RNase A bound (100 − per cent RNase bound). (2) Of the available (per cent unbound) RNase A molecules, the number which could bind to activated (metal–RNA complex) and normal (no metal bound) RNA sites was determined by multiplying, the per cent RNase unbound by, respectively, the per cent RNA bound and the per cent RNA unbound. (3) The total per cent effect is then found by multiplying 1 times the normal number of sites for RNase A, and 2 times the activated number of sites for RNase A; and summing the results (from the results of Eichhorn *et al.* (1969), and Figure 5, it appears that the activated rate is about 2 times the normal rate). For example, let us examine the Cu^{2+}–RNase A–RNA system for 10^{-4} M. The per cent unbound RNase is (100 − 7.5) = 92.5%. Of these 92.5% unbound RNase A molecules, (0.33) (92.5%) = 30.5% can bind to sites on RNA that have bound Cu^{2+}, while (0.67) (92.5%) = 62.0% can bind to sites on RNA without bound Cu^{2+}. Finally, the total activity is equal to (1)(62.0%) + (2)(30.5%) = 123%.

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Spin-Labeling Studies of Aspartate Transcarbamylase. I. Effects of Nucleotide Binding and Subunit Separation*

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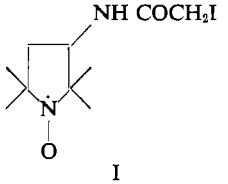
ABSTRACT: Aspartate transcarbamylase has been reacted with the spin-labeling reagent 4-α-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl and the effects of ligand binding, pH, and separation into subunits on the electron spin resonance spectrum of the labeled protein have been investigated. CTP and ATP binding cause broadening of the electron spin resonance spectrum while UTP and succinate (a substrate analog) and carbamyl phosphate have no effect. Increasing pH and decomposition into subunits by *p*-hydroxymercuribenzoate result in narrowing of the spectrum. It was also found that the course of the labeling reaction was affected by the presence of ligands. The conformational change measured by changes in the labeled protein electron spin resonance in the presence of CTP did not correlate well with the predictions of the simple allosteric models for heterotropic effects.

The spin-labeling technique, in which conformation changes in biopolymers are followed by means of changes in the electron spin resonance spectrum of a bound free-radical label, has been shown by McConnell and coworkers to be very useful in studying the allosteric transitions accompanying oxygen binding in hemoglobin (Ogawa and McConnell, 1967; McConnell and Hamilton, 1968; McConnell *et al.*, 1969). In these investigations the protein was reacted with the reagent *N*-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I) and a change in the electron spin resonance spectrum of the label bound at the β93 cysteine was observed which correlated with the degree of oxygenation in agreement with the predictions of the sequential model for allosteric transitions of Koshland *et al.* (1966) (referred to as KNF in this paper).



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The aim of the present work is to use this technique to study the binding of allosteric effectors in a more complex system which exhibits heterotropic as well as homotropic effects. An ideal protein for such a study is the regulatory enzyme aspartate transcarbamylase (ATCase) from *Escherichia coli* which exhibits all of the general features of allosterism, including cooperativity in substrate binding, and inhibition and activation through binding at sites distinct from the catalytic site. With the exception of hemoglobin it is the most extensively studied protein in regard to its allosteric properties and is available in gram quantities from a special strain of *Escherichia coli* by a relatively simple isolation procedure (Gerhart and Holoubek, 1967).

ATCase is the first enzyme concerned only with pyrimidine biosynthesis and is subject to feedback inhibition by the end product cytidine triphosphate (CTP), as a means of turning off synthesis of intermediates when levels of the end product are sufficiently high in the cell. It is activated in a manner analogous to the CTP inhibition by adenosine triphosphate (ATP). This has been suggested to be of importance in maintaining the balance between the supply of purines and pyrimidines in the nucleic acid synthesis pool (Gerhart and Pardee, 1962). Also binding of the substrates aspartate and carbamyl phosphate (CAP)[2] is cooperative through interactions between catalytic sites on different subunits.

Considerable progress has been made toward understanding the structure and mechanism of catalysis by ATCase in recent years. Gerhart and Schachman (1965) have shown that the enzyme can be separated by heat or mercurials into 2 distinct types of subunits, one of which binds only the substrates and is fully catalytically active (catalytic C) and another that has no activity and binds the inhibitor

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[2] Abbreviations used are: CAP, carbamyl phosphate, PMB, *p*-hydroxymercuribenzoate.