$^{65}$Zn(II), $^{115m}$Cd(II), $^{60}$Co(II), and Mg(II) Binding to Alkaline Phosphatase of Escherichia coli

STRUCTURAL AND FUNCTIONAL EFFECTS*

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Zn(II), Cd(II), Co(II) and Mg(II) binding to apoalkaline phosphatase of Escherichia coli and the relative stabilities of the resulting metalloenzyme complexes have been measured by equilibrium dialysis and metal exchange reactions using $\gamma$-emitting isotopes of these metals. At millimolar concentrations of these metal ions the alkaline phosphatase dimer binds three pairs of metal ions (A, B, and C sites). One of these pairs dialyzes readily without detectable change in the structure or function of the enzyme (C site). Of the remaining two pairs, the binding affinity of both for Zn(II) and Cd(II) is increased by formation of the phosphoenzyme intermediate $\text{Cd}(II)$ is bound less tightly to both A and B sites than Zn(II), and at pH 6.5 Cd(II) is induced to bind to the B sites by formation of the phosphate complexes. Mg(II), 5-10 mM, competes successfully with the IIB metal ions for the second or lower affinity pair of binding sites (B sites), although Mg(II) is a relatively poor competitor on an equimolar basis, especially for Cd(II). Binding of metal ions to the apoenzyme appears to be a cooperative process involving conformational changes in the protein which are not readily reversible. The initial binding of a pair of Zn(II) or Cd(II) ions to the apoenzyme is characterized by equilibrium constants of $10^{-7}$ to $10^{-5}$ M for Zn(II) and $10^{-7}$ to $10^{-5}$ M for Cd(II). Following the cooperative binding of all three pairs of metal ions, however, re-establishment of equilibrium by dialysis indicates binding constants of $<10^{-8}$ M for Zn(II) and $<10^{-8}$ M for Cd(II) at the sites of greatest affinity (A sites). Binding of Mg(II) or Cd(II) to the B site, once the A site is occupied, increases the phosphorylation rate of the Cd(II) enzyme by 20-fold. In the presence of saturating concentrations of Mg(II) complete activity is restored to the apoenzyme by 2 Zn(II) ions. In the absence of Mg(II) as many as 6 Zn(II) ions may be required before complete restoration is achieved. Roles for the A and B site metal ions in the catalytic mechanism are discussed.

The stochiometry of Zn(II) binding to Escherichia coli apoalkaline phosphatase has been the object of a number of studies. The results show the stochiometry of the rebinding of Zn(II) to the metal-free apoenzyme to vary from 2 to 6 or more g atoms of Zn(II)/mol of dimer depending on pH and buffer conditions (1-3). The Zn(II) content of the native enzyme reported in the literature has also varied between 2 and 4 g atoms/mol of enzyme dimer, depending on the mode of preparation (1, 4, 5). The lower values appear to correlate with the use of ammonium sulfate precipitation in the absence of Zn(II)-containing buffers. The average Zn(II) content of enzyme released from E. coli by osmotic shock after treatment of the cells with EDTA and the cold water wash loaded directly onto a DEAE-cellulose column, is 2.7 to 3.5 g atoms/mol (1).

Alkaline phosphatase has an absolute requirement for Zn(II), but in common with many phosphoryl transferases the Zn(II) enzyme can be further activated by Mg(II) (6). Studies of the reactivation of the apoenzyme by metal ions have also yielded variable results concerning the minimum stochiometry required for restoration of full activity with values reported from 2 to 4 g atoms Zn(II)/mol of enzyme dimer required to restore maximal activity (2, 7-11). Part of this variability appears to derive from the fact that restoration of the "native" state from metal ions and apoenzyme is not an instantaneous process and is subject to other variables in addition to the Zn(II) stochiometry per se. The presence of Mg(II) and phosphate are additional factors which affect the restoration of activity. The presence of Mg(II) has been shown to potentiate the restoration of activity to the apoenzyme by Zn(II) and the addition of Mg(II) to enzymes containing 2 Zn(II) or 2 Co(II) ions/mol of dimer has been reported to enhance the activity of the enzymes containing 2 metal ions to that observed with excess Zn(II) or Co(II) (3, 12). The native enzyme prepared by the usual procedures contains slightly more than 1 g atom of Mg(II)/mol of dimer and has been observed to bind $\sim 1.8$ g atoms Mg(II)/mol at free Mg(II) concentrations of 0.45 mM (3). The latter binding was cooperative with that of Zn(II) at pH values below 9, i.e. maximum Mg(II) binding required the presence of Zn(II).

Studies of metal binding to alkaline phosphatase have been most extensive for Zn(II) and much less extensive for the other first transition and IIB metal ions which bind to the active center. Even for Zn(II), data on metal binding as functions of a wide range of metal and protein concentrations in the presence and absence of Mg(II) and phosphate are not available. Recent $^{31}$P and $^{115}$Cd NMR studies from this laboratory examining both the unliganded enzyme ($^{115}$Cd NMR) and its phosphoenzyme intermediates ($^{31}$P and $^{115}$Cd NMR) have shown significant changes in chemical shift of the active site $^{115}$Cd ion as well as the $^{31}$P resonance of the phosphoseryl and noncovalent phosphoenzyme intermediates depending on metal ion stoichiometry (13-15). The most striking finding

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from the \( ^{31}P \) NMR spectra was a shift of phosphate binding stoichiometry from 1 to 2 mol of bound phosphate/mol of enzyme as the Cd(II) ion stoichiometry was increased from 1 to 4 mol/mol of dimer (15). The NMR studies are distinguished from earlier investigations of phosphate binding stoichiometry by requiring protein concentrations > mm, i.e. at least an order of magnitude greater than those used in most previous studies of alkaline phosphatase. At these relatively high protein, metal ion, and phosphate ion concentrations, some sites may be saturated which are not saturated with either metal ion or phosphate at lower concentrations. Likewise, cooperative binding between first transition ions and Mg(II) or phosphate may radically alter the NMR resonances. Hence, in order to interpret the \( ^{31}P \) and \( ^{111}Cd \) NMR signals correctly, a complete understanding of the thermodynamics of metal ion binding to alkaline phosphatase is required. The present studies using \( ^{65}Zn, ^{111}Cd, \) and \( ^{60}Co \) as radioactive labels in equilibrium dialysis studies were designed to obtain this basic information.

**MATERIALS AND METHODS**

**Enzyme Preparations.**—Alkaline phosphatase was isolated from *E. coli* (strain CW3747) as previously described (16). Enzyme concentrations were determined spectrophotometrically at 278 nm with \( E_{1}^{1 \%} \) = 0.72 (17). For molar calibrations a dimer, M, of 9,000 was used based on the most recent data on the complete amino acid sequence (18). Enzyme activity was measured by the release of p-nitrophenol from p-nitrophenyl phosphate (Sigma) in 1 m Tris-HCl, pH 8, 25°C. The native enzyme had a specific activity of 2,500 ± 5% units (micromoles of substrate hydrolyzed/h/mg of protein). All buffer solutions were prepared metal-free (1).

**Apophosphatase.**—Apophosphatase was prepared by a new method designed by Dr. Peter Gettins to prepare the concentrated NMR solutions. It uses ammonium sulfate to remove Zn(II) from the enzyme as described in detail in the following paper (19). This results in the rapid production, 5 days, of apoenzyme containing <3% of the original Zn and uncontamintated with chelating agents which have shown a tendency to bind to the apoenzyme (20, 21).

**Metal Binding Studies.**—Metal binding studies using the \( \gamma \)-emitting metal were performed by Coleman and Vallee (22). Dialysis tubing was Visking-Nojax casing designed by Dr. Peter Gettins to prepare the concentrated NMR solutions (1). Metal concentrations in samples were adjusted to a range of 2 to 10 μM.

**RESULTS**

Cadmium binds more tightly to alkaline phosphatase than the native zinc ion and in the presence of excess metal ion has fewer total binding sites at comparable free metal ion concentrations. Thus, the differential binding affinities of the several pairs of potential metal binding sites on alkaline phosphatase are most easily demonstrated with \( ^{111}Cd \) binding and these data will be presented first.

**\( ^{111}Cd \) Binding to Apoalkaline Phosphatase as Functions of Phosphate, Magnesium, pH, and Cd(II) Concentration**—

Many of the studies of Cd(II) alkaline phosphatase, particularly NMR studies, have been performed at pH 6.5 (13). At pH 6.5 Cd(II) is relatively weakly bound; slightly less than 2 Cd(II) ions are bound per enzyme dimer at enzyme and Cd(II) concentrations of 5 × 10⁻⁶ M or below (Fig. 1A). Equilibrium binding as the Cd(II) concentration is lowered to 10⁻¹ M shows the apparent dissociation constant for this pair to be \(~5 \times 10^{-7}\) M. The presence of 1 mM phosphate at pH 6.5 (5 × 10⁻⁵ M Cd(II)) induces the binding of a second pair of Cd(II) ions (Fig. 1A). The bound phosphate at this pH is present entirely as the phosphoseryl intermediate (E-P). A total of 4 Cd(II) ions/enzyme dimer are bound and the dissociation constants for both pairs are 2 × 10⁻⁶ M or less (Fig. 1A).

As would be expected, both pairs of metal binding sites on the alkaline phosphatase dimer bind Cd(II) more tightly at pH 8 than at pH 6.5, and at enzyme and Cd(II) concentrations of 5 × 10⁻⁵ M, 4 Cd(II) ions are bound per dimer even in the absence of the phosphate ligand (Fig. 1B). At pH 8, two groups of Cd(II) binding can be detected as a function of concentration, one pair with a dissociation constant, \( K_{d} \approx 5 \times 10^{-7} \) M, and one pair bound \(~10^{-9} \text{ less tightly}, K_{d} \approx 5 \times 10^{-6} \) M (Fig. 1B). The presence of 10 mM Mg(II) at pH 8.0 prevents the binding of the pair with \( K_{d} \approx 5 \times 10^{-6} \) M (Fig. 1C), while the pair with a \( K_{d} \approx 5 \times 10^{-6} \) M is undisturbed by Mg(II).

The pH dependency of Cd(II) binding from pH 6.5 to 9 and at enzyme and Cd(II) concentrations of 5 × 10⁻⁵ M is shown in Fig. 1D. In the presence of the phosphate ligand the enzyme binds 4 Cd(II) ions/dimer at all pH values tested, while in the absence of the ligand, binding rises from \(~1.5 \text{ Cd(II) ions at pH 6.5 to } 4 \text{ Cd(II) ions bound per dimer at pH 8.5 to 9.0. One of the two pairs of Cd ions bound to the dimer is displaced at all pH values by 10 mM Mg(II).}

While the unliganded enzyme at pH 6.5 binds only 2 Cd(II) ions/dimer at 5 × 10⁻⁵ M free Cd(II), if higher enzyme concentrations (1.5 × 10⁻⁴ M) are used in order to measure Cd(II) binding at higher free metal ion concentrations, binding can be increased to 6 gm atoms/mol of dimer at 10⁻⁴ M Cd(II) (Fig. 1A). These two additional pairs have binding constants between \(~10^{-5} \text{ and } 10^{-3} \) M which suggest kinetic dissociation constants, \( k_{\text{on}} \), on the order of \(~10^{5} \text{ to } 10^{7} \) s⁻¹. The latter value applying if binding is assumed to be diffusion controlled, i.e. \(~10^{5} \text{ to } 10^{7} \text{ s}^{-1} \). Such exchange rates between bound and free Cd(II) can easily be in the intermediate exchange range for \( ^{111}Cd \) NMR signals of exchanging species and can have dramatic effects on \( ^{111}Cd \) NMR signals (19).

In the experiments of Fig. 1A measuring binding of \( ^{111}Cd \) to the apoenzyme as well as similar ones reported below, equilibrium was defined operationally by counting the dialysis bags every 24 h until no further increase in enzyme-bound radioactivity was observed in successive measurements separated by 24 h. Except where noted (see \( ^{65}Zn \) below), equilibrium was achieved in 24–48 h and test experiments carried to 7 days showed no further increase. The experimental points are plotted at the free metal ion concentration in


FIG. 1. $^{115}$Cd binding to apoalkaline phosphatase as a function of [Cd(II)], [HPO$_4^{2-}$], [Mg(II)], and pH. A, enzyme-bound $^{115}$Cd/dimer versus [Cd(II)] at pH 6.5 (○); plus mm HPO$_4^{2-}$ (○); and following dialysis of samples labeled at $10^{-5}$ M Cd(II) (●). B, $^{115}$Cd/dimer versus [Cd(II)] at pH 6.5 (○) and pH 8.0 (●); plus 10 mM Mg(II) (○). C, $^{115}$Cd/dimer versus pH with no addition (○) or in the presence of 1 mM HPO$_4^{2-}$ (●) or 10 mM Mg(II) (●); 5 × $10^{-5}$ M $[^{115}$Cd(II)], 5 × $10^{-5}$ M enzyme. Points are missing at pH 9 because of the accumulation with time of a dialyzable $^{115}$Cd-containing species in the dialysate bag at the very alkaline pH when P, and Mg(II) and excess Cd(II) are present. In A, B, and C, enzyme concentration was 1 × $10^{-5}$ M in the lower concentration range, 5 × $10^{-5}$ M in the midconcentration range, 2 × $10^{-4}$ M in the high concentration range. All buffers were 0.01 M Tris-0.1 M NaCl, pH 4.4.

The surrounding 50-ml dialysate at the end of the dialysis as determined by the specific radioactivity of the final dialysate, except where noted. "Metal-free" buffer refers to buffer treated as described under "Materials and Methods" prior to the addition of the specific spectrographically pure metal ion under examination either by direct addition or by dialysis of labeled enzyme against metal-free buffer.

$^{115}$Cd(II) ↔ Cd(II)$^{2+}$ Exchange at the Active Center of Alkaline Phosphatase—Binding of a second pair of Cd(II) ions induced by formation of the phosphoryl enzyme at pH 6.5 (Fig. 1A) suggests that the phosphate ligand affects protein structure in the immediate vicinity of one pair of Cd(II) binding sites. Since the dissociation constant for both pairs is decreased (Fig. 1A), the affinity of the first pair must also increase on phosphate binding. One way to assess these changes is to measure metal ion self-exchange rates, i.e. exchange of $^{115}$Cd(II) on a fully metalated enzyme for nonradioactive active Cd(II) present in the dialysate at equimolar concentrations in a 50-fold volume excess. Since the stoichiometry of Me(II) on the enzyme remains constant during such exchanges, the observed rates are primarily expected to be a function of the kinetic dissociation constant, $k_{diss}$, of the Cd(II) ion from the enzyme complex. In the absence of phosphate at pH 6.5, the enzyme binds only one pair of $^{115}$Cd(II) ions and these exchange with external Cd(II) with a half-time of ~10 h (Fig. 2). Mg(II) (30 mM) at pH 6.5 has a slight effect on this exchange, lengthening the half-time by ~2 h (Table 1). If the enzyme is first labeled with 2 $^{115}$Cd(II) ions, placed in phosphate (1 mM) and Cd(II)(1 × $10^{-5}$ M), the enzyme binds an additional pair of cadmium ions. Only the initial pair is labeled. Hence the self-exchange measures the exchange of the first pair in the presence of the second pair and the phosphoseryl residue. The half-life of this exchange increases dramatically to ~70 h (Fig. 2). If both pairs of bound Cd(II) ions are labeled by incubation with $^{115}$Cd(II) in the presence of phosphate, the exchange now divides into two distinct phases. One pair of $^{115}$Cd(II) ions exchanges rapidly (complete exchange in ~20 h), while the second pair exchanges with a half-time of ~70 h, similar to the rate for the first pair added in the presence of phosphate in the previous experiment.

At pH 9.0 $^{115}$Cd(II) ↔ Cd(II) exchange studies of the 4 Cd(II) species can be carried out both in the presence and absence of phosphate ligand, since 4 Cd(II) ions are bound in both cases (Fig. 1D). If there is not rapid intramolecular exchange of the Cd(II) ions on the enzyme surface, then it should be possible to differentially label the two pairs of sites by adding either the first or the second pair as $^{115}$Cd. The results in the presence and absence of phosphate are shown in Fig. 2. In both cases the two pairs of Cd(II) complexes with the enzyme are differentiated. The first pair exchanges slower than the second, and both pairs are stabilized by phosphate.

$^{1}$Cd(II) without an isotopic superscript will be used to designate either total cadmium or Cd(II) solutions where no radioactive isotope is present, as in the exchange of an unlabeled solution of cadmium (Cd(II)) for a labeled cadmium ($^{115}$Cd(II)) on the enzyme. While technologically $^{115}$Cd is $^{115}$Cd (designating a metastable isotope), $^{115}$Cd will be used in this paper.


The exchange half-time approximately doubles for both sites in the presence of phosphate. At pH 9.0 the phosphate is present almost exclusively as the noncovalent complex (E.P)

The exchange reaction for the $^{115}$Cd.III.AP species in the presence of phosphate is shown in Fig. 4. The nonlinear semi-log exchange plots can be broken into two linear exchange plots whose half-times correspond within the error of the measurement to those observed for the first and the second pair of $^{115}$Cd.II ions measured separately by the experiments graphed in Fig. 3. Cd.III exchange at the Cd.III binding sites of alkaline phosphatase under a variety of conditions are tabulated in Table I along with estimates of the kinetic exchange constants of the complexes, $k_{exch}$. $k_{exch}$

That these Cd.III binding sites are shared by other transition and IIB metal ions binding to alkaline phosphatase and that one pair is shared by Mg.II is illustrated by the set of order of additions experiments listed in Table II. The competing metal ions were added first and then the enzyme dialyzed against $5 \times 10^{-5}$ m $^{115}$Cd.II to label the remaining binding sites. The stoichiometry of the $^{115}$Cd.II label was determined at 24 h and after 30 days. The latter stoichiometry reflects the slow exchange of $^{115}$Cd.II with the occupied metal binding sites. Addition of 2 Zn.II ions and phosphate block both pairs of Cd.II sites at both pH values, somewhat more effectively at pH 8. The fractional binding observed reflects a minimal exchange in 24 h. Without phosphate this exchange is more rapid. Mg.II (10 mM) added to the 2 Zn.II or 2 Mn.II enzyme blocks at least 50% of the second pair of Cd.II binding sites. The combination of 2 Mn.II 2 Mg.II and phosphate is particularly effective. This is in contrast to 2 Mn.II and phosphate which does not appear particularly stable, since over 3 g atoms of $^{115}$Cd.II are bound within 24 h, while 2 Zn.II ions in the same system completely block one pair of Cd.II sites, even after 30 days (Table II). This must reflect the relative stabilities of the mixed metal-enzyme-phosphate complexes (see “Discussion”).

$^{65}$Zn Binding to Apoalkaline Phosphatase as Functions of Phosphate, Magnesium, pH, and Zn.II Concentration—$^{65}$Zn(I) binding to the apoenzyme ($5 \times 10^{-5}$ m) in the presence and absence of phosphate is shown in Fig. 2. The fractional binding observed reflects a minimal exchange in 24 h. Without phosphate this exchange is more rapid. Mg(I) (10 mM) added to the 2 Zn(I) or 2 Mn(I) enzyme blocks at least 50% of the second pair of Cd(I) binding sites. The combination of 2 Mn(I) 2 Mg(I) and phosphate is particularly effective. This is in contrast to 2 Mn(I) and phosphate which does not appear particularly stable, since over 3 g atoms of $^{115}$Cd.II are bound within 24 h, while 2 Zn(I) ions in the same system completely block one pair of Cd(I) sites, even after 30 days (Table II). This must reflect the relative stabilities of the mixed metal-enzyme-phosphate complexes (see “Discussion”).
of a 50-ml volume excess of $5 \times 10^{-5} \text{ M} \text{Zn(II)}$ as a function of pH is shown in Fig. 5A. Even in the absence of phosphate ligand, 4 g atoms of Zn(II)/mol of dimer are bound at pH 6.5. This stoichiometry remains constant to pH 7.5. At higher pH further binding occurs and $\sim 5$ g atoms of Zn(II)/mol of dimer are bound at pH 9.0. While the Zn(II) stoichiometry at pH 6.5 is not altered by mM P, the binding of extra Zn(II) at alkaline pH begins at lower pH and reaches $\sim 6.5$ g atoms/mol of dimer at pH 9.0. Enzyme-bound phosphate is almost exclusively in the form of the noncovalent complex over this pH range in the case of the Zn(II) enzyme (19). Addition of 10 mM Mg(II) has little effect on the Zn(II) binding stoichiometry at any pH (Fig. 5B). On the other hand, if the phosphate ligand is added along with 10 mM Mg(II), the Zn(II) stoichiometry is depressed by $\sim 2$ g atoms/mol of dimer at all pH values (Fig. 5B). The Zn(II) binding occurring at higher pH does not appear to be influenced by Mg(II), since the binding curve moves upward toward higher pH values in the same manner, but begins at a lower stoichiometry (Fig. 5B). The Zn(II), above 4 g atoms/mol of dimer, dialyzes very quickly (see below).

At pH 6.5 a pair of Zn(II) ions bind to apoalkaline phosphatase with a binding constant of $\sim 5 \times 10^{-7} \text{ M}$, estimated from the Zn(II) concentration required for binding of an average of 1 Zn(II)/dimer (Fig. 5C). The second pair binding at pH 6.5 has a binding constant of $\sim 5 \times 10^{-6} \text{ M}$. In contrast to Cd(II) binding at pH 6.5, 1 mM phosphate or 10 mM Mg(II) have only marginal effects on the final Zn(II) equilibrium stoichiometry (Fig. 5C). An additional finding that distinguishes Zn(II) binding from Cd(II) binding is its time dependence. The Zn(II) stoichiometry versus concentration shown in Fig. 6B is that reached after 72 h of equilibration and the stoichiometry rises slowly over this period to reach this constant value after 72 h. For example, the Zn(II) stoichiometry at $10^{-6} \text{ M} \text{Zn(II)}$ is 0.56, 1.16, and 1.6 g atoms/mol of dimer at 24, 48, and 72 h, respectively. At pH 6.5 Zn(II) is bound much more tightly to the enzyme in the absence of phosphate than

**Fig. 5.** $^{65}\text{Zn(II)}$ binding to apoalkaline phosphatase as a function of $\text{Zn(II)}, \text{HPO}_4^{2-}, \text{Mg(II)}, \text{and pH}$. A, enzyme-bound $^{65}\text{Zn(II)}$/dimer versus pH, no addition (○); plus mM HPO$^{2-}$ (●); $5 \times 10^{-5} \text{ M} \text{Zn(II)}, 5 \times 10^{-3} \text{ M}$ enzyme. B, enzyme-bound $^{65}\text{Zn(II)}$/dimer versus pH; plus 10 mM Mg(II) (●), identical data was observed without Mg(II) (○); plus 1 mM P (●); plus 1 mM P and 10 mM Mg(II) (●), 5 $\times 10^{-5} \text{ M} \text{Zn(II)}, 5 \times 10^{-5} \text{ M}$ enzyme. C, enzyme-bound $^{65}\text{Zn(II)}$/dimer versus [Zn(II)] pH 6.5, (○) no addition; (●) plus 1 mM P; (●) plus 1 mM P and 10 mM Mg(II). Corresponding Cd(II) data in the absence of P, (——); plus P, (— —). D, enzyme-bound $^{65}\text{Zn(II)}$/dimer versus [Zn(II)], pH 6.5, (○) initial labeling; (●), following dialysis of samples labeled at $5 \times 10^{-5} \text{ M} \text{Zn(II)}$ against metal-free buffer, points are plotted at final free Zn(II) concentration. (○) two samples were further dialyzed against 10 mM Mg(II). Conditions as in Fig. 1.
is the larger Cd(II) ion. Binding of phosphate, however, stabilizes the Cd(II) enzyme-complexes to the point where they appear at least as stable as those of Zn(II) (Fig. 5C).

Metal ion binding to the apoenzyme as a function of metal ion concentration cannot be interpreted as a simple reversible equilibrium process. At pH 8, Zn(II) binding reaches 4 g atoms/mol of dimer at lower concentrations of free metal than at pH 6.5, but the apparent stability of the first pair of Zn(II) ions bound is not appreciably increased by alkaline pH, a somewhat surprising result. Once 4 Zn(II) ions are bound, if the samples, initially at $10^{-5}$ M free metal, are resuspended in metal-free buffer and allowed to re-equilibrate at $10^{-5}$ to $10^{-7}$ M free Zn(II), equilibrium is reached at a much higher stoichiometry of $^{65}$Zn(II), approximately 4 g atoms/mol of dimer. The stoichiometry does not fall to even 3 g atoms/mol of dimer until $3 \times 10^{-7}$ M free Zn(II) (Fig. 5D). This suggests that the binding of a metal ion to a second site on the monomer must induce conformational changes that stabilize both the first and second metal ion complexes. Homologous and heterologous metal exchange studies with $^{65}$Zn(II) and $^{114}$Cd(II) to be shown below also suggest such cooperative changes, since they indicate large differences in the comparative stability of the fully metalated Zn(II) and Cd(II) species, differences not suggested by the initial binding stoichiometry as a function of metal ion concentration (Figs. 1 and 5).

Achievement of equilibrium in the redialysis experiments was defined operationally by changing the 50-mI dialysate to "metal-free" buffer and following the increase of radioactivity in the dialysate and the loss of enzyme-bound radioactivity at successive 24-h periods until no further changes were observed. This took 7–10 days depending on enzyme concentration, and the free metal ion concentration is plotted as determined from the final specific activity of the dialysate. If equilibrium at a lower free metal ion concentration was desired, the enzyme-containing dialysis bag was resuspended in a second 50 ml of metal-free buffer and the process repeated, as for example the $^{65}$Zn(II)-enzyme sample in Fig. 5D containing 3 g atoms of Zn(II)/mol and a free [Zn(II)] of $3 \times 10^{-8}$ M. While the above protocol was adequate, individual test experiments were continued for as long as 4 weeks, but did not result in either further increase in the binding when starting with apoenzyme or in loss of metal ion from the enzyme that had initially been fully metalated. Thus the failure to reach the same "equilibrium" in the forward and reverse directions does not appear to be due to differences in the methodology, but seems to relate to kinetic barriers to conformational change in the initial partial binding reaction, barriers removed by binding of the full complement of metal ions which results in a more stable species (see "Discussion").

Having observed this "hysteresis" effect on metal-enzyme stability by dialyzing a fully metalated Zn(II) enzyme, we repeated the experiment on Cd(II),AP and found a similar but not as dramatic increase in stability of the fully metalated enzyme (Fig. 1A). Two g atoms of Cd(II) remain bound at $5 \times 10^{-6}$ M at pH 6.5, a free Cd(II) concentration at which little would bind in the forward arm of this hysteretic binding curve. Phosphate binding remains a relatively more effective stabilizing process for the Cd(II) ion.

$^{65}$Zn(II) ↔ Zn(II) Exchange at the Active Center of Alkaline Phosphatase—Exchange of $^{65}$Zn(II) bound at the active center of the enzyme ($5 \times 10^{-7}$ M) with equimolar stable Zn(II) in the dialysate at pH 8.0 is plotted in Fig. 6. Note that exchange is always from the 4 Zn(II) species. Two examples are shown. The first (solid symbols) is $^{65}$Zn(II),AP exchanged against stable Zn(II) in the absence of ligands or Mg(II). The exchange divides dramatically into two phases. One pair of $^{65}$Zn(II) ions exchanges relatively rapidly with a half-time of ~24 h, while the second pair exchanges very slowly with a half-time of ~840 h. The analogous slow exchange for the Cd(II),AP species even at pH 8.0 is ~15 h (Table I). The open symbols in Fig. 6 illustrate the exchange of a species initially labeled with the addition of 2 $^{65}$Zn(II) ions in the presence of 10 mM Mg(II) and 1 mM P. To insure placement of the Zn(II) ions at the slowly exchanging sites. This species was then dialyzed against 1 mM P, 1 X $10^{-5}$ M Zn(II), 10 mM Mg(II). The half-time of the slowly exchanging Zn(II) species has now increased to ~2500 h. Self-exchange rates of similar magnitude for the Zn(II),AP species are observed at pH 6.5 (Table I). The binding of the phosphate ligand (still mostly as E—P for the Zn(II) enzyme at pH 6.5) stabilizes the Zn(II) at both pairs of sites (Table I). Substantially more than 2 g atoms of $^{65}$Zn(II)/dimer remain after 500 h and suggest exchange half-times of greater than 1000 h. These rates should be compared to the Cd(II) exchange under the same conditions which has a maximum half-time of ~70 h for the slowest pair in the phosphoryl enzyme and only ~10 h in the unliganded species which remains Cd(II)AP at pH 6.5 (Fig. 1). In the same manner as Zn(II) blocks $^{114}$Cd(II) binding (Table II), Cd(II) can be shown to block $^{65}$Zn(II) binding (Table II). At $5 \times 10^{-7}$ M enzyme and Zn(II), approximately 4 g atoms of $^{65}$Zn(II) are bound to the apoenzyme per mol of dimer after 24-h dialysis in the presence or absence of 1 mM P. The prior addition of 2 Cd(II) ions/mol of dimer blocks a pair of these sites. Mg(II), 10 mM, in the presence of phosphate will also block a pair for 24 h if the Mg(II) and P, are added to the enzyme prior to dialysis. After prolonged dialysis $^{65}$Zn(II) exchanges with the Mg(II). A second pair of Cd(II) ions also blocks the second pair of potential Zn-binding sites, but even by 24 h of dialysis against $^{65}$Zn(II) significant exchange has occurred (Table II).

$^{114}$Cd(II) ↔ Zn(II) Exchange at the Active Center of Alkaline Phosphatase—Ordinarily the exchange rates would be expected to reflect the kinetic metal dissociation constants and Zn(II) would be expected to replace Cd(II) at least as fast as Cd(II) replaces itself and probably faster. Such is not the case as shown in Fig. 7. $^{114}$Cd(II)AP at pH 6.5 exchanges with equinolar Zn(II) with a half-time of ~16 h, the same order of magnitude as observed for Cd(II) self-exchange. In contrast, if $^{114}$Cd(II) ,AP-P is used (induced by adding 1 mM P, at pH
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Structural and Functional Effects of Mg(I1)—In the preparation of samples of both the Zn(I1)AP and Cd(I1)AP for NMR studies at enzyme concentrations >10^{-3} M (>100 mg/ml), if 10 mM Mg(I1) is added and the enzymes then dialyzed, the Mg(I1) content rapidly falls to ~2 g atoms/mol. Mg(I1) can be removed by extensive dialysis against metal-free buffer. The precise role of Mg(I1) in the structure and function of this enzyme with two or more pairs of potential metal binding sites is more difficult to assess than that of the IIB metal ions, since it requires the prior presence of a IIB metal ion at the most stable metal binding site and for which all three metal ions compete. If the Mg(I1) concentration is raised to 10 mM it can successfully compete for this pair of sites as illustrated in Figs. 1, 5, and 8. On an equimolar basis, however, it is a poor competitor, hence stoichiometric titrations which achieve maximal Mg(I1) effects are impossible. A functional effect of Mg(I1) on the phosphorylation of cadmium alkaline phosphatase is readily demonstrated. At ~4 mM concentrations of H^3PO_4^-, Cd(I1)AP phosphorylates relatively slowly (i.e. hours) at pH 6.5 as measured by the formation of E-Cd(I1)P (Fig. 9). Final equilirium stoichiometry in such reactions in our hands has always been ~1 mol of phosphate/mol of dimer for the Cd(I1)AP (Fig. 9). The rate, but not the final stoichiometry, of phosphorylation is dramatically enhanced by the addition of Mg(I1). The half-time of phosphorylation progressively decreases from 20–30 min at 0.1 mM Mg(I1) (not significantly different from that in the absence of Mg(I1)) to ~1 min at 5 and 10 mM Mg(I1) (Fig. 9).

In contrast to Mg(I1), the stoichiometric addition of a second pair of Cd(I1) ions brings about the identical rate enhancement of the phosphorylation step, but also raises the final stoichiometry of phosphorylation to between 1.5 and 2.0 mol of E-P/mol of dimer (Fig. 9). The increase in the stoichiometry of formation of E-P by the Cd(I1)AP was first detected in the NMR samples by integrating the 31P-NMR signal (15), an integration that consistently shows ~2 mol of E-P/mol of enzyme dimer. While in some samples, especially in the presence of excess Cd(I1), determination of E-P stoichiometry by acid precipitation has indicated ~2 mol/mol enzyme dimer (15), the acid precipitable ^31P is usually below 2

40Co(I1) Binding to Apoalkaline Phosphatase—At pH 8.0 ~2 g atoms of Co(I1) are bound per enzyme dimer at a free Co(I1) concentration of 10^{-6} M (Fig. 8). As the free Co(I1) concentration is raised to 10^{-3} M, the stoichiometry of enzyme-bound Co(I1) rises to ~10 g atoms/mol of dimer. This large stoichiometry of nonspecific metal ion binding at alkaline pH is not unique to Co(I1). At pH 8.0, if the Cd(I1) concentration is raised to 10^{-3} M, the stoichiometry is also ~10 rather than 6 as observed at pH 6.5 (Fig. 1). Enzyme-bound Co(I1) above 4 g atoms/mol of dimer is fairly loosely bound, since it is readily removed by dialysis against metal-free buffer and the stoichiometry falls to ~4 g atoms of Co(I1)/mol of enzyme dimer. In contrast to the earlier figures for Zn(I1) and Cd(I1), the points representing stoichiometry after dialysis are plotted on the x axis at the position corresponding to the original labeling concentration to illustrate the fraction of Co(I1) dialyzed from each sample. Final free Co(I1) concentration in each was <10^{-5} M. Of the 4 nondialyzable bound Co(I1) ions, two are removed by dialysis if 10 mM Mg(I1) is added to the dialysate.

Conditions: 5

Metal Ion Binding to Alkaline Phosphatase

40Co(II) binding to apoalkaline phosphatase versus [Co(II)] at pH 8.0. Enzyme-bound ^40Co(II)/dimer after 76 h of labeling (a). Same samples after 48 h of dialysis against metal-free buffer (b). Same samples after 48 h of dialysis against metal-free buffer containing 10 mM Mg(I1) (c). Conditions: 1 x 10^{-5} M enzyme from 10^{-5} to 10^{-3} M Co(II), 5 x 10^{-2} M enzyme from 10^{-5} to 10^{-2} M Co(II), and 2 x 10^{-4} M enzyme from 10^{-5} to 10^{-2} M Co(II), 0.01 M Tris-HCl 0.1 M NaCl pH 8.0, 4 °C.
Metal Ion Binding to Alkaline Phosphatase

Alkaline phosphatase can potentially bind three pairs of metal ions over a free metal ion concentration range from $10^{-8}$ M to $10^{-3}$ M (Figs. 1 and 5). The magnitudes of the binding constants of the successively more weakly bound pairs are not sufficiently different under many conditions to resolve the binding curves for each of the three pairs of metal ions. Because of this one might raise the question of whether metal ion binding to the apoenzyme produces a strictly homogeneous species (each type of site fully occupied) until all 6 metal ions are bound. While this is clearly a potential problem, several conditions of metal ion binding can be found which enable one to distinguish the properties of the different pairs during less than maximal metal ion binding. These distinguishing features will be used to designate the pairs of sites as A, B, and C in decreasing order of binding affinity. From the direct binding curves for $^{113}$Cd(II), apocatalase phosphatase binds approximately two Cd(II) ions at $5 \times 10^{-3}$ M Cd(II) and pH 6.5 (Fig. 1). Since the binding of the first two Cd(II) ions in the absence of phosphate is part of a smooth binding curve achieving a stoichiometry of 6 Cd(II) ions/dimer at $10^{-3}$ M Cd (Fig. 1A), this data alone cannot identify the first two as a unique pair. The following additional data has to be used to argue that these represent homogeneous A-site Cd(II). A second pair of Cd(II) ions is induced to bind by phosphate, hence one of the pairs is not binding in the $<10^{-3}$ M Cd(II) range. This latter pair involves binding to sites whose affinity is markedly increased by formation of the phosphoseryl intermediate (Fig. 1) and will be designated the B sites. The enzyme at pH 6.5 with only two Cd(II) ions bound per dimer shows a single $^{113}$Cd(II) NMR signal corresponding to both $^{113}$Cd(II) ions as will be detailed in the following paper (19). Thus, a unique pair of sites is occupied, which will be defined as the A sites.

Not unexpectedly both A- and B-site Cd(II) complexes are made more stable by raising the pH (Fig. 1), but only the less stable pair is successfully competed for by Mg(II) (Fig. 1C). Competition with Mg(II) is thus another operational definition of the B-site Cd(II) ions. Once the stable homogeneous 4 Cd(II) species is formed it is easy to demonstrate by the time dependence of the $^{113}$Cd(II) ↔ Cd(II) self-exchange that there are two types of site present characterized by differential exchange constants (Figs. 2–4, Table I). While both sites are stabilized by the presence of the phosphate ligand (either as E-P, pH 6.5 or E-P, pH 9.0), they are still distinguished by different exchange rates in the phosphate complexes and the sites can be differentially labeled by the order of addition of pairs of $^{113}$Cd(II) and stable Cd(II) ions (Fig. 3).

While the “on” constants for the formation of these enzyme-metal complexes may be smaller than the diffusion limit, it still seems reasonable that the slow step, and the one accounting for the major difference in relative stabilities of a series of metalloalkaline phosphatases or their phosphoenzyme forms, is a relatively small “off” constant. The kinetic constants characterizing the self-exchange reactions can be used to distinguish the Cd(II) complexes of the A and B sites. At pH 9.0 where both A and B sites are occupied in the unliganded enzyme the tight Cd(II) site has a $k_{on}$ of $4 \times 10^{-6}$ s$^{-1}$, while the second site has a $k_{on}$ of $8 \times 10^{-4}$ s$^{-1}$ (Table I). While formation of E-P (pH 6.5) or E-P (pH 9.0) stabilizes both A and B sites (Table I), the relatively large dissociation constant for the B site results in induction of binding to the B site by the phosphate ligand at low pH (Fig. 1A). There is a functional consequence of this, since binding of Cd(II) or Mg(II) to the

Although this number is somewhat variable depending on conditions.

**DISCUSSION**

Alkaline phosphatase can potentially bind three pairs of metal ions over a free metal ion concentration range from $10^{-8}$ M to $10^{-3}$ M (Figs. 1 and 5). The magnitudes of the binding constants of the successively more weakly bound pairs are not sufficiently different under many conditions to resolve the binding curves for each of the three pairs of metal ions. Because of this one might raise the question of whether metal ion binding to the apoenzyme produces a strictly homogeneous species (each type of site fully occupied) until all 6 metal ions are bound. While this is clearly a potential problem, several conditions of metal ion binding can be found which enable one to distinguish the properties of the different pairs during less than maximal metal ion binding. These distinguishing features will be used to designate the pairs of sites as A, B, and C in decreasing order of binding affinity. From the direct binding curves for $^{113}$Cd(II), apocatalase phosphatase binds approximately two Cd(II) ions at $5 \times 10^{-3}$ M Cd(II) and pH 6.5 (Fig. 1). Since the binding of the first two Cd(II) ions in the absence of phosphate is part of a smooth binding curve achieving a stoichiometry of 6 Cd(II) ions/dimer at $10^{-3}$ M Cd (Fig. 1A), this data alone cannot identify the first two as a unique pair. The following additional data has to be used to argue that these represent homogeneous A-site Cd(II). A second pair of Cd(II) ions is induced to bind by phosphate, hence one of the pairs is not binding in the $<10^{-3}$ M Cd(II) range. This latter pair involves binding to sites whose affinity is markedly increased by formation of the phosphoseryl intermediate (Fig. 1) and will be designated the B sites. The enzyme at pH 6.5 with only two Cd(II) ions bound per dimer shows a single $^{113}$Cd(II) NMR signal corresponding to both $^{113}$Cd(II) ions as will be detailed in the following paper (19). Thus, a unique pair of sites is occupied, which will be defined as the A sites.

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While the “on” constants for the formation of these enzyme-metal complexes may be smaller than the diffusion limit, it still seems reasonable that the slow step, and the one accounting for the major difference in relative stabilities of a series of metalloalkaline phosphatases or their phosphoenzyme forms, is a relatively small “off” constant. The kinetic constants characterizing the self-exchange reactions can be used to distinguish the Cd(II) complexes of the A and B sites. At pH 9.0 where both A and B sites are occupied in the unliganded enzyme the tight Cd(II) site has a $k_{on}$ of $4 \times 10^{-6}$ s$^{-1}$, while the second site has a $k_{on}$ of $8 \times 10^{-4}$ s$^{-1}$ (Table I). While formation of E-P (pH 6.5) or E-P (pH 9.0) stabilizes both A and B sites (Table I), the relatively large dissociation constant for the B site results in induction of binding to the B site by the phosphate ligand at low pH (Fig. 1A). There is a functional consequence of this, since binding of Cd(II) or Mg(II) to the
B sites accelerates the phosphorylation rate dramatically (Fig. 9). In view of the dramatic stabilization of both A and B-site Cd(Ii) by the phosphate ligand, it is not certain which site is the most stable to exchange in the phospho-derivatives. There is data from both ⁴¹Cd and ³¹P NMR to suggest that once the enzyme is phosphorylated the A site becomes most labile to exchange (19, 30).

The two pairs of sites found for Cd(Ii) also represent two pairs of Zn(Ii) binding sites, since Cd(Ii) binding is almost completely blocked by 4 Zn ions, a block which is made more effective by the phosphate ligand (Table II). The second pair of sites also appears to be shared by Mg(Ii), since addition of 10 mM Mg(Ii) and phosphate to a 2 Zn(Ii) enzyme partially blocks the binding of ¹¹¹Cd(Ii) to the second pair. There is apparently a special stability of the mixed Mn(Ii₃)Mg(Ii₂) phosphatezyme. While the Mn(Ii) enzyme has been extensively characterized by ESR and 2 Mn(Ii) ions shown to occupy unique low symmetry sites (27), 2 Mn(Ii) ions alone are not very effective in blocking ¹¹¹Cd(Ii) binding (Table II). In contrast the combination of Mn(Ii) and Mg(Ii) in the phosphatezyme is especially effective (Table II). This suggests that the stability of metal complexes at both sites is critically influenced by occupancy of the second site and by the presence of the phosphate ligand and perhaps by the properties of the metal ion as well, e.g., ionic radius. The ready formation and characterization of stoichiometric 4 metal enzymes with both Zn(Ii) and Cd(Ii) tends to emphasize the roles of these ions in the B sites. Yet under physiological conditions the concentrations of free metal ion in cell-free fluids (~0.01 mM for Zn(Ii) and 5-10 mM for Mg(Ii) (25, 26)) may favor Mg(Ii) as the B site metal ion.

X-ray diffraction studies of crystals of the Cd(Ii) phospho-enzyme in the presence of 50 mM Cd(Ii) have recently identified three closely spaced Cd(Ii) binding sites on each monomer, the second and third separated from the catalytic or A site by ~4 and ~5 Å, respectively (28). The catalytic or A site contains histidyl ligands and appears to correspond to that identified by Cu(Ii) ESR to contain three equivalent nitrogens as ligands to the Cu(Ii) ion (29). A second pair 3.9 Å away is close to seryl 102 and would appear to the site to which Cd(Ii) binding is induced by phosphate at low pH.

The dialysis of Zn(Ii) and ⁴¹Cd(Ii) into samples of the apoenzyme indicate rather large dissociation constants for even the more tightly bound metal ions, i.e., not less than 2 x 10⁻²⁻ M for Zn(Ii) (Fig. 5). This led us to consider the possibility that binding of the second pair of metal ions might result in conformational changes which further stabilized the first pair. Such a conformational change appears to take place, since if one re-establishes equilibrium by redialysis of the apo-Zn(Ii)AP species against metal-free buffer and measures the achievement of equilibrium by following radioactivity in the dialysate, a much smaller apparent dissociation constant is indicated, i.e., ~10⁻¹⁻ M even for the second pair (Fig. 5D).

Such data imply that binding of metal ions at one of the three sites in each monomer influences the dissociation constants of the metal ions at the other two sites. The phosphate ligand has an even more striking ability to stabilize these metal-enzyme complexes (Figs. 1, 2, and 6), a fact confirmed by the effect of phosphate binding on the ¹¹¹Cd NMR signals from these sites as shown in the following paper (19). In view of the close spacing of the three metal binding sites, less than 10 Å apart, and the direct coordination of the phosphate of E·P to the A site Cd(Ii) ion as shown in the following two papers (19, 30), it is not surprising that there is positive cooperativity between the three metal ion sites, particularly the first two (A and B), and the phosphate ligand. While behavior of this kind may not be unexpected for a multinuclear chelate complex undergoing formation of a mixed complex with phosphate, the cooperativity of metal binding to the alkaline phosphatase monomer can also be shown by the ¹¹¹Cd and ³¹P NMR methods (19, 30) to involve interactions between the subunits of the dimer. For example, the binding of phosphate to Cd(Ii)·AP is shown to be associated with migration of Cd(Ii) from the A site of one monomer to the B site of the phosphorylated active center of the opposite monomer, a migration which requires the presence of subunit-subunit interactions to produce the relative destabilization of the A site of one of the monomers (19).

The metal exchange reactions of the fully metalated phospho-enzyme are extremely slow (Figs. 2 and 6, Table I) and can account for the slow dissociation of metal ions in dialysis experiments involving fully metalated enzyme. The failure in many instances, however, to bind a full complement of metal ions to the apoenzyme except in the presence of phosphate or at higher metal ion concentrations than expected from the exchange experiments, may relate to the existence of several conformational states of alkaline phosphatase which are of similar stability, but interconvert very slowly. The kinetic barriers which separate these states can be overcome by additional binding of pairs of metal ions or the phosphate ligand. Once established, the final conformation does not appear to be readily reversed. On the other hand, if the apoenzyme is binding less than a full complement of metal ions two conformational states of the protein may exist simultaneously and not interconvert except on a time scale of days. While the radioactive metal binding cannot prove the existence of several conformational states, the chemical shifts, coupling, and exchange broadening of ¹¹¹Cd(Ii) NMR signals (and in many instances ³¹P NMR signals) can easily discriminate between different conformational states of the enzyme and show that several can exist in the same sample. These species interconvert very slowly if at all despite the fact that a pathway for interconversion must exist (19).

The catalytic roles of two closely spaced metal ions at the active center of alkaline phosphatase remain speculative at the present time. One of the roles of Zn(Ii) is clearly to produce conformational changes which result in phosphate binding as has been documented previously (16). Another possible role would be to generate the nucleophile, either coordinated OH⁻ or H₂O, required to hydrolyze the phosphoseryl intermediate. In addition, it seems highly probable, in common with phosphorly transfer enzymes in general, that charge neutralization at the negative phosphate diion is one of the functions of the metal ion in order to facilitate a nucleophilic attack of the seryl hydroxyl on the incoming phosphorus atom. Spatial relationships alone could account for a mechanism of advantage to separating these two functions by incorporating two metal ions. Indeed, binding of Cd(Ii) or Mg(Ii) at the B site accelerates phosphorylation, and as will be shown in the following paper (19) the phosphate of E·P coordinates one, but not both Me(Ii) ions at the active center. The charge/radius ratio of the particular pair of metal ions occupying the A and B sites may significantly influence the
topology and stability as well as the catalytic efficiency of the resultant metalloenzymes and may account for the slow turnover of the Cd(II) enzyme, the alteration in apparent pK_a of phosphorylation-dephosphorylation (see Ref. 19), and the differences in stoichiometry of phosphate binding (negative cooperativity) between the homogeneous IIB metal enzymes and the Mg(II) hybrids.

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