Effect of Magnesium on the Properties of Zinc Alkaline Phosphatase†

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ABSTRACT: Alkaline phosphatase of *Escherichia coli*, isolated by procedures which do not alter its intrinsic metal content, contains 4.0 ± 0.3 g-atoms of tightly bound zinc per mole ($K_d \ll 1$ μM) and 1.3 ± 0.2 g-atoms of magnesium per mole (Bosron, W. F., Kennedy, F. S., and Vallee, B. L. (1975), *Biochemistry 14*, 2275–2282). Importantly, the binding of magnesium is dependent both upon pH and zinc content. Hence, the failure to assign the maximal magnesium stoichiometry to enzyme isolated by conventional procedures may be considered a consequence of the conditions chosen for optimal bacterial growth and purification of the enzyme which are not the conditions for optimal binding of magnesium to alkaline phosphatase. Under the conditions employed for the present experimental studies, a maximum of six metal sites are available to bind zinc *and* magnesium, i.e., four for zinc and two for magnesium. Magnesium alone does not activate the apoenzyme, but it regulates the nature of the zinc-dependent restoration of catalytic activity to apophosphatase, increasing the activity of enzyme containing 2 g-atoms of zinc five-fold and that of enzyme containing 4 g-atoms of zinc 1.4-fold. Moreover, hydrogen–tritium exchange reveals the stabilizing effects of magnesium on the structural properties of phosphatase. However, neither the K_M for substrate nor the phosphate binding stoichiometry and K_I are significantly altered by magnesium. Hence, magnesium, which is specifically bound to the enzyme, both stabilizes the dynamic protein structure and regulates the expression of catalytic activity by zinc in alkaline phosphatase.

While specific participation of metal ions in enzymatic catalysis is now well known, it has become apparent only recently that some metalloenzymes contain discrete sets of metal-binding sites that are critical to the catalytic process, on the one hand, and essential to structure stabilization, on the other. Moreover, occupancy of the sites exercising these roles by the same or different metal atoms critically affects catalysis, structural stability, or both.

The importance of zinc both in catalysis and structure stabilization of *E. coli* alkaline phosphatase has been recognized for some time. Initial studies in this laboratory demonstrated that two zinc atoms are essential to catalytic activity (Plocke et al., 1962), and that an additional 2 g-atoms of zinc stabilize protein structure (Simpson and Vallee, 1968). However, the presence of stoichiometric quantities of magnesium (Plocke et al., 1962; Simpson et al., 1968; Bosron et al., 1975; Anderson et al., 1975) largely has been ignored; hence, its role has remained both unappreciated and undefined. Previous ambiguities in the metal content of phosphatase can be attributed to the conditions used for the isolation and purification of the enzyme, variations in the analytical methodology employed for metal analysis, and the molecular weight employed as the basis of molar stoichiometry (Bosron et al., 1975).

In order to delineate the effect of magnesium on phosphatase function, we have examined its equilibrium binding properties as well as its effect on enzymatic activity and protein conformation in the presence and absence of zinc. The present study demonstrates that enzyme-bound magnesium modulates both the catalytic and structural properties of phosphatase, which is therefore one of the most complex metalloenzymes yet known.

Materials and Methods

Preparation of Alkaline Phosphatase. Alkaline phosphatase was released by osmotic shock with EDTA[1] from the periplasmic space of *E. coli* C-90 and purified to homogeneity by DEAE-cellulose chromatography (Simpson et al., 1968). This procedure prevents both loss of intrinsic and contamination with extrinsic metals (Bosron et al., 1975). Highly purified enzyme contained 4.0 ± 0.3 g-atoms of zinc and 1.3 ± 0.2 g-atoms of magnesium and no other metals (Bosron et al., 1975). In addition, the enzyme contained 1.2 tightly bound inorganic phosphates per molecule (Bosron and Vallee, 1975). Its specific activity was 48 and 23 units when measured in 1 M Tris-Cl and 20 mM veronal–0.4 M NaCl, pH 8, 25 °C, respectively. The difference in activities in the two buffers arises from the ability of phosphatase to catalyze phosphoryl-group transfer to Tris (Wilson et al., 1964) in addition to its capacity to hydrolyze phosphate esters.

Apophosphatase was prepared by the method of Simpson and Vallee (1968). Purified phosphatase was dialyzed against three changes of 100 volumes of 10 mM 8-hydroxyquinoline-5-sulfonic acid (HQSA) in 10 mM Tris-Cl, pH 8, 23 °C, followed by three 24-h dialyses against metal-free buffer. The apoenzyme contained less than 0.02 g-atom of zinc and 0.04 g-atom of magnesium, as measured by atomic absorption spectrometry. The specific activity was 0.01 unit or 0.02% of that of the native enzyme. Apophosphatase prepared by this method does not contain bound phosphate (Bosron and Vallee, 1975). It sediments as a single symmetrical boundary in 10 mM Tris-Cl, pH 8, with an $s_{20,w} = 6.3$, identical to that of

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HQSA, 8-hydroxyquinoline-5-sulfonic acid; DEAE, diethylaminoethyl.

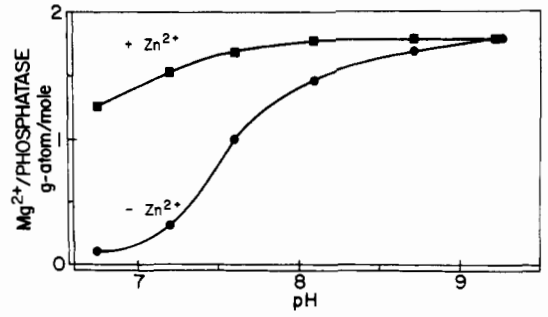

FIGURE 1: pH dependence of magnesium binding to phosphatase. Duplicate samples of apophosphatase, 10 μM, were dialyzed either against a tenfold molar excess of magnesium alone (●) or a tenfold molar excess of both zinc and magnesium (■) in metal-free 50 mM imidazole, pH 6.8–8.1, or ammediol, pH 8.1–9.2 for 24 h, 23 °C. Ionic strength of the buffers was held constant with NaCl.

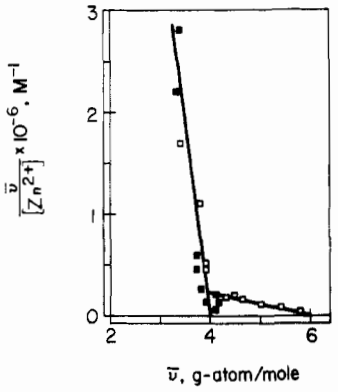

FIGURE 2: Zinc binding to phosphatase. Apophosphatase, 0.5–45 μM, was dialyzed against a tenfold molar excess of zinc (□) or a tenfold molar excess of both zinc and magnesium (■) for 24 h in metal-free 50 mM Tris-Cl, pH 8 at 23 °C.

native, dimeric phosphatase. Apophosphatase is stable for at least 1 month in 10 mM Tris-Cl, pH 8, 4 °C, and can be restored to full enzymatic activity by the addition of an excess of both zinc and magnesium.

Assays. All enzymatic activities were determined in 1 M Tris-Cl, pH 8.0, 25 °C, with 4-nitrophenyl phosphate, 1 mM, as substrate. A unit of activity is defined as the micromoles of substrate hydrolyzed per minute per milligram of protein, using a molar absorptivity of 1.68×10^4 for the 4-nitrophenolate ion at 400 nm. To ensure uniformity, activities of phosphatase, partially or fully restored with zinc and/or magnesium, were measured after 24-h incubation in 50 mM Tris-HCl, pH 8, 23 °C. Protein concentration was determined spectrophotometrically using $A_{278}^{1\%} = 7.2$ (Plocke et al., 1962), and all calculations involving molarity were based on a phosphatase molecular weight of 89 000 (Simpson et al., 1968).

Determination of Metals and Phosphate. Zinc and magnesium analyses were performed by atomic absorption spectrometry (Fuwa and Vallee, 1963; Wacker et al., 1965; Bosron et al., 1975). Phosphate was analyzed by the method of Chappelet-Tordo et al. (1974).

Equilibrium Dialysis. One-milliliter Plexiglas cells were soaked for 2 h in 8 N HNO_3, followed by storage in buffer. Cellulose membranes (Bolab, Inc.) were rendered metal free by boiling twice in 10 mM HQSA for 15 min, followed by boiling three times in water and storage in buffer at 4 °C. In all instances, individual phosphatase samples were dialyzed against metals or phosphate to equilibrium for 24 h at 23 °C while rotating at 3 rpm. Each side of the dialysis cell was analyzed at least twice for protein, metals, or phosphate.

Preparation of Reagents. Analytical grade chemicals were used throughout. 4-Nitrophenyl phosphate (Sigma 104) and Tris (Trizma) were obtained from Sigma Corp., spectrographically pure zinc sulfate and magnesium sulfate from Johnson-Matthey, Ltd., and phosphate standards from New England Reagent Laboratories. All solutions were prepared with deionized and distilled water. All buffers were extracted with 0.001% dithizon in CCl_4 and stored over Chelex-100 (Anderson and Vallee, 1975). Glassware and polyethylene containers were rendered metal free by treatment with nitric acid–sulfuric acid as described by Thiers (1957).

Results

Equilibrium Binding of Metals and Phosphate. The binding of magnesium to apophosphatase depends critically both on pH and zinc concentrations. Duplicate samples of apophosphatase, 10 μM, were dialyzed to equilibrium at 23 °C against either a tenfold excess of magnesium alone (Figure 1, circles) or against this same excess of both magnesium *and* zinc (Figure 1, squares). At pH 6.8, in the absence of zinc, only 0.1 g-atom of magnesium binds to apophosphatase. Increasing the pH above 6.8 markedly increases magnesium binding until at pH 9.2 a maximum of 1.8 g-atoms/mol are bound. The addition of a tenfold molar excess of zinc markedly facilitates magnesium binding (Figure 1, squares) increasing it 12-fold at pH 6.8, from 0.1 to 1.2 g-atoms/mol, and from 1.4 to a maximum of 1.8 g-atoms/mol at pH 8.2. Raising the pH from 8.2 to 9.2 does not further affect the extent of magnesium binding to phosphatase, which contains approximately 4 g-atoms of bound zinc.

The maximal binding of zinc to apophosphatase was determined by equilibrium dialysis while systematically varying the concentration of free zinc ions. Apophosphatase, 0.5 to 45 μM, was dialyzed for 24 h against a tenfold molar excess of zinc alone (Figure 2, open squares) or against this same excess of both zinc *and* magnesium (Figure 2, solid squares). Zinc binding is plotted in the form of one of the linearized equations for the law of mass action (Scatchard, 1949) as $\bar{\nu}$ vs. $\bar{\nu}/[Zn^{2+}]$ where $\bar{\nu}$ is the average g-atoms of zinc bound per mole of enzyme and $[Zn^{2+}]$ is the concentration of free zinc in equilibrium with the zinc-enzyme complex. In the presence of magnesium, the plot is linear throughout the range from 1.3 to 130 μM free zinc ions, $\bar{\nu}/[Zn^{2+}] = 2.8\text{–}0.03 \times 10^6\ M^{-1}$, and extrapolates to a maximum of 4.0 g-atoms of tightly bound zinc atoms/mol of enzyme, with a dissociation constant which is much less than 1 μM. The maximal number of g-atoms of metal bound is 5.8, i.e., 4.0 g-atoms of zinc plus 1.8 g-atoms of magnesium (Figures 1 and 2). At zinc concentrations less than 13 μM, $\bar{\nu}/[Zn^{2+}] > 0.3 \times 10^6\ \mu M^{-1}$, binding is identical both in the absence and presence of magnesium. However, increasing the concentrations of free Zn^{2+} from 13 to 130 μM in the absence of magnesium increases zinc binding to approximately 6 g-atoms/mol (Figure 2, open squares).[2] Magnesium readily displaces these additional two weakly bound zinc atoms.

Purified phosphatase has been shown to contain tightly bound phosphate, which is removed in the course of the preparation of apoenzyme with HQSA (Bosron and Vallee, 1975). In order to delineate the metal-ion dependence of phosphate binding, apoenzyme was dialyzed against phosphate in the

[2] Under these conditions, the enzyme remains dimeric with an $s_{20,w} = 6.3$.

TABLE I: Metal Dependence of Phosphate Binding to Phosphatase.[a]

Sample	Metal Added	Phosphate Bound (moles)
1	None	0
2	Mg^{2+}	0
3	Zn^{2+}	1.5
4	Zn^{2+}, Mg^{2+}	1.6

[a] Apophosphatase, 50 μM, in the presence and absence of 0.1 mM magnesium and/or 0.2 mM zinc was dialyzed against 0.5 mM phosphate for 24 h, 23 °C, in metal-free 50 mM Tris-Cl, pH 8. Inorganic phosphate was analyzed by the method of Chappelet-Tordo et al. (1974).

TABLE II: Kinetics of Zinc and Magnesium Phosphatases.[a]

Sample	g-atoms of Metal Added Mg^{2+}	g-atoms of Metal Added Zn^{2+}	Sp Act. (Units)	K_M (μM)	K_I (μM)
1	0	2	6 ± 2	4 ± 1	3 ± 1
2	0	4	39 ± 3	5 ± 1	3 ± 1
3	2.5	0	0		
4	2.5	2	30 ± 2	6 ± 1	4 ± 1
5	2.5	4	54 ± 3	6 ± 1	4 ± 1

[a] Apoenzyme, 11 μM, was incubated for 24 h prior to assay in metal-free 50 mM Tris-Cl, pH 8, 23 °C, containing the indicated molar ratios of metal ions. Reconstituted enzyme, 2.25 nM, was assayed in 5-cm cells in the presence of 0, 1, and 5 μM phosphate in 1 M Tris-Cl, pH 8, 25 °C. Kinetic constants for 4-nitrophenol phosphate (K_M) and phosphate (K_I) were calculated from Lineweaver-Burk plots.

presence and absence of magnesium and zinc. Neither apoenzyme nor phosphatase containing 1.3 g-atoms of magnesium binds inorganic phosphate (Table I, samples 1 and 2). However, both the enzyme containing 3.7 g-atoms of zinc and that containing 3.7 g-atoms of zinc *plus* 1.6 g-atoms of magnesium bind virtually identical quantities of phosphate, 1.5 and 1.6 mol/mol of enzyme, respectively (Table I, samples 3 and 4). Hence, phosphate binding appears to be dependent on the presence of zinc but independent of that of magnesium.

Effect of Metals on Enzyme Catalysis. Purified phosphatase catalyzes both the hydrolysis of phosphate esters and transfer of a phosphoryl group to suitable nucleophilic acceptors (Wilson et al., 1964). For all reconstitution experiments, the ratio of transferase to hydrolyase activity remained constant at 1.1, identical to that for the native enzyme. The enzymatic activities reported in Table II are the sum of both activities, as measured in 1 M Tris-Cl, pH 8, 25 °C. Magnesium alone does not restore catalytic activity to apophosphatase (Table II, sample 3), but it enhances the activity of enzyme reconstituted with varying molar ratios of zinc. The activities of magnesium-free phosphatase containing 2 or 4 g-atoms of zinc are 6 and 39 units, respectively (Table II, samples 1 and 2). Addition of magnesium increases the activity of 2-Zn phosphatase[3] by fivefold to 30 units, and raises that of the 4-Zn enzyme 1.4-fold to the maximum of 54 units (Table II, samples 4 and 5). Further addition of a tenfold molar excess of either

[3] The number of metal ions added to apophosphatase is indicated by an Arabic number preceding the metal symbol; i.e., 2-Zn phosphatase is the enzyme to which 2 g-atoms of zinc have been added.

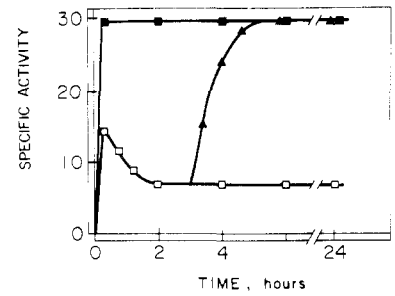

FIGURE 3: Effect of magnesium on 2-Zn phosphatase activity. Two gram atoms of zinc was added both to 2 μM apophosphatase (□) and to 2-Mg phosphatase which was preincubated with magnesium for 24 h (■) in metal-free 50 mM Tris-Cl, pH 8, at 23 °C. Magnesium, 2 g-atoms, was also added to the 2-Zn phosphatase after 3 h of incubation (▲). Enzymatic activity was determined at the times indicated. In all cases, the resultant enzymes contained 2 g-atoms of zinc, as determined by atomic absorption spectrometry subsequent to gel filtration on Bio-Gel P-4 in 50 mM Tris-Cl, pH 8, 23 °C.

metal to 4-Zn 2.5-Mg phosphatase in the course of incubation or assay does not increase activity above the maximum. Importantly, the K_M for 4-nitrophenyl phosphate and the K_I for phosphate with the 2- or 4-Zn phosphatases in the presence or absence of magnesium remain relatively constant at 4–6 μM and 3–4 μM, respectively (Table II). These data demonstrate that the principal effect of magnesium is on the rate of hydrolysis of phosphate ester, but not on kinetic constants for substrate or phosphate, and, further, that magnesium is required for achievement of maximal catalytic activity in all instances examined.

The reversible time-dependent loss of activity observed after addition of 2 g-atoms of zinc to apophosphatase (Brown et al., 1974) depends critically on the magnesium content of the enzyme. Addition of 2 g-atoms of zinc to apophosphatase increases enzymatic activity to 14 units within the first 5 min (Figure 3, open squares), while over the next 2 h enzymatic activity decreases to 7 units but remains constant thereafter. These activity changes are independent of the time of incubation of the apoenzyme in buffer prior to the addition of the 2 g-atoms of zinc. Even as the activity is changing, atomic absorption analyses subsequent to gel filtration indicate that 2 g-atoms of zinc remain bound throughout the 24-h of incubation.

However, addition of 2 g-atoms of zinc to phosphatase preincubated with 2 g-atoms of magnesium increases activity to 30 units within the first 5 min. The activity of this 2-Zn 2-Mg phosphatase remains constant thereafter (Figure 3, solid squares), unlike that of 2-Zn phosphatase (Figure 3, open squares). Moreover, the activity of 2-Zn phosphatase, constant at 7 units after 3-h incubation, also increases to 30 units after addition of 2 g-atoms of magnesium (Figure 3, triangles), indicating that the magnesium-stimulated activity of 2-Zn phosphatase is independent of the order of addition.

Discussion

It has become increasingly apparent that metal ions can serve various roles in different metalloenzymes. They can participate directly in catalysis, stabilize protein structure, and regulate enzymatic activity. Native *E. coli* alkaline phosphatase, a dimer of molecular weight 89 000, contains all three classes of metal ions and has been instrumental in defining these roles and their interrelationships. Two gram-atoms of zinc per dimer have been postulated to be involved in catalysis (Plocke et al., 1962), 2 more zinc atoms stabilize structure

(Simpson and Vallee, 1968), and 1–2 g-atoms of magnesium regulate catalytic activity (Anderson et al., 1975, 1976).

The zinc content of purified phosphatase has been reported to vary from 2 to 6 g-atoms/mol of enzyme (Plocke et al., 1962; Simpson et al., 1968; Harris and Coleman, 1968; Reynolds and Schlesinger, 1968; Csopak and Szajn, 1973; Bosron et al., 1975) and the magnesium content, when examined, has ranged from 1 to 2 g-atoms/mol (Plocke et al., 1962; Simpson et al., 1968; Bosron et al., 1975). Csopak and Szajn (1973) have suggested that previous discrepancies in the nature of the metal-dependent restoration of activity to apophosphatase and in the zinc content of the native alkaline phosphatase are due to residual EDTA bound to the enzyme as a consequence of its use in the course of some of the procedures employed for its isolation. However, the use of EDTA during purification of phosphatase both in this and previous studies does not alter the intrinsic metal content (Bosron et al., 1975; Anderson et al., 1975). Further, apoenzyme prepared with HQSA followed by dialysis for 72 h under metal-free conditions (Simpson and Vallee, 1968) does not contain any residuum of this chelating agent, as determined spectrophotometrically. Therefore, metal stoichiometry and other experimental results determined with enzyme prepared under these conditions cannot be attributed to the effects of residual chelating agent and certainly cannot serve as a basis for comparison with studies performed in the presence of added chelating agents (Hull and Sykes, 1976).

We have recently confirmed our earlier suggestion that magnesium modulates the catalytic activity of alkaline phosphatase (Plocke and Vallee, 1962). In addition, magnesium, which is specifically bound to phosphatase, stabilizes the structure of the enzyme, as judged by hydrogen–tritium exchange (Anderson et al., 1975), and induces changes in the spectral and paramagnetic properties of the cobalt-substituted enzyme (Anderson et al., 1976). The lack of prior confirmation of the importance of magnesium to the function of alkaline phosphatase of *E. coli* and the recognition of its importance to structure most likely is the consequence of the failure to monitor its presence in the enzyme.

The specific binding of magnesium to apoalkaline phosphatase depends both on pH and the cooperative effects of zinc binding. The binding of magnesium to apophosphatase rises from 0.1 g-atom/mol at pH 6.8 to a maximum of 1.8 g-atoms/mol at pH 9.2 with an apparent pK of 7.5 (Figure 1). Remarkably, the presence of zinc significantly increases magnesium binding 12-fold at pH 6.8 while shifting the pH at which the maximum is achieved from 9.2 to 8.2. This clearly demonstrates that, under optimal conditions both of pH and zinc concentration, magnesium binds to approximately two sites per dimer. Furthermore, the binding of magnesium and zinc to phosphatase is both cooperative and mutually dependent, reaffirming the essentiality of both metals to phosphatase structure and function.

Importantly, the magnesium content of native phosphatase, 1.3 ± 0.2 g-atoms/mol (Bosron et al., 1975), when isolated from *E. coli* near pH 7 at stationary phase, is consistent with the pH dependence of magnesium binding to enzyme containing approximately 4 g-atoms of zinc (Figure 1, pH 7). Thus, the failure to assign the maximal magnesium stoichiometry to enzyme isolated by conventional procedures may be considered a consequence of the conditions chosen for optimal bacterial growth, which are not the conditions for optimal binding of magnesium to alkaline phosphatase.

Based on the metal content of native phosphatase, we have previously suggested the existence of four zinc binding sites (Simpson et al., 1968; Bosron et al., 1975; Anderson et al., 1975). Both in the presence and absence of approximately 1.8 g-atoms of bound magnesium, a maximum of 4.0 g-atoms of zinc are bound with a dissociation constant that is much less than 1 μM (Figure 2). Thus, phosphatase maximally tightly binds 5.8 g-atoms of metal: 4.0 g-atoms of zinc and 1.8 g-atoms of magnesium (Figures 1 and 2). However, at alkaline pH and at high free-zinc concentrations, 10^{-4} M, i.e., conditions at the upper limit of those here employed, a reversible self-association of phosphatase to a tetramer containing 16 g-atoms of zinc/mol has been reported (Reynolds and Schlesinger, 1969b). In our hands, raising the free zinc concentrations from 13 to 130 μM, in the absence of magnesium, increases zinc binding from 4 to 6 g-atoms/mol (Figure 2, open squares), but under these conditions, the enzyme remains dimeric. Thus, in the presence of a relatively vast excess of zinc, an enzyme can be obtained that contains additional zinc atoms and, concomitantly, even changes to a tetramer. However, the conditions and results of those experiments do not relate to studies here performed either with the native dimeric enzyme or with the stoichiometrically reconstituted apoenzyme.

In assessing the meaning of such past and present work, it is critical to recall that the affinities of zinc and cobalt for the same metal-binding sites of the apoenzyme differ significantly. Spectral studies with cobalt phosphatase have demonstrated six metal binding sites per dimer; in the absence of magnesium, apophosphatase readily binds up to 6 g-atoms of cobalt (Anderson et al., 1976). Unlike the zinc-binding isotherm in the absence of magnesium but under similar conditions (Figure 2), the cobalt isotherm (not shown) does *not* differentiate between the binding affinities of these six metal sites. However, cobalt binds in excess of 4 g-atoms/mol at concentration of free Co^{2+} ions sixfold lower than that required to bind zinc in excess of 4 g-atoms/mol. Hence, given the same conditions, up to 6 g-atoms of cobalt but only 4 g-atoms of zinc bind tightly to apophosphatase.

Previous mechanistic deductions based on the spectra of enzymes substituted with from 2 to 4 g-atoms of cobalt, copper, or manganese must now be reexamined under conditions where the magnesium content of phosphatase is known (Anderson et al., 1975, 1976). Moreover, recent experiments designed to determine the effects of zinc, chelating agents, and phosphate on phosphatase structure (Hull and Sykes, 1976) have ignored the reported multiplicity of metal-binding sites (Bosron et al., 1975) and mutual interaction of magnesium and zinc (Anderson et al., 1975). Clearly, studies utilizing reconstituted enzyme whose metal content is not defined cannot be compared with those performed with enzyme fully reconstituted with the essential metal atoms or the native enzyme, and, hence, they cannot lead to mechanistic conclusions valid for native alkaline phosphatase.

Phosphate does not bind to either apo- or the magnesium phosphatase (Table I), while both the zinc enzyme and that containing both zinc and magnesium bind 1.5 to 1.6 mol of phosphate/mol. Further, the addition of magnesium does not alter the K_I for phosphate (Table II) nor does the addition of phosphate perturb the spectrum of the 4-Zn 2-Co enzyme in which cobalt is bound primarily to the magnesium sites (Anderson et al., 1976). Jointly, these data suggest that phosphate does not interact directly with enzyme-bound magnesium.

Magnesium alone does not confer catalytic activity on alkaline phosphatase (Table II), but it does regulate the zinc- or cobalt-induced restoration of activity and, perhaps, the metal-binding loci (Anderson et al., 1976). Under conditions where the enzyme contains only 2 g-atoms/mol of zinc, magnesium enhances activity fivefold to 30 units or 56% of that

which we have found maximal. By comparison, the addition of magnesium to 4-Zn phosphatase increases activity 1.4-fold to 54 units. Clearly, the coordinated interaction of both zinc *and* magnesium with *E. coli* alkaline phosphatase markedly influences and regulates the nature of the catalytic activity restored to the apoenzyme by zinc.

Previous reports of the total number of zinc atoms required to induce maximal phosphatase activity have varied from two (Simpson and Vallee, 1968; Csopak and Szajn, 1973) to four (Reynolds and Schlesinger, 1969a; Petitclerc et al., 1970; Trotman and Greenwood, 1971). The zinc-dependent restoration of activity has been found to be linear (Simpson and Vallee, 1968; Reynolds and Schlesinger, 1969a; Csopak and Szajn, 1973) or thought to be characterized by a pronounced lag (Petitclerc et al., 1970; Trotman and Greenwood, 1971). Importantly, in these studies the presence or absence of magnesium was not reported nor was its addition controlled quantitatively. Thus, the addition of magnesium to 2-Zn phosphatase results in 56% of the maximum activity (Table II, sample 4), a value similar to that obtained by Reynolds and Schlesinger (1969a). In other studies, the reactivation of phosphatase with 2 g-atoms of zinc, as determined by active-site titrations (Petitclerc et al., 1970), is similar to the 11% here obtained in the *absence* of magnesium (Table II, sample 1).

Not only is the activity dependent on both zinc *and* magnesium, but it is also a function of the chemical composition and concentration of buffers,[4] and the period of time during which these ions are incubated with apoenzyme prior to assay of the reconstituted phosphatases. For example, the restoration of activity, after addition of 2 g-atoms of zinc to apophosphatase, is time dependent, and falls off from 14 units at 5 min to 7 units after 2 h (Figure 3, open squares). Such a decrease in activity could come about in a variety of ways, which could include a redistribution of metals between catalytic, structural, and/or regulatory sites, slow conformational changes of the protein affecting activity, or all of these. Importantly, hydrogen–tritium exchange of 2-Zn phosphatase indicates such a slow conformational change (Brown et al., 1974; Anderson et al., 1975), since the decay curve approaches and then becomes coincident with that of the apoenzyme. Addition of magnesium *prior to* zinc prevents both of these potentially related phenomena (Figure 3, solid squares); moreover, addition of magnesium *after* incubation of phosphatase with 2 g-atoms of zinc totally reverses the loss of activity (Figure 3, triangles).

Previous uncertainties regarding the nature and number of zinc atoms required to restore maximal catalytic activity to apophosphatase can be attributed largely to (1) differences in the methods utilized for purification and for subsequent preparation of the apoenzyme, (2) the failure to monitor the presence of magnesium, and (3) the variation in conditions affecting the kinetics and nature of the reconstitution of apoenzyme with metals. Hence, it would seem necessary to reassess the assignment of catalytic or structural roles for metal atoms in alkaline phosphatase, based on the metal-dependent restoration of activity to apoenzyme (Simpson and Vallee, 1968; Reynolds and Schlesinger, 1969a; Petitclerc et al., 1970; Trotman and Greenwood, 1971; Csopak and Szajn, 1973). The present and previous data demonstrate that magnesium serves a major role in regulating the expression of catalytic activity and maintenance of the structural integrity of *E. coli* alkaline phosphatase. Specific substitution of metals with unique spectral properties (Anderson and Vallee, 1975; Anderson et al., 1976) or site-specific chemical modification of metal ligands (Tait and Vallee, 1966) might now result in more definitive assignments for each pair of the three metal-binding sites.

[4] The activities of phosphatase reconstituted partially with zinc alone differ when reconstitution is performed in 1 M Tris instead of 50 mM Tris (Table II) or 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (the latter two with identical results). While the reason for this difference is not clear, it might relate to the capacity of high concentrations of Tris to coordinate zinc.

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