Alkaline phosphatase of Escherichia coli (a dimeric zinc metalloenzyme of identical subunits) containing either two catalytic Zn(II) ions or two catalytic Cd(II) ions shows absolute negative cooperativity of phosphate binding as determined by $^{31}$P NMR of enzyme-bound phosphate species. With excess phosphate at millimolar concentrations, only 1 mol of noncovalent phosphate complex, $E\cdot P$ ($^{31}$P resonance at 5 ppm) is formed by the 2 Zn(II) enzyme at pH 8, while 1 mol of the covalent intermediate, $E\cdot P$ ($^{31}$P resonance at 8 ppm) is formed by the 2 Cd(II) enzyme at pH 6.3. In contrast, enzymes containing an additional pair of structural Zn(II) or Cd(II) ions and 2 Mg(II) ions form 2 mol of $E\cdot P$ or $E\cdot P$, respectively, as detected by the characteristic $^{31}$P resonances for these two intermediates. This change in phosphate binding stoichiometry induced by the pair of structural metal ions has been confirmed by $[^{31}$P]-phosphate labeling studies of the 2 Cd(II) and 4 Cd(II) enzymes carried out at enzyme concentrations identical to those of the NMR samples. Addition of 2 Cd(II) ions to the apodiphosphoryl enzyme, prepared by treatment of the 4 Cd(II) enzyme with chelating agents, results in dephosphorylation of one of the active sites, confirming that negative cooperativity of phosphate binding is an equilibrium property of the 2 metal enzyme. The varying content of structural metal ions in the enzyme as a function of changing pH, dilution, and preparative technique appears to account for the variation in phosphate binding stoichiometry reported for this enzyme. It is possible that metal ions in the environment act as allosteric activators of alkaline phosphatase in addition to the essential role of the 2 catalytic Zn(II) ions in the mechanism of action.

Alkaline phosphatase from Escherichia coli is a dimeric Zn(II) metalloenzyme with a molecular weight of 86,000 which catalyzes nonspecific hydrolysis of phosphate monoesters. Two tightly bound Zn(II) ions (one per identical subunit) have been shown to be required for phosphate binding and formation of the covalent phosphoenzyme intermediate (1). These Zn(II) ions, referred to as "catalytic," have binding constants near $10^{10}$ M$^{-1}$ at pH 7.0 (2-4) and occupy coordination sites of low symmetry (5-7). The protein is also capable of specifically binding an additional pair of "structural" Zn(II) ions and 1 to 2 Mg(II) ions which confer on the enzyme increased structural stability and catalytic activity (8-11). Although enzyme isolated by mild isolation procedures generally contains 4 Zn(II) and 1 to 2 Mg(II) (12), there has in the past been considerable variability in reported metal ion content for the native enzyme. Much of this variability can be explained by the differential affinity which the catalytic and structural metal ions have for the enzyme. Since the dissociation constants of the structural Zn(II) ions are estimated to be several orders of magnitude greater than those of catalytic Zn(II) ions (2, 9, 13, 14), it is likely that variable amounts of structural Zn(II) may be lost from the purified enzyme during dialysis, especially at low concentrations of enzyme and particularly at pH values below 7 where the binding is weaker (15).

The stoichiometry of ligand binding to alkaline phosphatase has also been characterized by uncertainty. Despite the fact that the enzyme is a symmetrical dimer containing two active sites located 32 Å apart across the 2-fold axis of the dimer (16), rapid flow kinetic studies at pH values below 5.5, following the burst kinetics resulting from phosphorylation of the seryl residue at the active site, have in general shown a burst of only 1 mol of alcohol/enzyme dimer (17-20). For this reason, the enzyme was characterized as being negatively cooperative in terms of its substrate interactions, only a single site reacting at a time, the other being inhibited, presumably via conformational changes induced by substrate binding at the first site. Several lines of evidence support this concept, although other data suggest that, under some conditions, the two subunits are independent in their interaction with substrate. Measurements of $^{32}$P, binding to the enzyme by equilibrium dialysis techniques have generally supported the concept of negative cooperativity in that only a single tightly bound ($K_d = 10^{-6}$ M) phosphate ion was observed and only 1 mol of covalent phosphoryl enzyme was isolated under conditions where this species is favored (1, 21, 22). Several spectroscopic studies involving replacement of the catalytic Zn(II) by transition metal ions (Co(II), Cu(II), and Mn(II)) have usually shown spectral changes which are complete following the addition of one eq of P/dimer (6, 7, 15, 23), although a phosphate stoichiometry of 2 mol/dimer has also been reported (19). Phosphate titration of the fluorotyrosine enzyme monitored by phosphate-induced changes in the $^{19}$F NMR spectrum also indicated that 2 phosphate ions/dimer were required to complete the spectral change (24). Direct observation of the covalent and noncovalent enzyme-phosphate complexes by $^{31}$P NMR has clearly shown that apo-enzyme reconstituted with 2 eq of Zn(II), Co(II), Cd(II), or Mn(II) can tightly bind a maximum of 1 mol of phosphate/dimer (25, 26). Similar $^{31}$P NMR measurements on enzyme of unspecified metal ion content, however, showed a phosphate stoichiometry as high as 2 mol/mol of dimer, although occasionally the binding of 1.5 mol/dimer was also observed (27).
Significantly, similar nonintegral ligand binding stoichiometries have also been reported by several other investigators using a variety of monitoring techniques (28, 29).

The present paper describes phosphate binding and enzyme phosphorylation studies on Zn(II) and Cd(II) alkaline phosphatases of varying metal ion content. The results indicate that there is a direct relationship between ligand binding stoichiometry and metal ion content. Binding of structural metal ions to the enzyme is shown to suppress the absolute negative cooperativity of phosphate binding exhibited by the enzyme containing only the 2 catalytic metal ions. The relatively low affinity of the enzyme for structural metal is therefore suggested to be the origin of much of the variability in the literature concerning both metal ion content and the ability of the dimer to bind 1 or 2 phosphate ions. In addition, the relationship between metal ion content and phosphate binding ability offers a reasonable explanation for the frequent reports of nonintegral ligand binding stoichiometry in alkaline phosphatase.

MATERIALS AND METHODS

Enzyme Preparations—Alkaline phosphatase was isolated from E. coli (strain CW 3747) as previously described (1). Enzyme concentrations were determined spectrophotometrically at 290 nm with E1%1 cm = 0.72 (30). For molar calculations, a molecular weight of 36,000 was used. Enzyme activity was measured by the release of p-nitrophenol from p-nitrophenylphosphate (Sigma) in 1 M Tris-HCl, pH 8, 22°C (1). The native enzyme had a specific activity of 5000 ± 200 units (micromoles of substrate hydrolyzed per h per mg of protein). All buffer solutions were prepared metal-free. Apophosphatase was prepared by dialysis of the native enzyme at 4°C against four changes of 5 X 10-6 M o-phenanthroline (100-fold volume excess) in 0.01 M Tris-HCl, 0.01 M NaOAc, 0.1 M NaCl, pH 6, for 48 h followed by eight changes of the same buffer for 4 to 6 days. Absorbance measurements at 260 and 280 nm confirm that this procedure completely frees the apoenzyme from possible contamination with bound o-phenanthroline. Concentration of apoenzyme to 0.1 ml was carried out in a metal-free Amicon ultrafilter using a PM-10 membrane. Me(II) phosphatases were prepared by direct addition of stoichiometric amounts of ZnCl2, CdCl2, and MgSO4 (spectrograde from Johnson Matthey Chemicals, Ltd., London) to the apoenzyme. 

Metal Ion Stoichiometry—In order to determine if variable metal content of the enzyme might be responsible for previous differences in phosphate binding stoichiometry as determined by 31P NMR (25-27), the 31P resonance of E-P was followed during phosphate titrations of Zn(II)2AP, Zn(II)4Mg(II)2AP, and Zn(II)4Mg(II)2AP. All additions were performed at pH 8.0 where E-P is the only species of enzyme-bound phosphate present and where the chemical shift of the 31P resonance has been previously assigned (22, 25, 26). At pH 8, addition of 1 mol of P; to Zn(II)AP is accompanied by the appearance of a major 31P resonance at 5 ppm, ~3 ppm further downfield than the resonance expected for P; (22, 26) (Fig. 2A). The two minor upfield peaks (< 10% of the total area) at 3 and 1.5 ppm may represent small amounts of a different conformer of E-P (see below) and free P; respectively. Upon addition of a 2nd mol of P;, a new resonance accounting for all of the 2nd eq of P; appears at the chemical shift position expected for free P; at this pH (Fig. 2B). The relative areas of resonances of different species (i.e. E-P and P;) cannot be used directly as an indication of the relative amounts of these species, since their T2 values differ and the spectra were not obtained under "fully relaxed" conditions (i.e. pulse repetition rate, ~4 T1). Comparison of the areas of a resonance corresponding to a single species in a series of titration experiments, however, is valid.

In contrast to the appearance of a homogenous resonance characteristic of E-P and limited to the 1st mol of P; added to Zn(II)2AP, if Zn(II)4AP is used, two 31P resonances, at -2 and 3 ppm, are observed (Fig. 2C). The presence of these two resonances is consistent with the presence of two distinct phosphate binding sites and indicates that Zn(II)4AP is not a single species in solution.

Formation of E-P by Zn(II) Alkaline Phosphatase as a Function of Metal Ion Stoichiometry—In order to determine if variable metal content of the enzyme might be responsible for previous differences in phosphate binding stoichiometry as determined by 31P NMR (25-27), the 31P resonance of E-P was followed during phosphate titrations of Zn(II)2AP, Zn(II)4Mg(II)2AP, and Zn(II)4Mg(II)2AP. All additions were performed at pH 8.0 where E-P is the only species of enzyme-bound phosphate present and where the chemical shift of the 31P resonance has been previously assigned (22, 25, 26). At pH 8, addition of 1 mol of P; to Zn(II)AP is accompanied by the appearance of a major 31P resonance at 5 ppm, ~3 ppm further downfield than the resonance expected for P; (22, 26) (Fig. 2A). The two minor upfield peaks (< 10% of the total area) at 3 and 1.5 ppm may represent small amounts of a different conformer of E-P (see below) and free P; respectively. Upon addition of a 2nd mol of P;, a new resonance accounting for all of the 2nd eq of P; appears at the chemical shift position expected for free P; at this pH (Fig. 2B). The relative areas of resonances of different species (i.e. E-P and P;) cannot be used directly as an indication of the relative amounts of these species, since their T2 values differ and the spectra were not obtained under "fully relaxed" conditions (i.e. pulse repetition rate, ~4 T1). Comparison of the areas of a resonance corresponding to a single species in a series of titration experiments, however, is valid.

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RESULTS

31P Resonances of the Enzyme-Phosphate Complex (E-P) and the Phosphoryl Enzyme (E-P)—31P NMR is an excellent method for quantitating both the amount of phosphate bound to alkaline phosphatase and the stability of the resultant covalent and noncovalent complexes, since distinct resonances are observed for both of these species which are well resolved from the resonance arising from free inorganic phosphate (25-27). This is demonstrated in Fig. 1 by the spectrum of the Zn(II) enzyme at pH 5.5 in the presence of excess phosphate. At pH values between 5 and 6, both E-P and E-P are present in significant concentrations at equilibrium and these well resolved resonances at 8.2, 3.0, and 0.0 ppm are observed which correspond to E-P, the phosphoryl intermediate resulting from phosphorylation of a specific serine residue at the active site, E-P, the noncovalent enzyme-phosphate complex, and the monophosphonic form of free inorganic phosphate, respectively (26).

Formation of E-P by Zn(II) Alkaline Phosphatase as a Function of Metal Ion Stoichiometry—In order to determine if variable metal content of the enzyme might be responsible for previous differences in phosphate binding stoichiometry as determined by 31P NMR (25-27), the 31P resonance of E-P was followed during phosphate titrations of Zn(II)2AP, Zn(II)4Mg(II)2AP, and Zn(II)4Mg(II)2AP. All additions were performed at pH 8.0 where E-P is the only species of enzyme-bound phosphate present and where the chemical shift of the 31P resonance has been previously assigned (22, 25, 26). At pH 8, addition of 1 mol of P; to Zn(II)AP is accompanied by the appearance of a major 31P resonance at 5 ppm, ~3 ppm further downfield than the resonance expected for P; (22, 26) (Fig. 2A). The two minor upfield peaks (< 10% of the total area) at 3 and 1.5 ppm may represent small amounts of a different conformer of E-P (see below) and free P; respectively. Upon addition of a 2nd mol of P;, a new resonance accounting for all of the 2nd eq of P; appears at the chemical shift position expected for free P; at this pH (Fig. 2B). The relative areas of resonances of different species (i.e. E-P and P;) cannot be used directly as an indication of the relative amounts of these species, since their T2 values differ and the spectra were not obtained under "fully relaxed" conditions (i.e. pulse repetition rate, ~4 T1). Comparison of the areas of a resonance corresponding to a single species in a series of titration experiments, however, is valid.

In contrast to the appearance of a homogenous resonance characteristic of E-P and limited to the 1st mol of P; added to Zn(II)2AP, if Zn(II)4AP is used, two 31P resonances, at -2 and 3 ppm, are observed (Fig. 2C). The presence of these two resonances is consistent with the presence of two distinct phosphate binding sites and indicates that Zn(II)4AP is not a single species in solution.

The abbreviations used are: Zn(II)_2AP, Zn(II)_4AP, and Zn(II)_4Mg(II)_2AP designate enzymes containing 2 catalytic Zn(II) ions, 2 catalytic and 2 structural Zn(II) ions, and 2 catalytic Zn(II) ions, respectively; AP, alkaline phosphatase; apoAP, the metal-free enzyme or apoalkaline phosphatase; Me(II)_2AP, Me(II)_4Mg(II)_2AP, and Me(II)_4Mg(II)_2AP designate the monophasphoryl and diphosphoryl enzymes respectively.

Fig. 1. 31P NMR spectrum of Zn(II)_2Mg(II)_2AP plus 4 eq of P; at pH 5.5. The enzyme concentration was 2.4 X 10^{-3} M in 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 5.5, plus 9.6 X 10^{-3} M phosphate.
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3.7 ppm, appear on addition of the first phosphate anion and both approximately double on the addition of the 2nd eq of $P_i$ (Fig. 2, C and D). Neither of these resonances has a chemical shift corresponding to free $P_i$. It is only on the addition of a 3rd eq of $P_i$ that a substantial resonance appears at 1.5 ppm, characteristic of $HPO_4^{2-}$ under these conditions (Fig. 2E). We have previously shown that additional metal ions can induce heterogeneity in the 31P resonance from $E-P$ and the resonances in Fig. 2, C and D correspond to those previously reported and ascribed to the existence of relatively stable "conformers" of $E-P$ (26, 27).

On the other hand, if 2 Mg(II) ions are added to Zn(II)$_2$AP before the phosphate, then a relatively more homogeneous resonance is observed for $E-P$ (2.9 ppm), which also doubles in amplitude on addition of a 2nd mol of $P_i$. The addition of a 3rd mol of $P_i$ generates a resonance corresponding to that of free $P_i$ (Fig. 2, F to H).

The mechanism by which an additional pair of metal ions induces the binding of a 2nd mol of phosphate is clearly a complex one and may involve the induction of further conformational changes in the protein dimer. 31P NMR studies in progress show that the structural metals can influence the equilibrium between $E-P$ and $E-P$ observed at a given pH value. Since this reflects the rates of phosphorylation and dephosphorylation of the enzyme, such alterations can clearly affect the observed catalytic activity. If 1-$\mu$l aliquots of the NMR samples are removed and assayed for activity during the metal ion titrations, the activities are -300 for Zn(II)$_2$AP, -2100 for Zn(II)$_4$AP, and -2800 for Zn(II)$_4$Mg(II)$_2$AP, expressed as micromoles/h/mg of protein. These data on apoenzyme prepared by chelex treatment at 4°C where 90% of the original activity is recovered on the addition of 2 Zn(II) ions in the presence of Mg(II). It is possible that the 2nd pair of metal ions or Mg(II) induce further structural changes required to re-form the native structure. The formation of the metalloenzyme in vivo involves processing of the pre-enzyme (32), subunit association, and metal ion binding, a sequence which must involve conformational changes in the protein. These structural alterations may not be identical with those induced on the addition of 2 Zn(II) ions to the apoenzyme dimer. Bock and Kowalsky (33) have recently reported a hysteresis effect accompanying the regeneration of activity to the apo-enzyme. They report that once full activity is regenerated by the readdition of 4 or more metal ions, the Zn(II) content can then be reduced to less than 2.5 Zn(II) ions/dimer without loss of activity.

Stoichiometry of Phosphorylation of Cd(II) Alkaline Phosphatase—In view of the above demonstration of the dependence of the stoichiometry of $E-P$ on the Zn(II) content of the enzyme, it was of interest to determine whether a similar dependence could be observed for the formation of the covalent reaction intermediate, $E-P$. In order to examine the properties of the covalent intermediate, $E-P$, it has been convenient to use the Cd(II)-substituted enzyme rather than the native Zn(II) enzyme, since the Cd(II) enzyme has the property of being entirely in the phosphorylated form at pH 6.5 (1).

The detailed studies of the Cd(II) enzyme have been previously published (1, 22, 26), and only the 31P NMR spectra showing the formation of 1 mol of $E-P$ (resonance at 8 ppm) by Cd(II)$_2$AP is repeated here (Fig. 3A). Any additional $P_i$ added to the monophosphoryl enzyme gives rise to a 31P resonance at the chemical shift expected for inorganic phosphate under these conditions (Fig. 3B). The data in Fig. 3, A and B are typical of the NMR evidence supporting the negative cooperativity of the enzyme containing only the pair of catalytic metal ions.

On the other hand, when the structural Cd(II) ions and Mg(II) are added to form Cd(II)$_4$Mg(II)$_2$AP, titration of the enzyme with phosphate results in the appearance of a 31P resonance corresponding to $E-P$ on the addition of both the 1st and 2nd eq of $P_i$ (Fig. 3, C and D). With the 4 Cd(II)
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Cd(II)2AP

Cd (II)4 Mg (II)2AP

FIG. 3. 31P NMR titrations of Cd(II) alkaline phosphatases of varying metal ion content with phosphate at pH 6.5. Spectra A and B were obtained following the addition of 1 and 2 eq of phosphate, respectively, to 1.9 x 10^-3 M apoaikaline phosphatase reconstituted with 2 eq of CdCl2. Spectra C, D, and E were obtained following the addition of 1, 2, and 3 eq of phosphate, respectively, to 1.9 x 10^-3 M apoaikaline phosphatase reconstituted with 4 eq of CdCl2 and 2 eq of MgSO4. The resonance at 29.3 ppm is due to the methylphosphonate standard. Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5.

It should be noted again that peak areas of the E-P and P1 resonances in Fig. 3 do not reflect relative concentrations due to their different T1 values. A T1 determination on the sample used for Fig. 3E yielded T1 values for E-P and P1 of 2.3 and 0.9 s, respectively. Therefore, under the sampling conditions used in Fig. 3E, the area of the E-P resonance will be attenuated compared to the P1 signal. When the spectrum in Fig. 3E was repeated under fully relaxed conditions (45° pulse, 9.6-s pulse repetition rate), the intensity of the E-P resonance was quantitated and found to correspond to 1.9 equivalents of phosphate.

Similar phosphate titration experiments were also carried out with the Zn(II) enzyme of pH 5.5. Under these conditions, the bound phosphate is present in roughly equimolar amounts as E-P and E-P (see Fig. 1). Again, a phosphate binding stoichiometry of 2 was observed for the fully metallized Zn(II)2Mg(II)2AP (results not shown). Unlike the sensitivity of the E-P chemical shift to the presence and identity of the bound metal ions, the E-P resonance position is found to be essentially invariant no matter whether the catalytic metal binding sites are occupied by Zn(II) or Cd(II), or whether additional Zn(II), Cd(II) or Mg(II) occupy the structural site(s). This observation may reflect a different spatial orientation adopted by the covalently and noncovalently bound addition of 1, 2, and 3 eq of phosphate, respectively, to 1.9 x 10^-3 M apoaikaline phosphatase reconstituted with 4 eq of CdCl2 and 2 eq of MgSO4. The resonance at 29.3 ppm is due to the methylphosphonate standard. Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5.

Fig. 4. Covalent incorporation of [32P]Pi into Cd(II) alkaline phosphatase at pH 6.5. O, Cd(II) alkaline phosphatase, [Cd(II)]/[alkaline phosphatase] = 2.10; ●, Cd(II)2Mg(II) alkaline phosphatase, [Cd(II)]/[alkaline phosphatase] = 6.45, [Mg(II)]/[alkaline phosphatase] = 3.87. Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5, 1.19 x 10^-3 M alkaline phosphatase, 7.13 x 10^-3 M K2HPO4/32P-O4, 25°C.
phosphate with respect to the enzyme’s metal binding sites. Radiolabeling studies have never been performed under the conditions of the $^{31}$P NMR experiments, and since many of the apparent discrepancies in the literature involve $^{32}$P-labeling of the enzyme, we have carried out a $^{32}$P-labeling study maintaining the concentrations of protein and phosphate the same as those present in the NMR tube. $[^{32}\text{P}]$Phosphate binding to the enzyme has generally been performed by equilibrium dialysis (1), but we have reported one study on the phosphorylation of the Cd(II) enzyme which involves the direct addition of small aliquots of $\text{HO}^{32}\text{P}^{2-}$ to a small volume of enzyme (1). We have thus added $\text{HO}^{32}\text{P}^{2-}$ to the enzyme under conditions of the NMR experiments, and after incubation for up to 75 h, 100-μl aliquots were dried on filter paper, and treated with perchloric acid for the assay of E-P as previously described in detail (1). With direct formation of E-P at high enzyme concentrations, 10,000 cpm from a 100-μl aliquot represents 1 mol of E-P against a background of 50 cpm. The results are shown in Fig. 4. Under these conditions, phosphorylation of Cd(II)$_2$AP is time-dependent and slowly approaches 1 mol of E-P/mol of dimer (Fig. 4). We have previously observed less than complete phosphorylation of the cadmium enzyme in the direct test tube phosphorylations at lower concentrations of enzyme ($10^{-5}$ to $10^{-4}$ M) (1). The same phenomenon appears more evident at the $10^{-3}$ M concentrations used here and a distinct time dependence is pres-

**Fig. 5.** Demonstration by $^{31}$P NMR of the reversible, metal ion dependent formation of di- and monophosphoryl Cd(II) alkaline phosphatase. A, Cd(II)$_2$AP-P; B, ApoAP-P; C, ApoAP-P, plus 2 eq of Cd(II); D, Cd(II)$_2$Mg(II)$_2$AP-P$_2$; E, ApoAP-P$_2$; F, plus 2 eq of Cd(II), 4.5 h later; G, Sample F 11 h later; H, Sample F 23 h later; I, Sample F 105 h later; J, Sample J plus 4 eq of Cd(II) and 2 eq of Mg(II). Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5, $3.2 \times 10^{-3}$ M alkaline phosphatase, metal ion, and phosphate stoichiometries as indicated. Apophosphoryl alkaline phosphatases were generated from the metallophosphoryl enzymes by dialysis versus o-phenanthroline. The resonance at 29.3 ppm is that of methylphosphonate, an external standard.
ent. The origin of the effect is not clear. In contrast, when 2 additional structural Cd(II) ions and 2 Mg(II) ions are added to the protein, otherwise under exactly the same reaction conditions, 2 mol of E-P/mol of dimer are isolated (Fig. 4), confirming the conclusions from the observation of the $^{31}P$ resonance (Fig. 3).

**Formation of Diphosphoryl Apoalkaline Phosphatase**—
The preparation of apophosphorylated alkaline phosphatase from the monophosphoryl enzyme, Cd(II)$_2$AP-P, by rapid removal of the Cd(II) ions with chelating agent has been previously described (22). The $^{31}P$ resonance of the apophosphoryl enzyme is $\sim 2$ ppm upfield from the resonance of the metallophosphoryl enzyme and the $^{31}P$ NMR spectra of these two species are shown in Fig. 5, A and B. In contrast to all metallophosphoryl enzymes, the apophosphoryl enzyme is stable over the pH range 2 to 10 (22). The original resonance of the stable metallophosphoryl enzyme can be regenerated by the readdition of 2 Cd(II) ions to the monophosphoryl apoenzyme at pH 6.5 (Fig. 5C).

Preparation of the diphosphoryl apoenzyme from Cd(II)$_2$Mg(II)$_2$AP-P, by the above techniques proved also to be possible with a shift of all of the $^{31}P$ resonance $\sim 2$ ppm upfield on removal of all metal ions (Figs. 5, D and E). In contrast to the addition of 2 Cd(II) ions to the monophosphoryl apoenzyme, when two Cd(II) ions are added back to the apodiphosphoryl enzyme, the original resonance is not completely restored. The downfield resonance splits and a small resonance appears at the chemical shift expected for free P, (Fig. 5F). The latter resonance grows slowly over $\sim 100$ h. Simultaneously, resonance intensity at $\sim 6$ ppm disappears (Fig. 5, F to I). Rephosphorylation of the second site can be induced by readding the structural Cd(II) ions and Mg(II) to the sample in the NMR tube which causes the free phosphate resonance to disappear and the resonance of E-P to double in amplitude (Fig. 5J).

**DISCUSSION**

Since inorganic phosphate is a substrate for alkaline phosphatase capable of phosphorylating the seryl residue at the active site, transferring to an alcohol acceptor, or undergoing hydrolysis, binding of the inorganic phosphate anion to the active site stoichiometry of 2 by NMR. The same cannot be said, however, for the active site stoichiometries (7). In these studies, the observed ligand binding stoichiometry for Structural Zn(II) and other metals (Cd(II) and Mg(II)) is scarce, the dissociation constant for structural Zn(II) at neutral pH is estimated to be about 1 $\mu$m (2, 9, 14). Below pH 7, binding is considerably weaker as reflected by the susceptibility of the structural metal ions to removal by dialysis (15). Despite this relatively weak binding, occupation of the structural metal binding sites following addition of stoichiometric amounts of Me(II) to apoenzyme is fairly well assured at the high enzyme concentrations (~1 mM) used in the NMR experiments. Thus, there was no difficulty in demonstrating a phosphate binding stoichiometry of 2 by NMR. Indeed, active site stoichiometries have been observed by these methods which vary from 1 to 2 per dimer. The range and irreproducibility of these values are understandable considering the sensitivity of structural metal ion binding to
experimental conditions such as pH, metal ion concentration, temperature, and ionic strength.

Since the pH dependence of the equilibrium between E-P and E-P for the Zn(II) enzyme predicts 100% formation of E-P only near pH 5, the low enzyme concentrations and acid pH required for demonstration of full burst kinetics due to rate-limiting dephosphorylation of E-P (17-20) make it doubtful that, under such conditions, the enzyme contains a full complement of structural metals, since equilibrium dialysis shows them to be gone by pH 5.5 (15). This must be kept in mind in interpreting the kinetic results. The burst kinetics observed at alkaline pH for phosphate-free enzyme due to rate-limiting dissociation of E-P have recently also been reported to show a maximum amplitude approaching 1 mol/mol of dimer (34), suggesting that low concentrations of enzyme (assay levels) may also be associated with some dissociation of structural metal ions even at alkaline pH.

The relationship between the metal ion content of alkaline phosphatase and the expression of negative cooperativity does not clarify the physiological or mechanistic reasons for negative cooperativity, except to suggest the additional dimension of allosteric modulation of alkaline phosphatase activity by the metal ion concentration of the medium. The in vivo metal ion content of the enzyme is difficult to determine and is expected to depend on the pH and metal ion concentration of the particular cell or body fluid where the enzyme is located. When isolated under conditions which minimize loss of metal ions, the enzyme is found to contain 4 g atoms of Zn(II) and 1.3 g atom of Mg(II) per mol of dimer (12). The additional presence of endogenous phosphate may stabilize metal binding to the isolated enzyme.

Mammalian body fluids generally have a zinc concentration slightly greater than 10^{-5} M, while some specific tissues have concentrations near 10^{-3} M, based on figures for zinc content per g, wet weight. The latter values must reflect concentration of the ion by certain cells (35). Zn(II) contents of E. coli vary depending on the Zn(II) concentration of the medium (31). In high zinc medium (31), the organism takes up about 12% of the total zinc, and ~60% of this is present in the cell compartment subject to osmotic shock (possibly only the periplasmic space) (31). This component ranges from a minimum of ~0.007 µmol to a maximum of ~0.2 µmol of Zn(II)/g, wet weight, of cells, the latter in high zinc medium (31). Calculated on a whole cell basis, the latter concentration is ~0.2 mM. If this zinc is confined to the periplasmic space the concentration could be higher. Since highly variable concentrations of Zn(II) exist in nature, it is possible that the structural Zn(II) ions (or possibly other ions in the environment) may have roles as allosteric activators of alkaline phosphatase in addition to the essential role of the catalytic Zn(II) in the mechanism of action. This may be a physiologically significant feature of an enzyme likely to be located in regions subject to ion gradients.

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