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Interaction of Alkaline Phosphatase of *E. coli* with Metal Ions and Chelating Agents*

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The chelating agent 1,10-phenanthroline inhibits *E. coli* alkaline phosphatase by removal of zinc; the inhibition is reversed on addition of Zn^{++} ions. Dialysis of the enzyme against the chelating agent results in an inactive zinc-free apophosphatase to which activity is completely restored on addition of Zn^{++} ions. Among a number of other metal ions tested Co^{++} and Hg^{++} restore activity, though only partially. Much higher concentrations of Mg^{++} ions bring about a similar effect, probably by a different mechanism. Cd^{++}, Co^{++}, Pb^{++}, and Cu^{++} ions inhibit the enzyme, presumably by displacement of the native Zn^{++} ion. Be^{++} ions also inhibit the alkaline phosphatase of *E. coli* as has been observed with other phosphatases. Activity of the native enzyme is enhanced by increasing ionic strength.

Purified preparations of *E. coli* alkaline phosphatase contain about 2 gram atoms of zinc per mole of enzyme protein (Plocke *et al.*, 1962). The purified enzyme is inhibited by a number of agents which form stable complexes with zinc ions in solution. The effectiveness of these inhibitors is closely related to the magnitudes of the stability constants of their zinc complexes. Zinc, therefore, seems to be involved in the action of the enzyme.

The present report considers the mechanism by which one of these agents, 1,10-phenanthroline, inactivates the enzyme through removal of zinc. The zinc-free apophosphatase can be reactivated completely by the addition of Zn^{++}, and partially by Co^{++} and Hg^{++} ions.

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METHODS AND MATERIALS

Alkaline phosphatase of E. coli was prepared as previously described (Plocke *et al.*, 1962).

Protein concentration was determined by the absorbancy at 278 mμ, a molar absorbancy index (a_M) of 5.6×10^4 being used (Plocke *et al.*, 1962).

All metals were determined by emission spectrography (Vallee and Hoch, 1955); zinc was also measured by means of the dithizone method (Vallee and Gibson, 1948).

Enzymatic activity was determined in 1 M Tris, *p*H 8.0, at 25°, with 10^{-3} M *p*-nitrophenyl phosphate (Garen and Levinthal, 1960; Plocke *et al.*, 1962). Where specified, 0.02 M veronal buffer was substituted for 1 M Tris.

Reagents.—The method of cleaning glassware and the preparation of metal-free water has been described previously (Thiers, 1957). Tris (Sigma 121) and sodium diethylbarbiturate (veronal) were dissolved in metal-free distilled water, adjusted to neutral *p*H with HCl, and extracted with dithizone in carbon tetrachloride to remove contaminating metal ions. Excess dithizone was removed by extractions with c.p. carbon tetrachloride. The extracted solutions were adjusted

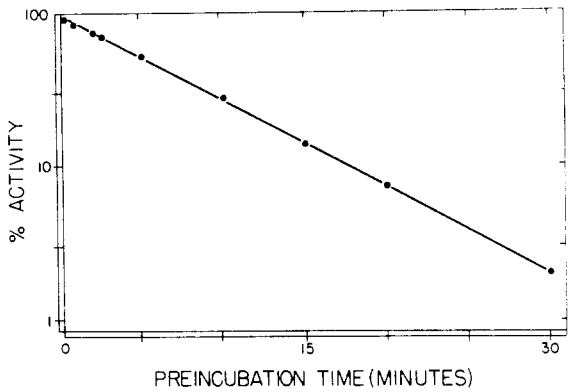

FIG. 1.—Time-dependent inhibition of *E. coli* alkaline phosphatase with 1,10-phenanthroline. 2×10^{-7} M enzyme was incubated with 2×10^{-3} M 1,10-phenanthroline in 1 M Tris buffer, *p*H 8.0, 25°, for the times indicated. For assay, aliquots were diluted 30-fold into a reaction mixture containing 2×10^{-3} M 1,10-phenanthroline and 1×10^{-3} M *p*-nitrophenyl phosphate in 1 M Tris buffer, *p*H 8.0, 25°. Activity as per cent of the control is plotted on a logarithmic scale.

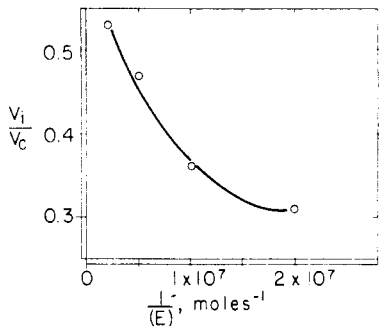

FIG. 2.—Effect of enzyme concentration on the time-dependent inhibition of *E. coli* alkaline phosphatase by 10^{-4} M 1,10-phenanthroline. The enzyme was incubated for 1 hour in the presence of 1×10^{-4} M 1,10-phenanthroline in 1 M Tris, *p*H 8.0, 25°, in concentrations ranging from 5×10^{-8} M to 5×10^{-7} M. Aliquots were then diluted for assay to result in a constant enzyme concentration of 3.3×10^{-9} M in the reaction mixture, which contained 1×10^{-4} M 1,10-phenanthroline, 1×10^{-3} M *p*-nitrophenyl phosphate, 1 M Tris buffer, *p*H 8.0, 25°. Inhibited activity, V_i; control activity, V_c.

to the final *p*H with either metal-free acid or base.

Dialysis tubing was Visking-Nojax casing which had been freed of metals according to the method of Hughes and Klotz (1957).

1,10-*Phenanthroline* was the crystalline monohydrate (G. F. Smith Company) and was used without further purification.

Standard metal solutions were prepared from weighed amounts of spectrographically pure salts of Zn^{++}, Co^{++}, Mn^{++}, Ni^{++}, Cd^{++}, Hg^{++}, Mg^{++}, and Cu^{++} (Johnson and Matthey Co., Ltd.) dissolved in metal-free water. In addition, a weighed amount of reagent grade ferrous ammonium sulfate, Fe^{++} $SO_4(NH_4)_2SO_4 \cdot 6H_2O$, was dissolved in metal-free water just before use.

RESULTS

Inhibition by 1,10-Phenanthroline.—Incubation of *E. coli* alkaline phosphatase with 1,10-phenanthroline results in inhibition of enzymatic activity (Fig. 1). When 2×10^{-7} M enzyme is incubated with 2×10^{-3} M 1,10-phenanthroline, activity is lost at a first order rate, and is completely abolished after 40 minutes of incubation. The level of activity is a function of both the inhibitor concentration and the time of incubation. When both of these are constant, the degree of inhibition is also a function of the concentration of enzyme in the incubation mixture (Fig. 2).

The addition of zinc and certain other divalent metal ions to the enzyme inhibited by 1.10-phenanthroline reverses this inhibition. Activity, which is completely abolished by incubation for 40 minutes with 2×10^{-3} M 1,10-phenanthroline, is restored instantaneously and almost completely on addition of 2×10^{-3} M Zn^{++} ions (Table I), and partially on addition of 2×10^{-3} M Cu^{++} ions under the same conditions. Mg^{++} ions have no effect.

TABLE I

REVERSAL OF 1,10-PHENANTHROLINE INHIBITION OF ALKALINE PHOSPHATASE OF *E. coli* BY METAL IONS

2×10^{-7} M enzyme was incubated in the presence of 2×10^{-3} M 1,10-phenanthroline in 1 M Tris, *p*H 8.0, 25°, for 40 minutes, then diluted 30-fold into an assay mixture containing 2×10^{-3} M 1,10-phenanthroline and metal ions as shown below. The assays were performed in 1 M Tris, *p*H 8.0, 25°, with 1×10^{-3} M *p*-nitrophenyl phosphate as substrate. Control activity in the absence of inhibitor is V_c; inhibited activity, V_i.

Metal Ion (2×10^{-3} M)	V_i/V_c
None	0.00
Zn^{++}	0.92
Cu^{++}	0.37
Mg^{++}	0.00

Dialysis of the enzyme against 1,10-phenanthroline results in concomitant loss of zinc and enzymatic activity (Table II). Under the conditions employed, only 6% of the original zinc and 2% of the original activity of the native enzyme remain after dialysis.

Metals other than zinc are also removed by dialysis against the chelating agent. Spectrographic data in Table II on metals other than zinc, obtained on enzyme dialyzed against 1,10-phenanthroline, represent the *maximum* concentrations of each element which could have been present. None of these elements were actually detected in the sample; the calculations of these maximum concentrations given are based on the limits of detection for each element.

On addition of a 3-fold molar excess of zinc ions to the dialyzed enzyme in Tris buffer, activity

TABLE II

METAL CONTENT OF *E. coli* ALKALINE PHOSPHATASE BEFORE AND AFTER DIALYSIS AGAINST 1,10-PHENANTHROLINE (OP)

(Metal content in μg per gram protein)

6 ml of 2.5×10^{-5} M enzyme was dialyzed for 120 hours *vs.* 4 changes of 5×10^{-3} M 1,10-phenanthroline (250 ml) in 0.01 M Tris, *p*H 7.0, followed by 4 changes of metal-free 0.01 M Tris, *p*H 7.0. Activity was measured on a 60-fold dilution of enzyme in 1 M Tris, *p*H 8.0, 25°, with 1×10^{-3} M *p*-nitrophenyl phosphate as substrate.

Metals	Before Dialysis	After Dialysis[a] Against OP
Zinc	1980	120
Magnesium	300	<20
Iron	149	<50
Calcium	173	<50
Aluminum	20	< 2
Copper	38	Not done
Nickel	42	<10
Manganese	99	<10

[a] The activity of the enzyme after dialysis against 1,10-phenanthroline was 2% of the original activity, as compared to 6% of the initial zinc content.

is immediately restored to nearly the same level which is exhibited by the native zinc enzyme (Table III A). Molar ratios of Zn^{++} to enzyme in excess of 3 to 1 do not result in further increases in activity, although at lower ratios correspondingly diminished restoration of activity is found. When added in a 3-fold molar excess under the same conditions none of the other divalent metal ions employed restore activity significantly (Table III A).

Since the stability constants of metal-veronal complexes are lower than those of the corresponding Tris complexes (*vide infra*), the concentration of free metal ions should be greater in veronal than in Tris buffer. Hence, in veronal buffer, apophosphatase-metal complexes might form whereas Tris might prevent their formation. When 1 M Tris in the assay is replaced by 0.02 M veronal, addition of Zn^{++} ions again restores activity almost completely, and significant restoration of activity also results on addition of Co^{++}, Hg^{++}, and Mg^{++} ions, in contrast with the results obtained with Tris (Table III B). At a Zn^{++} to enzyme ratio of 7 to 1, restoration of activity is nearly complete; higher concentrations of Zn^{++} ions do not bring about greater restoration of activity. Co^{++} and Hg^{++} ions, however, only restore activity partially and at relatively *low* molar ratios of these ions. Maximal restoration of activity, 27%, is obtained at a Co^{++} to enzyme ratio of 3 to 1; Co^{++} ions in excess of this ratio inhibit, and at a ratio of 30 to 1 even the original residual activity is abolished. A Hg^{++} to enzyme ratio of 1 to 1 restores 10% of the control activity, the maximum observed; higher concentrations of Hg^{++} ions inhibit.

TABLE III

RESTORATION OF ACTIVITY TO ALKALINE APOPHOSPHATASE ON ADDITION OF METAL IONS

To the apoenzyme, 10^{-5} M in 0.01 M Tris, *p*H 8.0, 0°, were added metal ions to yield the molar ratios of metal to enzyme indicated below. For assay, dilution was made into 1 M Tris or 0.02 M veronal and 1×10^{-3} M *p*-nitrophenyl phosphate at 25°. For assay in Tris, a 30-fold dilution was made for all metal ion additions except Zn^{++}, for which a 300-fold dilution was required. For assay in veronal, a 60-fold dilution was made throughout. Activities are given as per cent of the activity of the native zinc enzyme employed as a control.

Addition of Metal (M)	*A*[a] 1 M Tris		*B*[a] 0.02 M Veronal		
Enzyme (M)	3:1	1:1	3:1	10:1	30:1
	Activity (%)		Activity (%)		
Zn^{++}	86	13	62	89	89
Co^{++}	5	13	27	8	0
Hg^{++}	3	10	4	2	+[b]
Mg^{++}	3	2	5	10	16
Mn^{++}	1	2	2	2	+
Ni^{++}	3	4	2	2	+
Fe^{++}	4	2	4	2	+
Cu^{++}	2	6	3	3	+
Cd^{++}	2	4	3	1	+

[a] Activity of the apoenzyme, without added metal, in Tris = 2%, in veronal = 3%, of the activity of the native enzyme. [b] + = Activity not measured.

In contrast, significant increases in activity on addition of Mg^{++} ions are not observed until the ratio of Mg^{++} to enzyme becomes 10 to 1. Maximal restoration of activity to the extent of 16% of the control activity is attained at a Mg^{++} to enzyme ratio of 30 to 1; Mg^{++} ions in excess of this amount do not affect activity further.

The effect of metal ions added to the native zinc-containing enzyme has also been studied; in this instance large excesses of metal ion and long periods of incubation were employed (Table IV). Activity was inhibited in all instances. The metal ions are listed according to the apparent order of their effectiveness as inhibitors.

The absolute reaction velocities observed in 0.02 M veronal buffer are only about 20% of those observed in 1 M Tris under the standard assay conditions. As shown in Figure 3, enzyme activity is a function of the ionic strength. Activities were measured with varying concentrations of Tris, NaCl, and $MgSO_4$ in the reaction mixture. When NaCl and $MgSO_4$ were employed, 0.01 M Tris was added to control the *p*H. Ionic strength was estimated from the relationship $\mu = {}^1/_2 \Sigma c_i z_i^2$, where c_i represents the molar concentration of each ionic species and z_i its corresponding charge; Tris was presumed to be 50% ionized at *p*H 8.0. In general, increasing ionic strength increases the activity. At equivalent ionic strength the effect is, however, notably greater for Tris than for the other two electrolytes.

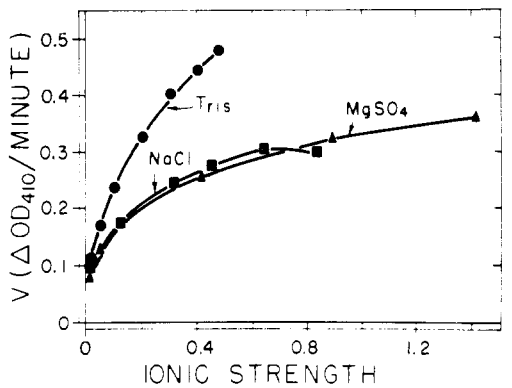

FIG. 3.—Activity of *E. coli* alkaline phosphatase as a function of ionic strength and buffer ion. The basic assay mixture contained 1×10^{-8} M enzyme, 1×10^{-3} M *p*-nitrophenyl phosphate, and 0.01 M Tris buffer, *p*H 8.0, 25°. In addition, Tris-Cl, ●; NaCl, ■; $MgSO_4$, ▲ were added to result in the final ionic strength, as indicated.

TABLE IV

INHIBITION OF *E. coli* ALKALINE PHOSPHATASE ON PREINCUBATION WITH 10^{-4} M METAL IONS

The enzyme, 5×10^{-7} M, was incubated with 10^{-4} M metal ion in 0.05 M Tris, *p*H 7.0, at 0° for the time indicated. For assay, the mixture was diluted 30-fold in 0.05 M Tris, *p*H 8.0, 25°. The approximate times required for establishment of equilibrium are shown. Inhibited activity, V_i; control activity, V_c.

Metal	Incubation Time (Hr.)	V_i/V_c
Be^{++}	18	0.20
Cd^{++}	74	0.56
Co^{++}	144	0.59
Pb^{++}	74	0.75
Cu^{++}	74	0.82
Hg^{++}	74	0.98

DISCUSSION

Spectrochemical analyses and inhibition data have shown that zinc is a component of alkaline phosphatase of *E. coli*, firmly bound to the protein and essential to its activity (Plocke *et al.*, 1962). The present experiments confirm these conclusions; in particular, the concurrent removal and restoration of zinc and activity demonstrate an absolute requirement of zinc for enzymatic function (Tables II and III). These analytical results strongly suggest that inhibition of enzymatic activity by 1,10-phenanthroline proceeds through the removal of zinc rather than through the formation of a mixed complex. The dependence of the inhibition both on time (Fig. 1) and on enzyme concentration (Fig. 2) is consistent with this view (Felber *et al.*, 1962).

In 1 M Tris buffer, the addition of zinc ions to the zinc-free apophosphatase restores activity almost completely, while other divalent metal ions fail to do so (Table III A); this suggests a highly specific and selective interaction between the apophosphatase and zinc. The substitution of veronal for Tris buffer, however, shows this apparently absolute specificity to be an artifact of the conditions employed for restoration of activity. When veronal buffer is substituted in the assay mixture, not only do Zn^{++} ions restore activity as before, but certain other ions, notably Co^{++}, Hg^{++}, and Mg^{++}, effect partial restoration of activity.

The failure to observe activity with metal ions other than zinc in 1 M Tris may be attributed to competition between the buffer anion and the apoenzyme for the added metal ions. The magnitude of the stability constants, log K_L, for the complexes of Tris and metal ions of the first transition series are of the order of 2 or 3 (Hall *et al.*, 1962). The concentration of Tris buffer, exceeding that of the enzyme by a factor of 10^7, would therefore allow for successful competition of Tris with the apoenzyme for any metal ion which would form a complex with the apoenzyme of a stability constant, K_E, of 9 or lower. Data obtained on metal ion inhibition of carboxypeptidase indicate a lower affinity of veronal for metal ions than that of Tris (Coombs *et al.*, 1962). Also, since the concentrations of veronal used in these experiments are 50-fold lower than those of Tris, metal ions such as Co^{++} and Hg^{++} would be able to bind to the apoenzyme more readily in the presence of veronal. The absolute specificity of the apoenzyme for zinc in Tris buffer is thus only apparent and reflects a stability constant of the zinc-apoenzyme complex greater than that of complexes of other metals with this protein.

Zinc ions in larger amounts are required for full restoration of activity in 0.02 M veronal buffer than in 1 M Tris at *p*H 8 (Table III). Although the reason for this is not readily apparent, it might be conjectured that in veronal buffer zinc hydroxides are formed; thus the added zinc would be less readily available to the apoenzyme than in Tris buffer. The latter would prevent the formation of hydroxides through complex formation; the zinc-Tris complex might more freely exchange zinc with the apoenzyme than the zinc hydroxides, allowing full restoration of activity at a lower concentration of added zinc.

A maximal activity of 16% results on addition of Mg^{++} ions when in 30-fold excess over the concentration of the enzyme. Since this is a much greater amount than that required for maximal activity with Co^{++} and Hg^{++} (Table III), a different mechanism for the activation by magnesium is suggested. Magnesium ions prefer oxygen donor groups, whereas Zn^{++}, Co^{++}, or Hg^{++} ions prefer sulfur, nitrogen, or sulfur-nitrogen ligands (Williams, 1959). Thus Hg^{++}, Zn^{++}, and Co^{++} would be likely to replace one another in binding to S or S–N sites, whereas Mg^{++} ions would not tend to bind to these. The present experiments are not decisive concerning the mode of action of Mg^{++} ions, since the "zinc-free"

enzyme on which the experiment was performed still contained 6% of zinc which was resistant to removal; this leaves the possibility that magnesium effects an enhancement of the activity associated with this 6% of zinc phosphatase (Table III).

Such a conclusion is certainly consistent with the observed activation of the native zinc enzyme by Mg^{++} and other ions (Fig. 3). Mahler (1961) has suggested that divalent metal ions may affect the activity of phosphatases indirectly through a reduction of the negative charges on the phosphate moiety of the substrate which, in turn, would favor the access of hydroxyl groups of water, required for hydrolysis. Such metal phosphate complexes might account for the increase in the reaction velocity of the native enzyme as a function of ionic strength (Fig. 3). The addition of magnesium ions to the apophosphatase might similarly reduce negative charges at the enzyme surface favoring the access of substrate.

The reversal of the 1,10-phenanthroline inhibition by metal ions supports the suggestion that the enhancement of activity by Mg^{++} requires the native zinc enzyme (Table I). Both Zn^{++} and Cu^{++} ions reverse the 1,10-phenanthroline inhibition, but Mg^{++} ions do not. Since activity in this experiment is lost completely, it may be supposed that zinc is entirely removed from the enzyme and a $[Zn(OP)_3]^{++}$ complex is formed[1]; Cu^{++} ions will dissociate this complex, freeing zinc ions to restore the active phosphatase. In contrast to Cu^{++} ions, Mg^{++} ions do not react with 1,10-phenanthroline and hence do not liberate zinc from the phenanthroline complex; thus restoration of activity is not observed under these circumstances.

During the dialysis against 1,10-phenanthroline not only zinc but other metals, including magnesium, are removed. The removal of magnesium may be a nonspecific consequence of the extensive dialysis conditions employed in these experiments. Even though both these ions are removed the addition of zinc ions alone is sufficient to restore activity; magnesium ions do not therefore seem to be mandatory for activity. The presence of magnesium in the final preparations of the enzyme had previously led to a discussion of the essentiality of this ion in the activity of the alkaline phosphatase of *E. coli* (Plocke *et al.*, 1962). The present data suggest that Mg^{++} affects activity nonspecifically. The inhibition of the enzyme by Be^{++} ions is analogous to that described for other phosphatases (Klemperer *et al.*, 1949; Grier *et al.*, 1949).

Many of the properties of alkaline phosphatase discussed, especially the time-dependence and reversibility of inhibition by 1,10-phenanthroline and the reversibility of zinc removal, bear close similarity to the behavior of carboxypeptidase A of bovine pancreas, another zinc enzyme (Vallee *et al.*, 1958; Felber *et al.*, 1962; Coombs *et al.*, 1962). Nitrogen and sulfur groups have been identified as the donor groups participating in binding of zinc to carboxypeptidase A. Also, the stability constants for a series of metallocarboxypeptidases have been measured and reflect the nature of these donor groups (Coleman and Vallee, 1961). Incubation of native zinc carboxypeptidase with the metal ions of this series results in inhibition of activity through displacement of zinc as a function of the sequence of the stability constants. Thus, the order of effectiveness of inhibition was found to be $Cd^{++} > Pb^{++} > Cu^{++}$. Under nearly identical conditions, these ions inhibit alkaline phosphatase in the same order of effectiveness (Table IV) suggesting perhaps a similar mechanism of zinc displacement. The similarities in the behavior of alkaline phosphatase and carboxypeptidase suggest that the considerations and experimental procedures developed in the course of studies on the active site of carboxypeptidase may prove pertinent for similar efforts in regard to alkaline phosphatase.

[1] OP = 1,10-phenanthroline.

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