The Interaction of Metal Ions with Polynucleotides and Related Compounds

XIII. THE EFFECT OF METAL IONS ON THE ENZYMATIC DEGRADATION OF RIBONUCLEIC ACID BY BOVINE PANCREATIC RIBONUCLEASE AND OF DEOXYRIBONUCLEIC ACID BY BOVINE PANCREATIC DEOXYRIBONUCLEASE I

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SUMMARY

The activity of ribonuclease increases with increasing concentration of added divalent metal ions until the attainment of an optimal metal concentration, beyond which the addition of further metal ions inhibits the reaction. When either the optimal metal concentration or the enzymatic activity at that concentration is plotted versus the atomic number of the transition metals, the resulting curves can be correlated with the stability constants of complexes of the metals. These results are in line with metal ions reacting at two types of sites, one activating and the other inhibiting. Crystalline bovine pancreatic ribonuclease is affected in the same way as ribonuclease A. The activation of deoxyribonuclease I has been reinvestigated. Metal ions generally activate this enzyme, but the relative activating ability of the various metal ions is not readily correlated with complexing ability, as in the case of ribonuclease. Co(II) activates deoxyribonuclease I better than the metals that have generally been used for this purpose.

In a search for specific cleavage sites that can be used for nucleotide sequence determination, we have been interested in the use of metal ions in the depolymerization of polynucleotides (1-3). The specificities of nuclease enzymes have been under investigation in many laboratories for similar purposes. It appears reasonable that relatively nonspecific enzymes can be made more specific in the presence of metal ions that have preferential base-binding capabilities.

Before such a hypothesis can be tested, it is necessary to examine the effect of metal ions on the activities of the enzymes. The present work represents a systematic investigation of this simple phenomenon, which has turned up some surprises. Ribonucleases, including the well known bovine pancreatic ribonuclease (EC 2.7.7.16), are known to be inhibited by metal ions (4-17), but previous studies have provided little evidence that activation can also occur, although there have been isolated reports of such activation (18-21). Our results indicate that such activation is generally observed, under appropriate conditions, and, perhaps even more surprisingly, the relative activation effects of different metals follow a logical sequence that is rarely found in metal enzyme activation. Bovine pancreatic deoxyribonuclease I (EC 3.1.4.5) is well known as a metal-activated enzyme (22-26); but, although a sophisticated study of metal involvement was recently conducted (26), no systematic comparison of the effects of various metal ions, especially the transition metal ions, seems to have been previously carried out. The results of the present investigation show that the activity of this enzyme can be considerably enhanced by the use of activators other than those commonly used.

RESULTS AND DISCUSSION

Ribonuclease—Bovine pancreatic ribonuclease (4, 12, 27-29) can be obtained in a crystalline form which can be chromatographed into a number of fractions (30-34), of which ribonuclease A is the most widely studied and the best characterized (27). We have determined the effect of the various first transition metal ions, as well as of magnesium, on the activity of the enzyme (Fig. 1A). We have also studied the effect of copper(II) and zinc(II) on the activity of ribonuclease A (Fig. 1B). It is immediately apparent that the effect of magnesium on ribonuclease A is the same as on the mixed crystal. This similarity in the behavior of the two preparations is perhaps not unexpected in view of the similarities in the primary structures of ribonuclease A and B (35), for example, and lends some support to the possibility that the ribonuclease fractions contained in the mixed crystal are virtually conformational isomers; the presence of the metals...
pancreatic ribonuclease, five times recrystallized, and ribonuclease A. RNA, 2 \times 10^{-5} \text{ M (P)}, was treated with the nitrates of Co(II), Mn(II), Ni(II); Cu(II); Zn(II); and \Delta, Mg(II). After treatment with the enzyme at pH 5.0 (0.05 M acetate buffer), activities were measured by the absorbance of the solution left after precipitation with uranyl trichloracetate, and the activities were compared to the activity obtained in the absence of divalent metals, the latter taken as 100\% activity. (The concentration of polynucleotide is expressed as the concentration of phosphate present in the sample.)

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FIG. 1. The effect of metal ions on the activity of A, bovine pancreatic ribonuclease, five times recrystallized, and B, ribonuclease A. RNA, 2 \times 10^{-5} \text{ M (P)}, was treated with the nitrates of Co(II), Mn(II), Ni(II); Cu(II); Zn(II); and \Delta, Mg(II). After treatment with the enzyme at pH 5.0 (0.05 M acetate buffer), activities were measured by the absorbance of the solution left after precipitation with uranyl trichloracetate, and the activities were compared to the activity obtained in the absence of divalent metals, the latter taken as 100\% activity. (The concentration of polynucleotide is expressed as the concentration of phosphate present in the sample.)

FIG. 2. The dependence of ribonuclease activity upon the presence of divalent metal ions from the first transition series. (The omission of Fe(II) has been noted by a blank space.) Curve A, metal ion concentration at which maximum activity is attained; Curve B, activity at the metal concentration producing maximum activity. Points are obtained from Fig. 1.

may indeed induce a common conformation on all of the components of the mixture.

It is apparent that all of the metal ions studied, with the exception of copper, considerably increase the activity of the enzyme at appropriate metal ion concentrations, and that even copper ions produce a slight activation at low copper concentration.

All of the metal ions have little effect on enzyme activity at 10^{-2} \text{ M} concentration. With increasing metal concentration, activation occurs. All metal ions produce maximal activation at some optimal metal ion concentration, above which the extent of activation decreases, and eventually, with high enough metal concentrations, inhibition occurs until finally the enzyme is rendered completely inactive.

Thus, all of the metal ions produce similar curves. The differences between the behavior of these metals are graphically illustrated in Fig. 2. Curve A of this figure reveals that the concentration of metal ion required for maximum activity decreases from manganese to copper (going from left to right in the first transition series) and then increases again between copper and zinc. A similar correlation is obtained by plotting (Fig. 2, Curve B) the actual extent of the activation at the concentration of maximal activation. Both of these curves are in line with the well known Irving-Williams series for the stability of complex compounds. The stabilities of the metal complexes fall in the order: Mn < Co < Ni < Cu > Zn (36–38).

The shape of the curves in Fig. 1 can be explained very simply. One type of metal-binding site brings about activation when occupied by the metal. At low metal ion concentrations this site is bound. As the metal ion concentration is increased, some metal ions also lodge on a second type of site where they produce an inhibitory effect. The activity presumably rises with the addition of the initial increments of metal ions that bind primarily to the activating site, then levels off as an appreciable number of metal ions bind to the inhibiting site, and finally decreases when the inhibiting metal binding becomes the predominating influence.

The correlation of Fig. 2 Curve A with the Irving-Williams series (37) is also readily explained. Copper(II), the metal with the strongest ligand affinity, could be expected to activate at the lowest concentration by reacting most readily with the activating site. It could also be expected to inhibit at quite low concentration by reacting most readily with the inhibiting site. Logically, the weakest complexing metal, manganese, would require higher concentrations to react with both activating and inhibiting sites (39, 40). Actually, the differences between the metal ions are explained primarily by differences in inhibition, as inspection of Fig. 1 indicates.

As logical as this correlation appears to be, it is rarely found in enzymatic processes; in fact, we are unaware of any other case in which the metal ion activation or inhibition of an enzyme correlates so well with metal complex stability. The DNAse activation discussed below does not fall in line in this way. The correlation is, however, a common one for nonenzymatic metal catalyses and has been explained as above in such reactions (39, 40).

The correlation of Fig. 2 Curve B with the Irving-Williams series is perhaps more surprising than that of Fig. 2, Curve A. There is no a priori reason for anticipating that the extent of activation, a kinetic phenomenon, should be related to a thermodynamic phenomenon. The apparent reason for the trend appears to be that the activating ability of all the metals is quite similar at concentrations lower than those at which inhibition becomes dominant. The metals differ primarily in the inhibition effects. Thus, manganese is able to produce much higher activation than copper since the former inhibits at much higher concentration than the latter.

The significance of the correlation of the influence of metals on this particular enzymatic reaction with the stability of the metal complexes is probably that activation occurs by reaction of the metal with one type of site and that inhibition occurs by
reaction of the metal with one other type of site. Multiple activation and inhibition sites are quite possible, but if there are multiple sites, they must be identical or very similar. If they were dissimilar, such a family of curves could not be anticipated.

The question may arise whether the correlations depicted in Fig. 2 are coincident with a selected set of experimental conditions and therefore of no fundamental significance. For this reason we carried out another set of experiments with the transition elements, this time with only half the concentration of substrate as in Fig. 1. The results are shown in Fig. 3. It can be observed that the decrease in substrate concentration decreases the absolute values of the activities, but has no effect upon the shapes of the curves or upon the positions of the curves for the various metals relative to each other. If the results of Fig. 3 are plotted as in Fig. 2, similar curves are obtained. The Irving-Williams correlation is therefore not an artefact arising from a choice of experimental conditions, but must be considered a characteristic of the metal activation and inhibition of ribonuclease.

Although the present data indicate that one type of activating site and one type of inhibiting site can account for the observed metal ion effects, no conclusions about the nature of these sites can be drawn from them. The interaction of metal ions with both the enzyme and the substrate have, however, been thoroughly studied elsewhere.

The primary structure of ribonuclease A has been known for some time (27-29), and its tertiary structure has been to some extent elucidated crystallographically (41). This information on the structure of ribonuclease A has been used in turn in the study of copper(II) and zinc(II) binding to the enzyme in an attempt to understand the mechanism of metal ion inhibition (42-44). It is of interest to attempt to correlate our results with these metal binding studies.

All of the binding studies point to the existence of more than one binding site, although the number and nature of the sites do not appear to have been conclusively delineated. Spectrophotometric and titration studies have implicated four similar sites, probably the four histidine residues and adjacent chelating peptide groups (42). Chemical reactions of the histidine residues are suppressed in the presence of the metals, thus, likewise implicating histidine interaction (43). Copper(II) ions may also bind to a fifth "stronger" site, postulated to be the terminal amino group of the protein chain (44).

If the metal activation and inhibition of ribonuclease proceeded by binding to the enzyme, we would suppose that metal ions on the terminal amino group would have no effect, since the amino-terminal lysine can be cleaved from the molecule (45) or deaminated (46) or acetylated (47) with little effect on the activity of the enzyme. Two of the histidine residues, 12 and 119, are apparently active (41, 48, 49); whereas the other two, 48 and 105, are not. We could postulate that metals binding to one of the two groups of sites will activate, while binding to the other will inhibit.

The fact that the concentrations of metal ions that produce the effects noted here are very high, of the order of magnitude of the concentration of substrate, suggests the likelihood, nevertheless, that the activation, at least, is due to binding to substrate, rather than to the enzyme.

Metal ions bind to two types of sites on nucleic acid molecules, the phosphates and the electron donor atoms on the bases (50, 51). We have recently obtained evidence that the differences in the effects of various metal ions on the conformation of DNA can be explained by differences in the relative affinities of metal ions for the phosphate and base sites. Low concentrations of metal ions favor phosphate binding, while higher concentrations favor base binding. The stabilities of the metal-phosphates are relatively similar, while the stabilities of metal-base complexes vary greatly.

As has been noted above, the activity plots in Figs. 1 and 3 are approximately superimposable for concentrations in the activation range; the differences appear during the inhibition stage. Since the tendency of the metal ions to bind to phosphate does not vary greatly in this series of metals, we are inclined to believe that the activation phenomenon is affected by metal ions binding to the phosphate groups on the RNA substrate. Metal ions alone can bring about the cleavage of the phosphodiester bonds in RNA and other polynucleotides, although, of course, at rates that cannot be compared with the enzymatic rates (1, 2). It does appear reasonable, however, that metal ions can aid the enzyme by acting in concert with it. The metal ions can also be useful in neutralizing the charges on the phosphates of the substrate. We believe that the similarity in the activating ability of the metals constitutes evidence that substrate binding is involved in this activation.

Since higher metal ion concentrations produce binding to the bases, it is possible that the inhibition effect is due to this type of substrate binding. Indeed, the nonenzymatic degradation of RNA by metal ions binding phosphate is inhibited by metal ions binding to the bases (3). The nonenzymatic and the enzymatic reactions are not necessarily analogous in this respect, however, and inhibition through binding to a histidine site on the
It appears that this trend is duplicated with many nucleases, including bovine pancreatic. Observed inhibitory effects are usually diminished with decreasing pH (12, 52-54). Thus, the activations observed in Figs. 1 and 2 would probably not be observed at higher pH values.

One more question that needs to be resolved about this metal activation of ribonuclease is why the enzyme is not generally considered to be metal-activated, even though numerous studies on the effect of metals on the enzyme have been carried out.

Most previous studies on the effect of metal ions on this enzyme have been conducted only incidentally to a consideration of other aspects of the enzymatic reaction in which the investigators were primarily concerned. Generally, the reaction was studied at only one pH (the pH optimum in the absence of metal) or at only one metal ion concentration, and frequently buffers were used which would tend to obscure the effects noted in Fig. 1. When these factors are taken into consideration, the results of other investigators are not in conflict with the results reported here; in fact they fall pretty much in line with those of Fig. 1.

Although it is difficult to compare the results in the literature of metal effects on bovine pancreatic ribonuclease, because the results are derived from experiments under many conditions, there are some studies on the effect of pH on the activity of some spleen and liver nucleases that show very clearly how it is possible to miss a metal activation effect by working at one pH rather than another. In Fig. 4 we have replotted data obtained by Hilme (5) for curve A and Mauer and Greco (11) for curves B and C.

It should be emphasized, of course, that the results of Figs. 1 and 3 show that there is no absolute dependence of the enzyme activity on the presence of metals; the enzyme functions perfectly well with no added metal ions, and indeed the metal activation never amounts to more than approximately doubling the activity without metal. This is not an enzyme that requires metal ions for activity, but metal ions under certain conditions activate it, and this metal activation turns out to be a rather interesting phenomenon.

Deoxyribonuclease—In contrast to the rather mild effect of metals on pancreatic ribonuclease, the commonly used DNA nuclease, bovine pancreatic DNAse I, requires metal ions for activity (22-26). It does not function without metal ions and is generally considered a metal-activated enzyme. Magnesium(I1) ions have usually been used for activation. There have been previous studies on the substitution of other metal ions for magnesium. One of the earlier such studies was by Miyaji and Greenstein (23), who reported that Mg(I1), Co(I1), and Mn(I1) all lead to approximately the same activity. Wiberg (24), on the other hand, carried out an investigation in which Mg(I1) and Mn(I1) have been compared over a range of metal ion concentrations and concluded that Mn(I1) is a better activator than Mg(I1). This variation of effects can be explained by the use of different conditions, e.g. pH, anions present, ionic strength, etc.

We have determined the activation of DNase I by the same metal ions used in the RNase study and over the same metal ion concentration range. The results are shown in Fig. 5. The finding that Mn(I1) is a better activator than Mg(I1) is confirmed, but Fig. 6 shows that the metal of choice for the activation of the deoxyribonuclease is Co(I1), which is over twice as good as Mn(I1) and over 5 times as good as the still commonly used Mg(I1).
The relative activating ability of DNase by the various metal ions resembles such comparative curves for other enzymes much more than the curves of Fig. 1. There is no clearcut correlation of activating ability with the complexing ability of the metal. The metal with the strongest complexing activity of those investigated, Cu(II), does show a maximum (though quite low) activating effect at low concentration, and the activating ability of Cu(II) then decreases with increasing metal concentration. The activating ability of all the other metals either increases with metal concentration over the whole concentration range or comes to a plateau. Quite possibly, lower activation or even inhibition might occur with these metals if it were experimentally possible to use even higher concentrations (which are precluded because of precipitation).

Evidently a metal ion has to occupy an activating site or sites in order for the enzyme to function. There is evidence for an inhibitory site or sites with Cu(II) and possibly with Cd(II). The failure of the other metal ions to fall into the Irving-Williams series is an indication that the curves cannot be explained by one type of activating site and one type of inhibiting site, as in the case of the ribonuclease curves (see "Addendum").

Comparison of Ribonuclease and Deoxyribonuclease—A comparison of Figs. 1 and 5 provides us indeed with what is to be expected of the influence of metals on an enzymatic reaction in which there is one type of activating and one type of inhibiting site (Fig. 1) and one reaction in which such competing processes are less clearly defined (Fig. 5). Experience shows that Fig. 5 is much more typical of enzymatic reactions than Fig. 1, indicating that metal activation of enzymes is frequently a more obscure process than the activation of ribonuclease A.

EXPERIMENTAL PROCEDURE

DNA, DNase I and the ribonuclease recrystallized five times were obtained from Sigma; ribonuclease A and the yeast RNA used as substrate were from Worthington. All of the inorganic chemicals were reagent grade.

Ribonuclease activity was determined by incubating RNA \((2 \times 10^{-4} \, \text{M})\) in 0.05 M acetate buffer, pH 5, with 5 \(\mu\text{g}\) (in 5 \(\mu\text{l}\)) of enzyme in a water bath for 10 min at 25\(^\circ\), adding an equal volume of MacFadyen's (55) reagent (2.5% trichloracetic acid and 0.25% uranyl acetate), and measuring the absorbance of the material remaining soluble after 30 min at 25\(^\circ\) at 260 nm on a Cary model 14 spectrophotometer. Activity in the absence of added metal was taken as 100\%, and the activity in the presence of metal was calculated accordingly. Absorbances ranged from 0 to 2. For the crystalline enzyme the measured absorbance corresponding to 100\% activity was 0.80 ± 0.08; for the ribonuclease A fraction it was 1.14 ± 0.05. Precipitates were present in the solutions containing the highest concentration of metal; slight haze was evident at the next lower concentration. All other solutions had no traces of colloidal properties. We do not believe that the precipitation interfered with the data, since the curves of Fig. 1 (and the data plotted in Fig. 2) are well defined without the last two points, and these latter points do not deviate from the curves drawn without incorporating them. The absolute values of the activities at the highest metal concentration are, however, questionable.

DNA was prepared in 0.01 M NaNO\(_3\) as previously described (56). For the determination of DNase activity, a modification of the Kunitz (57) procedure was used. DNA \((5 \times 10^{-4} \, \text{M})\) in NaNO\(_3\) \((3.3 \times 10^{-3} \, \text{M})\) and acetate buffer \((\text{pH} 5, 0.1 \, \text{M})\) was equilibrated at 37\(^\circ\) for 15 min, 0.6 \(\mu\text{g}\) of enzyme was added (total volume then was 3 ml), and the reaction was allowed to proceed 10 min in the absence or presence of the metals. (Since the reaction with Cu(II) ions was exceedingly sluggish, 0.3 mg of enzyme was used, and the reaction was allowed to proceed for 10 hours. All other conditions were the same.) Perchloric acid \((1.5 \, \text{M})\) was then added, the mixture chilled in ice for 10 min, and the supernatant from centrifugation read at 260 nm in a Cary model 14 spectrophotometer.

The reaction times used for the comparison of enzymatic rates with various metals were determined from preliminary experiments in which the indicated quantities of reagents were allowed to react for extended time periods. It was found that the plot of the change in absorbance with time was generally linear up to and beyond 10 min (at all metal concentrations) in the presence of all metal ions except cobalt. The absorbance measurement after 10 min was therefore selected as an optimum time for the comparison of the enzymatic activities. Since the reaction with cobalt was very rapid, the absorbance versus time plot had leveled off somewhat at 10 min. The 10 min points were nevertheless used even for cobalt, to give a proper comparison with the other metals, with some of which lower time intervals would have produced only limited reaction. Thus, the cobalt curve in Fig. 5 does not reveal a strictly accurate activity, but the deviation from linearity is not great, and the conclusions reached are not affected by this circumstance. Indeed, the fact that a leveling of the activity is achieved at lesser times than with other metals is in line with the statement that cobalt is the best activator of the DNase reaction. Among the metal ions studied, copper ions give very much slower reaction than any of the others. For this reason, the much longer time interval was required to study activity in the presence of copper.

Addendum—An article has just appeared (by Melgar and Goldthwait (58)) that explains the lack of correlation between the Irving-Williams series and the effect of metal ions on DNase I. Apparently, different metals can affect the enzymatic mechanism in different ways; as these authors have shown, some metals cleave native DNA by a single hit mechanism, whereas others produce a double hit mechanism, in which both DNA chains are cleaved simultaneously. One cannot expect the metal ions to follow the Irving-Williams series or any other logical order, when their effects on an enzyme are completely different. The reason for the family of curves that are produced with ribonuclease is therefore dependent upon the same type of mechanism operating with all metal ions.

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XIII. THE EFFECT OF METAL IONS ON THE ENZYMATIC DEGRADATION OF RIBONUCLEIC ACID BY BOVINE PANCREATIC RIBONUCLEASE AND OF DEOXYRIBONUCLEIC ACID BY BOVINE PANCREATIC DEOXYRIBONUCLEASE I
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