Zn(II)-$^{113}$Cd(II) and Zn(II)-Mg(II) Hybrids of Alkaline Phosphatase

$^{31}$P AND $^{113}$Cd NMR

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Peter Gettins* and Joseph E. Coleman

From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510

Methods have been developed for the addition of different metal ion species to the three distinct pairs of metal sites (A, B, and C) found in the dimer of alkaline phosphatase. This allows the preparation of hybrid alkaline phosphatases in which A and B sites of each monomer contain two different species of metal ion or the A and B sites of one monomer contain the same species of metal ion, while the adjacent monomer contains a second species. The following hybrids have been characterized in detail: (Zn(II), Cd(II))$_2$ alkaline phosphatase, (Zn(II), Mg(II))$_2$ alkaline phosphatase, (Cd(II), Zn(II))$_2$ alkaline phosphatase, (Cd(II), Cd(II))$_2$ alkaline phosphatase, and (Zn(II), Cd(II))$_2$ alkaline phosphatase.

$^{31}$P and, where appropriate, $^{113}$Cd NMR have been used to monitor the behavior of the covalent (E-P) and noncovalent (E-P) phosphor intermediates and of the A and B metal ions. From the pH dependencies of the E-P $\equiv$ E + P, it is clear that A site metal is the dominant influence in the dephosphorylation of E-P and may have a coordinated water molecule, which ionizes to ZnOH$^-$ at a low pH providing the nucleophile for dephosphorylation. A site metal also serves to coordinate phosphate in the E-P complex. B site metal has a much smaller effect on dephosphorylation rates, although it does dramatically alter the P$^-$ dissociation rate, which is the rate-limiting step for the native enzyme at alkaline pH, and is probably important in neutralizing the charge on the phosphoseryl residue, thus potentiating the nucleophilic attack of the OH$^-$ bound at A site. Phosphate dissociation is slowed markedly by replacement of B site zinc by cadmium. There is clear evidence for long range effects of subunit-subunit interactions, since metal ion and phosphate binding at one active center alters the environments of A and B site metal ions and phosphoserine at the other active site.

$^{113}$Cd when substituted for zinc at the metal binding sites of the zinc metalloenzyme alkaline phosphatase of Escherichia coli shows a maximum of three separate $^{113}$Cd(II) binding sites on each monomer as indicated by three separate $^{113}$Cd NMR resonances (153, 72, and 3 ppm) in the phosphorylated $^{113}$Cd(II)$\cdot$AP dimer (1). The crystal structure of Cd(II)AP has now confirmed that there are three closely spaced Cd(II) ions (A, B, and C) at each active center of the dimeric enzyme (2) (see Fig. 1 of preceding paper (24)).

From previous NMR studies it is known that A site metal serves to coordinate the phosphate group in the noncovalent Michaelis complex E-P, whereas any charge neutralization by B site must be less direct (3). Studies of the pH dependence of the equilibria E-P $\equiv$ E + P, indicated that an additional role of the metal ions is to generate suitably positioned OH$^-$ at a low enough pH to be mechanistically useful (3). The metal ion responsible for this, however, could be either A or B, since A site metal is no longer coordinated to phosphate at the point where OH$^-$ would be required for dephosphorylation of the noncovalent E-P intermediate. The phosphate dissociation rate from the E-P complex is also influenced by both the A and B site metal ions. We report here the properties of several mixed metal hybrids of alkaline phosphatase in which A and B sites contain different metal ions (zinc, cadmium, or magnesium). The $^{31}$P chemical shifts and $^{113}$Cd-O-$^{31}$P coupling of the E-P and E-P intermediates and changes in the pH dependence of the E-P equilibrium for these different hybrids reveal much about the function of the separate metal binding sites. In addition, the hybrid containing 2 each of cadmium and zinc, one monomer containing zinc and the other cadmium, was prepared with a view to investigating further the phenomenon of subunit-subunit interaction, since the $^{31}$P signals from the intermediates are distinct for the Zn(II) and the Cd(II) monomers.

MATERIALS AND METHODS

Enzyme Preparation—Alkaline phosphatase was isolated from an overproducing strain of E. coli, CW3747, by osmotic shock and purified as previously described (4). Enzyme concentrations were determined spectrophotometrically at 278 nm using $E_{10}$ = 0.72 (5) and a molecular weight of 94,000 for the dimer calculated from the amino acid sequence (6). The activity of the enzyme was determined by the hydrolysis of p-nitrophenyl phosphate in 1 M Tris-HCl at pH 8, 22 °C (7). Native enzyme had a specific activity of 2,500 ± 500 µmol of substrate hydrolyzed/h/mg of enzyme. All buffer solutions used were made metal-free by treatment with dibutyl ether (8) and contained 0.01 M Tris-HCl, 0.01 M sodium acetate, and 0.1 M NaCl. Apoenzyme was prepared by dialysis against 2 liters of 2 M (NH$_4$)$_2$SO$_4$, pH 9, with two changes of dialysate followed by dialysis against 3 × 2 liters of desired buffer to remove ammonium sulfate. Concentration of samples was carried out at 4 °C in a metal-free Amicon ultrafiltration cell using a PM30 membrane. The desired metal ion compositions were achieved by addition of stoichiometric amounts of stock solutions of ZnSO$_4$ or $^{113}$Cd (CH$_3$COO)$_2$, the latter prepared from 95% isotopically enriched metal oxide (Oak Ridge National Laboratory). Metal ion concentrations of reconstituted metalloenzyme were

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$\ddagger$ Present address, Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, TN 37232.

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RESULTS

(Zn(II)ACd(II)B)2 Alkaline Phosphatase—As with preparation of all mixed metal or mixed isotope hybrids of alkaline phosphatase there are two major difficulties in achieving the desired metal ion distribution. Firstly, the proximity of the A and B sites to one another (4 Å) and their rather similar affinities for zinc and cadmium results in facile intersite metal ion rearrangement at rates which vary as a function of precise experimental conditions. Secondly, in the case of zinc the affinities for A and B sites, while different, are both sufficiently high to produce metastable kinetic distribution of zinc when less than sufficient metal is added to saturate both types of site. Several attempts to produce Zn(II)Cd(II) hybrids have therefore produced largely heterogeneous products rather than the desired species. An example of this is given in Fig. 1. Two equivalents of zinc were added to apoenzyme at pH 8 in the presence of 10 mM MgCl2; the latter was present to block B sites to Zn(II), since magnesium binds to B but not A sites. The sample was dialyzed against buffer at pH 6 in the presence of 1 mM Pi. To this was added 2 eq of 113Cd(II) and 1 eq of phosphate. The 110Cd NMR spectrum showed no discernible resonances, while the 31P spectrum shown in Fig. 1. has four peaks. The most upfield resonance is a composite of free P, and a peak with the chemical shift of E-P formed by the Zn(II)Mg(II)2AP (see below). Further downfield, the largest resonance has the chemical shift of E-P formed by the Zn(II)2Mg(II)2AP (3). In the region of normal E-P resonances (~8 ppm), there is a broad peak, which appears to be a composite of several resonances. The fourth most downfield resonance is a singlet at 12.6 ppm. A resonance at this position has previously been shown to be characteristic of the E-P resonance of Cd(II)AP, but in the latter case it is a doublet due to a 30 Hz 110Cd-O-31P coupling from direct coordination of the phosphate oxygen to the A site metal ion (3, 9). In addition, for Cd(II)AP no E-P intermediate forms below pH 8. Thus this appears to be an E-P in which the phosphate is coordinated to Zn in a Zn(II)Cd(II)AP hybrid. From the area of the resonance, it represents no more than 20% of the total phosphate bound.

A more successful preparation of the Zn(II)Cd(II)AP hybrid started from Zn(II)Cd(II)AP rather than the apoenzyme and employed removal of metal ions to a stoichiometry of Zn(II)AP by dialysis against o-phenanthroline at pH 5.7. The advantage of this method lies in the slightly greater affinity of A site for Zn(II) so that Zn(II) is left preferentially in A sites. After dialysis and removal of o-phenanthroline, an average stoichiometry of 1.5 eq of zinc/dimer was found. This was brought back to 2 eq/dimer by adding 0.5 eq of Zn(II). Assuming a statistical distribution of the final 0.5 eq and a pairwise binding, as has always been found (10-12) a final composition of approximately 19% Zn(II)AP, 69% Zn(II)MAAP, and 12% apotalkaline phosphatase should result. Addition of 2 eq of 113Cd would thus not be expected to give a pure Zn(II)Cd(II)AP hybrid, although this should be the major species. Resonances from homo-cadmium and homo-zinc species would also be expected. The 31P spectrum at pH 6.2 (Fig. 2) shows five resonances, the most upfield is due to free P, the resonance at 4.3 ppm is from E-P formed by the Zn(II)AP species, the resonance at 8.7 ppm is in the position expected for E-P formed by Cd(II)AP, while a second E-P resonance is present at 8.0 ppm and the fifth resonance is again a singlet at 12.66 ppm. The pH of the sample was then changed in steps of about 0.5 unit, both lowered and raised and the 31P and 113Cd spectra recorded. The changes in the 31P spectra show that the pair of resonances at 8.0 ppm and 12.66 ppm are related; their amplitudes vary inversely (Fig. 2). If these amplitudes are plotted as a function of pH, two curves similar in shape to those describing the E-P = E-P equilibria for Zn(II)AP and Cd(II)AP are found (3), although the pH at which [E-P] = [E-P] is shifted to a far lower pH, 6.2, than for Cd(II)AP of any stoichiometry (Fig. 3). The inverse loss of the E-P resonance at 8.0 ppm and gain in the E-P resonance at 12.66 identifies the former as the resonance of E-P formed by the hybrid. Above pH 8.0 a resonance from inorganic phosphate begins to appear as E-P dissociates from the hybrid. This latter behavior of the Zn(II),Cd(II)AP hybrid is much like the homogeneous Zn(II) species, but is exhibited by the Cd(II) species only above pH 9 or 10.

110Cd spectra of this sample have detectable resonances only at low pH (Fig. 4). At pH 5.8 the most prominent resonance is at 30 ppm under conditions where most of the hybrid is in the form of E-P. The chemical shift of this resonance identifies it as almost certainly arising from 110Cd bound at B site. The upfield perturbation must arise from the unique conformation of the metal sites in the Zn(II)Cd(II) hybrid in the E-P form. The resonances numbered 1, 2, and 3 arise from A, B, and C site Cd(II) ions in the small Cd(II)E-P contaminant. Raising the pH causes the broadening and disappearance of the major cadmium resonance (Fig. 4). This is probably due to an exchange broadening mechanism in the E-P form. Despite this, the species once formed is very stable and requires extensive incubation with excess Cd(II) before there is significant displacement of Zn(II) from this hybrid.

Using a DANTE pulse sequence (13) to achieve selective inversion of the P resonance, the exchange rate between E-P and P has been determined.4 The value for 1/2π is 2-4 s−1, which is too slow for phosphate association/dissociation to be

4 P. Gettins and J. E. Coleman, unpublished data (manuscript in preparation will contain data on inversion transfer assay of phosphate dissociation rates for a variety of metalalkaline phosphatases).
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**FIG. 2.** 80.9 MHz $^{31}$P NMR spectra of 1.80 mM alkaline phosphatase containing 2 eq each of Zn(II) and $^{114}$Cd(II) and excess inorganic phosphate as a function of pH. Each spectrum is the average of 10,500 scans collected at 293 K with a 30° pulse, a repeat time of 0.8 s and 2 watt broad band proton decoupling. Line broadening of 10 Hz was used.

**FIG. 3.** Relative equilibrium concentrations of phosphorus species for (Zn(II)$_2$Cd(II)$_2$) alkaline phosphatase as a function of pH. The concentrations of each species, E-P, E-P, and P$_i$, were determined from integration of the relevant resonances of the $^{31}$P spectra in Fig. 2. The solid lines are for visual aid only.

The modulation responsible for the exchange broadening of the cadmium resonances.

Cooling the sample to 4 °C in an attempt to slow down the exchange and sharpen the resonances had no effect. Addition of a second pair of cadmium ions at pH 9 also resulted in no large increase in resonance intensity, whereas a subsequent pair produced a very prominent resonance at 105 ppm which represents free Cd(II), the chemical shift of which may be modified by exchange with enzyme-bound Cd(II).

(Cd(II)$_2$Zn(II)$_2$)$_2$ Alkaline Phosphatase—In contrast to being able to generate a stable (Zn(II)$_2$Cd(II)$_2$)$_2$AP hybrid, the reverse (Cd(II)$_2$Zn(II)$_2$)$_2$AP hybrid appears to be unstable and readily undergoes metal ion rearrangement. It is very easy to generate Cd(II)$_2$AP with cadmium occupying both A sites. This can be achieved by addition of 2 eq of cadmium to apoenzyme below pH 7 as has been well documented (14, 15), since the relative affinities of A and B sites at this pH and only in the absence of phosphate greatly favor A site occupancy. Subsequent addition of 2 eq of zinc, however, either before or after addition of phosphate, results in only transient appearance of unusual $^{31}$P resonances attributable to the desired hybrid. Three $^{31}$P spectra collected successively after addition of 2 eq of Zn(II) to a Cd(II)$_2$AP sample to which 2 eq of phosphate had been added 2 h prior to zinc addition are shown in Fig. 5. Phosphate increases metal ion affinities at both A and B sites (11). The resonances at 8.4 ppm and 4.2
with chemical shifts of 3.4 ppm and 4.2 ppm expected for E·P formed by \( \text{Zn(II)}_2\text{Mg(II)}_2\text{AP} \) and \( \text{Zn(II)}_1\text{AP} \) species, respectively (9). Peak 3 is inorganic phosphate whose chemical shift is influenced by the presence of \( \text{Mg(II)} \).

Raising the pH causes the affinity of phosphate to fall (3) so that the free \( \text{P} \), resonance grows above pH 8 as the E·P resonance declines. The complete pH profile for E·P, E·P, and P, formed by \( \text{Zn(II)}_2\text{Mg(II)}_2\text{AP} \) is shown in Fig. 7. At pH 5.4 only a very small resonance due to E·P is visible at 8.54 ppm. From this it can be estimated that the pH at which \([E·P] = [E·P]_0\) is close to pH 4 compared with pH 5 for \( \text{Zn(II)}_2\text{AP} \) and \( \text{Zn(II)}_2\text{Mg(II)}_2\text{AP} \) (3).

\( \text{Zn(II)}_2\text{Zn(II)}_2\text{Cd(II)}_2\text{Cd(II)}_2\text{AP} \) Alkaline Phosphatase—Both \( {\text{\textsuperscript{111}}\text{Cd}} \) and \( {\text{\textsuperscript{31}}\text{P}} \) NMR have previously given strong experimental evidence for the existence of subunit-subunit interaction in alkaline phosphatase (1, 3). In order to get further evidence of modulation of one active center by changes at the active center of the opposite monomer, we prepared enzyme in which the A and B sites of one subunit contained only zinc, while the other contained only cadmium. It was hoped that it might be possible to follow the effects of E·P on E·P interconversion in one subunit on the phosphate binding properties of the other, since zinc and cadmium alkaline phosphatases not only give distinguishable E·P and E·P resonances, but also have different pH profiles for the equilibria E·P = E + P. There are ranges of pH where zinc alkaline phosphatase would be expected to exist solely as E·P and cadmium alkaline phosphatase would exist solely as E·P, i.e. between pH 6 and 7.

The hybrid was prepared in the following way. Two equivalents of cadmium were added to apoenzyme at pH 6 followed by phosphorylation using 2 eq of inorganic phosphate. Under these conditions it is known that cadmium initially binds only to A sites of each monomer (1, 14, 15) and that phosphorylation of only one subunit then slowly occurs. Subsequent to phosphorylation there is a rearrangement of cadmium to give a dimer containing one phosphorylated subunit with both A and B sites occupied by cadmium and one subunit devoid of cadmium or phosphate (1). \( {\text{\textsuperscript{111}}\text{Cd}} \) and \( {\text{\textsuperscript{31}}\text{P}} \) spectra (not shown) were recorded during the 6-day phosphorylation and subsequent migration to confirm the expected rearrangement (1, 14). The pH was then raised to 7.8 and 2 eq of zinc added. Both \( {\text{\textsuperscript{31}}\text{P}} \) and \( {\text{\textsuperscript{111}}\text{Cd}} \) spectra were recorded and are shown in Fig. 8. Prior to Zn(II) addition the A and B site cadmium resonances in the monophosphoryl enzyme occur at 142 and 66 ppm, respectively. The enzyme is wholly in the form of E·P as shown by the single bound \( {\text{\textsuperscript{31}}\text{P}} \) resonance at 7.8 ppm (Fig. 8a). After Zn(II) has been added, both spectra change dramatically. In addition to the appearance of a new \( {\text{\textsuperscript{31}}\text{P}} \) resonance at 4.3 ppm due to E·P formed on the zinc-containing monomer, there is a shift downfield of 0.8 ppm in the E·P resonance from the cadmium-containing subunit (Fig. 8b). In the \( {\text{\textsuperscript{111}}\text{Cd}} \) spectrum both A and B resonances are shifted downfield by Zn(II) binding in the adjacent subunit; the A site resonance moves by 4 ppm to 146 ppm and the B site resonance moves by 26 ppm to 76 ppm. Once formed at pH 6, the phospho(\( \text{Cd(II)}_1\text{Cd(II)}_2\text{AP} \) (Zn(II),Zn(II)AP) is reasonably stable, but it is not stable to large manipulations of the pH. If it is titrated to pH 9 and back the \( {\text{\textsuperscript{31}}\text{P}} \) spectra show scrambling of Zn(II) and Cd(II) between subunits and the \( {\text{\textsuperscript{111}}\text{Cd}} \) resonances are lost at pH 7.6, a property characteristic of the \( \text{Zn(II)}_2\text{Cd(II)}_2\text{AP} \) hybrid (in one subunit) documented above.

DISCUSSION

Stability of Mixed Metal Alkaline Phosphatase—From the attempts described above to prepare well-defined mixed metal
hybrids of alkaline phosphatase, it is clear that some are unstable because of high and relatively similar metal ion affinities for both A and B sites or because of higher affinity of a particular metal ion species for the adjacent site rather than the one it occupies, (Cd(II)\_A Zn(II)\_B)\_2 AP for example. Zinc(II) binds more tightly to A site than does Cd(II) or Mg(II) (11), which is reflected in both (Zn(II)\_A Cd(II)\_B)\_2 AP and (Zn(II)\_A Mg(II)\_B)\_2 AP being indefinitely stable. Cadmium(II) binding is generally more labile than Zn(II) binding, allowing a solvent molecule to coordinate the A site metal ion.

The Role of A Site Metal Ion in Generation of a Metal-coordinated Hydroxide Ion—The pH dependence of the E-P \( \rightleftharpoons \) E-P equilibrium is determined by a metal-dependent ionization which we have previously assigned to the formation of OH\(^-\) from water coordinated to one of the two metal ions, A or B, adjacent to serine 102 (2). The hydroxide may be the nucleophile for dephosphorylation of serine 102. For (Zn(II)\_A Cd(II)\_B)\_2 AP the usual pattern of shift in equilibrium from E-P to E-P to E + P is seen as the pH is raised (Fig. 3). The midpoint for the E-P \( \rightleftharpoons \) E-P conversion is about pH 6.2 compared with pH 5 for Zn(II)\_A or Zn(II)\_A Mg(II)\_B AP, and pH 4 for (Zn(II)\_A Mg(II)\_B)\_2 AP (Fig. 7). In contrast the midpoint of this conversion is pH 8.7 for Cd(II)\_A AP and pH 10 for Cd(II)\_B AP. These data suggest that A site metal has the greatest influence on the activity-linked pK\(_{as}\). The profound effect of the metal ion species in A on the value of this pK\(_{as}\) as deduced from these pH shifts in the E-P \( \rightleftharpoons \) E-P equilibrium suggested assignment of this pK\(_a\) to A site metal-bound water (3). The metal ion in B site has a less significant effect on this equilibrium, e.g. Mg(II) moves the midpoint to lower pH, while Cd(II) raises it, both ~1 pH unit relative to Zn(II) in B site. On this basis it would be expected that (Cd(II)\_A Zn(II)\_B)\_2 AP would exhibit a midpoint closer to pH 8.7 than to 6, i.e. determined principally by Cd(II) in A site. Although this could not be rigorously tested because of the instability of this hybrid, the \(^{31}\text{P}\) resonance assigned to this hybrid at pH 6.5 corresponds to E-P rather than E-P (Fig. 5), which would require a midpoint > pH 7 as predicted.

\(^{31}\text{P}\) NMR data suggested that A site metal ion, initially coordinating phosphate in the E-P complex, develops an open coordination site after formation of E-P, since phosphate appears to move out of the first coordination sphere of the A site metal ion on phosphorylation of serine 102. This would allow a solvent molecule to coordinate the A site metal ion. Thus in the phosphoryl native enzyme, Zn(II) in A site is postulated to coordinate a solvent molecule with a relatively low pK\(_{as}\) (7 to 8). The midpoint of the E-P \( \rightleftharpoons \) E-P equilibrium is several pH units lower than the pK\(_a\) because of the ratio of kinetic constants, \( k_{\text{phosphorylation}}/k_{\text{dephosphorylation}}\). The proximity of B site metal to A site (4 Å (2)) would explain why there are secondary modifications of the pK\(_a\) of the coordinated solvent by the metal ion occupying the B site.

Influence of the B Site Metal Ion on the Conformations of the E-P and E-P Intermediates—The chemical shifts of the noncovalent E-P complexes of the three enzyme species containing Zn(II) in A sites and either Mg(II), Zn(II), or Cd(II) in B sites show large variation, reflecting the different metal in B site. E-P resonates at 1.8 ppm, 4.3 ppm, and 12.6 ppm for species containing Mg(II), Zn(II), or Cd(II), respectively at B site with A occupied by Zn(II) in all cases (Figs. 1, 2, and 6).

One might attribute such a range of chemical shifts to direct
coordination of the phosphate in E-P to the different species of metal ion occupying B site. Then, is, however, no model system data suggesting such variation in chemical shifts in the case of coordination compounds containing phosphate groups. Chemical shift changes on coordination are generally small, are similar for several metals, and reflect displacement of the proton (17). Current data clearly support coordination of phosphate only to the A site metal ion. When homogeneous Cd(II)-containing enzymes (113Cd(II)AP or 113Cd(II)AP) are studied, the 31P resonances of E-P show coupling (~30 Hz) to only one of the two 113Cd(II) ions, A and B, at the active center (3). Heteronuclear decoupling experiments and studies on enzymes containing mixed 113Cd (spin 1/2) and 115Cd (spin 0) have shown that coupling arises from 113Cd(II) at the A sites. This conclusion is supported by the observation from the present work that when Zn(II) occupies the A site and 113Cd(II) the B site, no coupling is seen in the E-P resonance (Fig. 2). Thus it appears much more likely that the range of 31P chemical shifts observed for E-P is a function of the species of metal ion occupying B site is due to conformational differences around phosphate induced by occupancy of the adjacent B site only 3.9 A away.

Phosphate of E-P is anchored by coordination to the A site metal ion and possibly also by interaction with Arg 166 which swings down toward the A site metal ion (2). Thus one may picture E-P as a complex in which phosphate is relatively fixed, possibly allowing small conformational changes induced by the B site metal ion to cause significant distortion of phosphate bond angles, a feature known to induce large downfield 31P shifts (18). On the other hand, the phosphate group of the phosphoserine may not be a rigidly held moiety. The 31P resonance of E-P is not coupled to 113Cd, hence the phosphate does not appear coordinated and the 31P chemical shift is relatively insensitive to the nature of the metal ion at either A or B sites being 8.0, 8.6, 8.4, and 8.5 ppm for (Zn(II),Cd(II))3AP, (Zn(II),Zn(II))3AP, (Cd(II),Cd(II))3AP, and (Zn(II),Mg(II))3AP, respectively.

**Influence of B Site Metal Ion on Dissociation of Inorganic Phosphate from the Active Center**—The rate-limiting step in the hydrolysis of phosphate monoesters by AP at acid pH is dephosphorylation of E-P, while at alkaline pH where the enzyme is maximally active, dissociation of product, P, is rate-limiting (3). The substitution of Cd(II) for Zn(II) in both A and B sites not only shifts the midpoint of the E-P = E-P equilibrium to alkaline pH, but also dramatically lowers the phosphate dissociation rate (3), while enzyme turnover is reduced by 2 to 3 orders of magnitude (19). At pH 9 the dissociation rate of P, from the Cd(II) enzyme is ~1 s⁻¹ (9), which is still too fast for dissociation to be rate-limiting and suggests that dephosphorylation remains rate-limiting for Cd(II)AP even at pH 9. If only B site zinc is replaced by cadmium to form the (Zn(II),Cd(II))3AP hybrid, P, dissociation falls dramatically, from a value of 20–50 s⁻¹ calculated for (Zn(II),Mg(II))3AP at alkaline pH (20–22) to 2–6 s⁻¹ as determined by saturation transfer. Slow P, dissociation is accompanied by the unusual downfield shift in the E-P resonance, from the near-free value of 3.4 ppm for (Zn(II),Mg(II))3AP to values between 12 and 13.4 ppm when cadmium occupies B site. An altered phosphate conformation and a reduced phosphate dissociation rate may both be manifestations of small changes in the active site brought about by replacement of the native B site Zn(II) with the larger Cd(II) ion. We are undertaking measurements of the anisotropy in the chemical shift of the 31P signals of the enzyme in the solid state to determine if the downfield shift does represent a change in O—P—O bond angles.

**Subunit-Subunit Interactions**—The Zn(II)2Cd(II)3AP hybrid which contains one all-zinc and one all-cadmium monomer provides evidence for subunit-subunit interactions. Initial chemical shifts of the A and B site 113Cd(II) ions and the 31P chemical shift of E-P in the phosphorylated monomer of the unsymmetrical dimer are unique (1, 3) (Fig. 8a). Zinc binding to both A and B sites of the adjacent vacant subunit then causes substantial chemical shift changes in E-P, and in the nuclei of Cd(II)A and Cd(II)B of the first subunit, even though these nuclei are ~30 Â from the Zn(II) (2). The final shifts are similar to those found for homogeneous Cd(II)AP (3). These shifts suggest that conformational changes that occur. At one active center are transmitted across the monomer-enzyme interface to the other active center, an effect presumably contributing for the site metal ion than for A site metal ion (Fig. 8). The structural basis for this may be that the single His ligand to the B site metal ion (His 370) and one of those to the A site metal ion (His 372) precede a segment of polypeptide (residues 374 to 386) which is only of three regions of the monomer that penetrate the monomer-enzyme interface to within 20 Â of the B site of the opposite monomer (2). It is known that apomonomer formed either by treatment of the dimer with formamide (23) or by dissociation at low pH in the presence of guanidine hydrochloride (10), binds only 1 eq of zinc (or cobalt). Furthermore association of the Zn(II) monomers to reform dimeric alkaline phosphatase is slow unless additional zinc is added, whereas the Zn(II) dimer is readily reconstituted. Thus, not only are the metal binding properties of the monomers influenced by subunit-subunit interactions, but the metal ions themselves appear to be intimately involved in providing the necessary conformational requirements for some monomer-monomer interactions.

**SUMMARY**

The characteristics of the hybrid metal phosphatases provide a much clearer picture of the functions of the separate metal ions. The A site metal ion, which is zinc(II) in the native enzyme, serves to coordinate the phosphate group in the noncovalent complex. As the phosphate is transferred to serine 102, its coordination site on A site metal ion would become available to solvent. Phosphorylation of serine 102 can proceed slowly in the absence of B site metal ion, but is accelerated by its presence (1, 11). This is probably due to additional charge neutralization of the negative phosphate group as it moves toward serine 102 and therefore closer to the B site metal ion. The metal ion at B site could be close enough to the seryl oxygen to potentiate the formation of seryl $O^-$, although there is no evidence as yet for direct coordination. Presumably the noncovalent complex with a phosphomonoester as substrate, E-ROP, would be analogous. Dephosphorylation of the phosphoserine is inefficient and rate-limiting. The new 31P resonance from E-P in the zinc monomer also has the same chemical shift as E-P in Zn(II)AP (3). These shifts suggest that conformational changes at one active site are transmitted across the monomer-enzyme interface to the other active center, an effect more pronounced for B site metal ion than for A site metal ion (Fig. 8). The structural basis for this may be that the single His ligand to the B site metal ion (His 370) and one of those to the A site metal ion (His 372) precede a segment of polypeptide (residues 374 to 386) which is only of three regions of the monomer that penetrate the monomer-enzyme interface to within 20 Â of the B site of the opposite monomer (2). It is known that apomonomer formed either by treatment of the dimer with formamide (23) or by dissociation at low pH in the presence of guanidine hydrochloride (10), binds only 1 eq of zinc (or cobalt). Furthermore association of the Zn(II) monomers to reform dimeric alkaline phosphatase is slow unless additional zinc is added, whereas the Zn(II) dimer is readily reconstituted. Thus, not only are the metal binding properties of the monomers influenced by subunit-subunit interactions, but the metal ions themselves appear to be intimately involved in providing the necessary conformational requirements for some monomer-monomer interactions.
determined by the A site metal ion and thus differs most between Zn(II)_{4}AP and Cd(II)_{2}AP.

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