Allosteric Interactions between Metal Ion and Phosphate at the Active Sites of Alkaline Phosphatase as Determined by $^{31}$P NMR and $^{113}$Cd NMR*

(Received for publication, April 5, 1977)

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$^{113}$Cd and $^{31}$P NMR have been used to determine the effect of variations in the nature and stoichiometry of bound metal ion and pH on the allosteric interactions (negative cooperativity) induced on association of phosphate with the dimeric zinc metalloenzyme Escherichia coli alkaline phosphatase. At pH 8.0, successive additions of an extra two Zn$^{2+}$ ions and one Mg$^{2+}$ ion to the Zn$_2^{2+}$ enzyme result in structural alterations of the noncovalent phosphate complex (E-P) reflected in a progressive upfield chemical shift of the $^{31}$P resonance from 5.1 ppm (Zn$_2^{2+}$ enzyme) to 4.2 and 3.5 ppm, respectively. Variation of metal ion content does not alter the stoichiometry of tight phosphate binding in all cases a single phosphate ligand is bound per protein dimer. Phosphate binding to the Co$^{2+}$ enzyme at pH 8.0 (where tightly bound and free P$_i$ exist under slow exchange conditions) and pH 6.5 (where P$_i$ is in fast exchange) are similarly consistent with the tight association of one P$_i$/dimer. Observation of the NMR of both the ligand and metal ion nuclei in the covalent phosphate complex (E.P) at pH 6.5 confirms these results. Two equivalents of Cd$^{2+}$/dimer are required to generate the maximum of 1 eq of phosphoryl enzyme/dimer, observed as a characteristic low field position (~8.0 ppm) resonance in the $^{31}$P NMR spectrum. The $^{113}$Cd NMR spectrum of the unliganded enzyme shows the Cd$^{2+}$ ions to exist in identical environments, since a single resonance is present 170 ppm downfield from the standard, 0.1 M CdCl$_2$. Covalent phosphorylation at a single site results in appearance of two resonances of equal intensity at 142 and 55 ppm. The presence of 1 mol of excess phosphate does not alter the stoichiometry of phosphoryl enzyme formation or the altered environments of the metal ions. These results are consistent only with the existence of negative homotropic interactions between the subunits induced on ligand (phosphate) binding resulting in conformational alterations at both active centers of the dimeric enzyme.

$^{31}$P NMR has been shown to be a powerful means of exploring the chemical nature of the phosphate complexes of the Zn$^{2+}$ metalloenzyme, Escherichia coli alkaline phosphatase (1-3). Initial studies have identified the phosphorus chemical shifts of the noncovalent complex of the enzyme, E-P, with the product, inorganic phosphate and the covalent intermediate, E-P, resulting from the phosphorylation of the hydroxyl group of serine 99 by phosphate or substrate. The low field position of the phosphorus resonance for E-P, ~8 ppm, suggests that the enzyme phosphoserine is a phosphate ester of unusual geometry (1, 4, 5).

Since $^{31}$P NMR can monitor simultaneously all forms of phosphate in the solution, it is potentially the best method for determining the stoichiometry of the various distinct forms of enzyme-bound phosphate. Phosphate binding to alkaline phosphatase has been shown to be metal ion-dependent (6). Therefore the stoichiometry of phosphate binding may be expected to depend on the metal ion stoichiometry. Conversely the presence of phosphate or the phosphoryl group might be expected to influence metal ion binding to the apoenzyme. The present paper explores in detail with $^{31}$P NMR methods the chemical nature of enzyme-bound phosphate as functions of phosphate, metal ion (Zn$^{2+}$, Cd$^{2+}$, and Co$^{2+}$), and magnesium concentrations. Using $^{113}$Cd NMR, the chemical nature of the active center metal ion has been explored both in the absence and presence of phosphate ligand. Evidence is presented consistent with the existence of stable structural isomers of enzyme-phosphate complexes as detected by differences in the chemical shifts of resonances assigned to tightly associated phosphate. The distribution of the enzyme in these conformational forms is controlled by the metal ion stoichiometry. Thus, the appearance of multiple E-P resonances can be related to the mode of preparation of the enzyme and 

* This work was supported by Grants AM 09070-13 and AM 18775-02 from the National Institutes of Health and by Grant PCM76-82231 from the National Science Foundation. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C Section 1734 solely to indicate this fact.

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its consequent metal ion content. Under all conditions the enzyme-bound phosphate stoichiometry remains constant at 1 mol/mol of enzyme dimer. Thus binding of ligand at one site of the symmetrical dimer must prevent ligand binding at the second site. This negative cooperativity is unambiguously confirmed by \(^{115}\text{Cd} \) NMR which shows the two Cd\(^{2+}\) ions to occupy identical chemical environments in the unliganded enzyme, but two different environments when a single phosphate is covalently bound to the dimer.

**MATERIALS AND METHODS**

Enzymes and Chemicals-Crystalline alkaline phosphatase was prepared from *Escherichia coli* (strain CW 3747) as described by Applebury et al. (6). Enzyme concentrations were determined spectrophotometrically at 278 nm with \(E_{1}^{1}% = 0.72 \) (7). For molar calculations a molecular weight of 86,000 was used. Buffer solutions, HCl, and NH\(_{4}\)OH were prepared metal-free (8). Apo- and Me\(^{2+}\) phosphatases were prepared as previously described (6, 8). For the NMR studies, the Co\(^{2+}\) and Cd\(^{2+}\) enzymes were prepared by the addition of slightly less than 2 eq of Me\(^{2+}\)/apoenzyme dimer. In the case of the cadmium enzyme 86 atom % of \(^{115}\text{Cd} \) salts (Oak Ridge Laboratories) were added to solutions of the apoenzyme, followed by concentration to a volume of \(\sim 1 \) ml. Titration of apokaolin phosphatase with Me\(^{2+}\) ions as followed by several spectroscopic techniques shows that the first two metal ions added are tightly bound at the sites occupied by the catalytically active Zn\(^{2+}\) ions of the native enzyme (6-9, 12). All NMR samples and equipment were prepared metal-free following procedures previously described (1).

**NMR Techniques**-Fourier transform \(^{31}\text{P} \) and \(^{115}\text{Cd} \) NMR spectra were obtained on an extensively modified Bruker HFX-90 MHz spectrometer operating at 96.44 and 19.96 MHz, respectively, at \(95 \pm 2 \) °C (13, 14). D,O in a 3-mm co-axial capillary insert was used as an external field frequency lock. All \(^{31}\text{P} \) NMR spectra were obtained under conditions of proton noise decoupling. \(^{115}\text{Cd} \) NMR spectra were obtained in the absence of proton noise decoupling because of the negative value of the nuclear magnetic moment for \(^{115}\text{Cd} \) and the negative value of the nuclear Overhauser enhancement on correlation time. A spectral width of 5000 Hz was used throughout to maximize the S/N improvement from a 5000 Hz bandwidth crystal filter. Typical parameters for \(^{31}\text{P} \) NMR spectra include an acquisition time of 0.2 s, a pulse delay of 0.2 s, and a pulse width of 80°. For \(^{115}\text{Cd} \) NMR spectra they were as follows: acquisition time, 0.2 s; pulse delay, 0.2 s; pulse width, 70°. For all spectra shown an interpolation expansion routine was employed providing a resolution of 1.2 Hz/point for \(^{31}\text{P} \) and 5.0 Hz/point for \(^{115}\text{Cd} \). Measurements were made on 1.0-ml samples contained in 10-mm sample tubes fitted with Vortex plugs to confine the solution within the transmitter coil. Typical spectra required from 40,000 to 200,000 transients (4 to 24 h) depending on nucleus and protein concentration. \(^{31}\text{P} \) chemical shifts were determined relative to external 85% H\(_{3}\)PO\(_{4}\). \(^{115}\text{Cd} \) chemical shifts are expressed relative to the resonance position of 0.1 M CdCl\(_{2}\). Signs for chemical shift values reflect the IUPAC standard nomenclature, increasing numbers to low field.

**RESULTS AND DISCUSSION**

**Stoichiometry of Zn\(^{2+}\) and Bound Phosphate**—Despite investigation by a variety of experimental methods, the stoichiometry of ligand (i.e. substrate, inhibitor, or product) binding to alkaline phosphatase has remained controversial. Under conditions of moderate ionic strength, \(K_{d} \approx 10^{-3} \) M/ enzyme dimer is observed (6, 9, 16, 19). Interactions of phosphate with this enzyme. An equimolar mixture of apokaolin phosphatase and inorganic phosphate at \(pH 8 \) shows a single sharp \(^{31}\text{P} \) resonance at the chemical shift position of P, in the absence of protein at the pH and ionic strength used (Fig. 1A), confirming that there is negligible specific interaction between the apoenzyme and inorganic phosphate. Addition of 1 eq of Zn\(^{2+}\) to the mixture of apoenzyme and inorganic phosphate results in the appearance of at least two additional broadened lines at lower field (Fig. 1B). Under these conditions the observed line broadening enzyme-bound phosphate and thus did not reveal whether binding of a second ligand is different from the first. All of the above studies have suggested that negative homotropic interactions exist between the two identical active sites on the dimer of alkaline phosphatase, interactions presumably mediated by conformational changes induced (or selected for) by the interaction of substrate or product with the enzyme and propagated across the monomer-monomer interface.

Several recent reports have suggested that 2 eq of phosphate bind to the enzyme and that binding is characterized by a single dissociation constant (3, 20, 21). While there is considerable variation in concentration in the several studies, the suggestion of phosphate contamination as a comprehensive explanation for apparent negative cooperativity (20) cannot apply to experiments in which the apoenzyme has negligible affinity for phosphate (6).

Phosphorus chemical shifts of the various forms of enzyme bound phosphate span \(-6 \) ppm, hence \(^{31}\text{P} \) NMR is a particularly good method to evaluate the subtle differences in the interactions of phosphate with this enzyme. An equimolar mixture of apokaolin phosphatase and inorganic phosphate at \(pH 8 \) shows a single sharp \(^{31}\text{P} \) resonance at the chemical shift position of P, in the absence of protein at the pH and ionic strength used (Fig. 1A), confirming that there is negligible specific interaction between the apoenzyme and inorganic phosphate. Addition of 1 eq of Zn\(^{2+}\) to the mixture of apoenzyme and inorganic phosphate results in the appearance of at least two additional broadened lines at lower field (Fig. 1B). Under these conditions the observed line broadening...
requires that enzyme species containing one Zn\textsuperscript{2+} ion have at least a transitory existence. Modulation of bound P\textsubscript{i} resonance from these species may result from direct transfer of P\textsubscript{i} between proteins of variable metal ion content or may be governed by the rate of metal ion exchange between protein forms. These alternatives, both plausible, cannot be distinguished from the present results. The line width and chemical shift of the peak for the remaining free P\textsubscript{i} indicate that it is not an intermediate in this exchange process.

Addition of a second equivalent of Zn\textsuperscript{2+} converts most of the \textsuperscript{31}P intensity into a narrowed line at the resonance position expected for E·P (1) (Fig. 1C). The resonance of low amplitude at higher field reflects a slight excess of P\textsubscript{i} beyond a stoichiometry of one P\textsubscript{i}/reconstituted dimer. A content of two Zn\textsuperscript{2+} ions/dimer has been shown to be the minimum metal content required for activity (6, 10, 22). The above sequence of resonances shifts is consistent with the postulate that 2 mol of Zn\textsuperscript{2+}/mol of dimer are required to induce the tight binding of 1 mol of P\textsubscript{i}, in the noncovalent complex, E·P (6).

The line width and relaxation times of resonances assigned to phosphate which is not tightly bound to the enzyme (assignment based on the observed chemical shift) can be satisfactorily explained on the basis of exchange mechanisms operative between the forms of P\textsubscript{i}, present in a given sample (see below). However, at the concentrations of P\textsubscript{i} employed in these studies (\approx 1 mM) binding of P\textsubscript{i} at protein sites of greatly reduced affinity is suggested by radiolabelling studies (6, 17) and cannot be definitively ruled out on the basis of the NMR data.

An additional 2 g at of Zn\textsuperscript{2+} and 1 to 2 g at of Mg\textsuperscript{2+} have been shown to stabilize the structure of alkaline phosphatase (23-25). Therefore, the effect of additional Zn\textsuperscript{2+}, P\textsubscript{i}, and Mg\textsuperscript{2+} on the enzyme phosphate noncovalent complex was examined. Addition of a further 2 eq of Zn\textsuperscript{2+} has no effect on the distribution of free and bound phosphate (Fig. 1D). There is a 1-ppm upfield shift of the resonance of E·P induced by the excess Zn. This would appear to reflect a modulation of enzyme structure by the binding of the extra zinc to sites on the molecule which stabilize the structure. These may be related to the partially occupied Zn\textsuperscript{2+} sites observed in the electron density map of the enzyme at 3 A resolution (26). When a second equivalent of phosphate is added to the enzyme containing four Zn\textsuperscript{2+} ions, additional intensity appears at the chemical shift position of free P\textsubscript{i} (Fig. 1E).

Addition of Mg\textsuperscript{2+} to the system does not alter the relative stoichiometry of the bound and free phosphate (Fig. 1F). Mg\textsuperscript{2+} does induce a substantial upfield shift in the resonance of E·P which moves from 4.2 to 3.5 ppm. This shift would appear to reflect further changes in enzyme structure induced by enzyme-bound Mg\textsuperscript{2+}, an ion which stabilizes the protein against heat denaturation (23, 25) and also activates the enzyme (25).

Exchange of P\textsubscript{i} Bound at Active Site of Zn\textsuperscript{2+} Enzyme —For the series of spectra shown in Fig. 1 the rate of chemical exchange between E·P and free P\textsubscript{i} is clearly slower than the chemical shift difference (\approx 800 s\textsuperscript{-1}). In this slow exchange limit it is possible to estimate the life-times of these phosphate species and thus the dissociation rate constant, \(k_{-1}\). As determined from the line width of the E·P resonance in Fig. 1C, \(k_{-1}\) is 60 ± 20 s\textsuperscript{-1}. This value is consistent with the value of \(k_{-1}\) determined from stopped flow reaction kinetics (27) and with the value of \(k_{-1}\) (10 to 20 s\textsuperscript{-1}) reported by Hull et al. (3) using \textsuperscript{31}P NMR. The precision of such determinations of rate constants is not sufficient, however, to determine to what extent dissociation of E·P contributes to the steady state rate. It is likely that the rates of phosphorylation of the enzyme, dephosphorylation of E·P, and dissociation of E·P are all of similar magnitude at alkaline pH.

Values of the spin-lattice relaxation time, \(T_1\), were determined for the resonances shown in Fig. 1F using the progressive saturation method. Both the resonances for E·P and free P\textsubscript{i} have equivalent \(T_1\) values of 1.8 ± 0.2 s. Thus in terms of relaxation rates the fast exchange condition \(\tau_{\text{rel}} \ll \tau_1\) in E·P is likely that the rates of phosphorylation of the enzyme, dephosphorylation of E·P, and dissociation of E·P are all of similar magnitude at alkaline pH.

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Dephosphorylation of the Cd\textsuperscript{2+} enzyme is slow relative to the rate of formation of E .P (Fig. 2). Similar to the spectrum shown in Fig. 1A, the \textsuperscript{31}P resonance of 2 eq of inorganic phosphate in the presence of 1 eq of apoenzyme occurs at the chemical shift of free inorganic phosphate at this pH and shows no evidence of specific interaction with the protein (Fig. 2A). On the addition of 1 eq of Cd\textsuperscript{2+} to this mixture a new resonance appears at 8.07 ppm, assigned to the phosphoserine group in the Cd\textsuperscript{2+} enzyme (Fig. 2B) (1). There is a reciprocal loss of resonance from the peak for inorganic phosphate. Addition of a second equivalent of Cd\textsuperscript{2+} to the enzyme increases the amplitude of the resonance which is now equivalent to the peak remaining at the resonance position for inorganic phosphate. Thus the cadmium enzyme forms a maximum of 1 eq of E-P:enzyme dimer and two Cd\textsuperscript{2+} ions/dimer are required to induce formation of 1 eq of E-P. Addional P\textsubscript{i} shows no evidence (see below) of interaction at the enzyme active center. Thus \textsuperscript{31}P NMR of the cadmium phosphoryl enzyme strongly suggests that, even though two identical Cd\textsuperscript{2+} binding sites, separated by 32 Å, are present at the active centers, on phosphorylation of the serine at one active site the other cannot interact with phosphate. This strongly supports the hypothesis that negative homotropic interactions between the subunits of the initially symmetrical dimer occur on interaction with ligand phosphate.

The rate of chemical exchange between E-P on the zinc enzyme and free phosphate is estimated to be ~60 s\textsuperscript{-1}. In contrast, exchange of E-P with free P\textsubscript{i} requires hydrolysis of E-P. For the Cd\textsuperscript{2+} enzyme at pH 6.5 the rate constant for the hydrolysis of E-P is <0.01 s\textsuperscript{-1} as shown by \textsuperscript{18}O exchange studies (6). The resonance for free phosphate in the presence of the phosphorylated Cd\textsuperscript{2+} enzyme (Fig. 2C) is broadened (45 Hz) relative to the corresponding resonance in the presence of the apoenzyme at pH 6.5 (Fig. 2A). Because of the large difference in rate constants describing the formation of E-P and E-P, the line width of free P\textsubscript{i} reflects lifetime broadening, controlled by the slowest association step in the overall process. Free P\textsubscript{i} in solution is in equilibrium with E-P via the E-P complex; the latter present in negligible steady state concentration for the Cd\textsuperscript{2+} enzyme at pH 6.5 (Fig. 2). Association of P\textsubscript{i} with the enzyme to form E-P, based on the results obtained with the Zn\textsuperscript{2+} enzyme, the major intermediate formed at alkaline pH is E-P (6, 27). Initial studies reported previously have shown that the phosphate in E-P is bound close enough to the Co\textsuperscript{2+} for the NMR line to be extensively broadened by the paramagnetic component of the relaxation (1). Thus the disappearance of the resonance for P\textsubscript{i} can be used to follow the binding of phosphate close to the active site metal ion.

The spectrum of a mixture of apoenzyme and 1 eq of P\textsubscript{i} at pH 8.0 is shown in Fig. 3A along with an external marker of methyl phosphonate. At pH 8.0 free phosphate in the presence of the apoenzyme has a significantly narrower line width than at pH 6.5 (compare Figs. 3A and 4A), perhaps reflecting less interaction of the phosphate dianion with the protein, thus resulting in less efficient relaxation as compared to the monoanion. The \( T_1 \) of the phosphate resonance is sufficiently long so that, at the pulse repetition rate employed, complete recovery does not occur. On addition of 1 eq of Co\textsuperscript{2+} at pH 8.0, there is a concomitant reduction in both \( T_1 \) and \( T_2 \) of free P\textsubscript{i} (Fig. 3B), sufficient to enhance the resonance amplitude compared to that of Fig. 3A. However, the resonance observed following the Co\textsuperscript{2+} addition can be shown to represent only half the phosphate present. The resonance of Fig. 3B is directly comparable to that observed on addition of 1 eq of P\textsubscript{i}.
to the reconstituted Co\(^{2+}\) enzyme after the full complement of phosphate is bound (Fig. 3D), and is half the amplitude of the latter.

On the addition of a second equivalent of Co\(^{2+}\) to the enzyme, the phosphate resonance is now broadened to several hundred Hz (Fig. 3C). This resonance must represent the \(E\cdot P\) complex of the Co\(^{2+}\) enzyme. Thus 2 mol of Co\(^{2+}\)/mol of enzyme dimer are required for 1 mol of phosphate to be bound to the dimer close enough to the metal ion for the resonance to be broadened by paramagnetic relaxation. This requires that the oxygen of the phosphate be within the first or second coordination sphere of the metal ion

When a second equivalent of P\(_i\) is added to the enzyme containing two Co\(^{2+}\) ions and one bound phosphate anion, the resonance of the second equivalent appears at the chemical shift of free inorganic phosphate (Fig. 3D). The resonance is broadened to the same extent as the resonance in the presence of one Co\(^{2+}\) ion (Fig. 3B), but is twice the amplitude, indicating that these resonances represent 1.0 and 0.5 eq, respectively, of free phosphate in slow exchange with the phosphate of \(E\cdot P\). If the exchange rate is <100 \(s^{-1}\) (similar to that for the Zn\(^{2+}\) enzyme), then the observed broadening can be entirely accounted for by the exchange broadening. Thus no significant amount of the second equivalent of P\(_i\) can be bound to a site within the first or second coordination sphere of the Co\(^{2+}\) ion. Hence, once 1 mol of \(E\cdot P\) is formed at one Co\(^{2+}\) site on the enzyme, the second site cannot form the same complex. These data reflect the same phenomenon of negative cooperativity for \(E\cdot P\) formation by the Co\(^{2+}\) enzyme as described above for the formation of \(E\cdot P\) by the Cd\(^{2+}\) enzyme (Fig. 2).

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**Fig. 3.** \(^{31}\)P NMR spectra of phosphate binding to Co\(^{2+}\) alkaline phosphatase at pH 8.0. Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 8.0. All spectra were obtained with a constant concentration of methyl phosphonate as an external standard (\(\delta = 29.4\) ppm). A, 1.65 \(\times 10^{-3}\) M apoalkaline phosphatase (apo AP), 1.60 \(\times 10^{-4}\) M K\(_2\)HPO\(_4\), 1.55 \(\times 10^{-3}\) M Co\(^{2+}\); B, 1.59 \(\times 10^{-4}\) M apoalkaline phosphatase, 1.55 \(\times 10^{-4}\) M K\(_2\)HPO\(_4\), 1.54 \(\times 10^{-2}\) M Co\(^{2+}\); C, 1.54 \(\times 10^{-2}\) M apoalkaline phosphatase, 1.50 \(\times 10^{-2}\) M K\(_2\)HPO\(_4\), 3.02 \(\times 10^{-3}\) M Co\(^{2+}\); D, 1.49 \(\times 10^{-3}\) M apoalkaline phosphatase, 2.90 \(\times 10^{-3}\) M K\(_2\)HPO\(_4\), 2.91 \(\times 10^{-3}\) M Co\(^{2+}\); Co\(^{2+}\) added as Co\(^{2+}\)Cl\(_2\).

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**Fig. 4.** \(^{31}\)P NMR spectra of phosphate binding to Co\(^{2+}\) alkaline phosphatase at pH 6.5. Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5. All spectra were obtained with a constant concentration of methyl phosphate as an external standard (\(\delta = 29.4\) ppm). A, 1.80 \(\times 10^{-3}\) M apoalkaline phosphatase (apo AP), 1.80 \(\times 10^{-3}\) M K\(_2\)HPO\(_4\), 1.76 \(\times 10^{-3}\) M apoalkaline phosphatase, 1.77 \(\times 10^{-3}\) M K\(_2\)HPO\(_4\), 1.73 \(\times 10^{-3}\) M Co\(^{2+}\); C, 1.73 \(\times 10^{-3}\) M apoalkaline phosphatase, 1.75 \(\times 10^{-3}\) M K\(_2\)HPO\(_4\), 3.40 \(\times 10^{-3}\) M Co\(^{2+}\); D, 1.70 \(\times 10^{-3}\) M K\(_2\)HPO\(_4\), 3.37 \(\times 10^{-3}\) M K\(_2\)HPO\(_4\), 3.31 \(\times 10^{-3}\) M Co\(^{2+}\); E, 1.62 \(\times 10^{-2}\) M apoalkaline phosphatase, 3.22 \(\times 10^{-3}\) M K\(_2\)HPO\(_4\), 3.16 \(\times 10^{-3}\) M Co\(^{2+}\); 0.06 \(\times 10^{-3}\) M apoalkaline phosphatase added. Co\(^{2+}\) added as Co\(^{2+}\)Cl\(_2\).
Phosphate Binding to Co²⁺ Enzyme, pH 6.5—The $^{31}$P spectra observed when the corresponding titration is conducted at pH 6.5 are altered. Addition of 1 eq of Co²⁺ results in line broadening ($\Delta v_{pp} \sim 120$ Hz) greater than that observed under comparable conditions at pH 8.0 (compare Fig. 4B and 3B). This suggests that the free phosphate may now be in rapid exchange with E·P. This conclusion is further supported by a doubling of the line width ($\sim 225$ Hz) of the observed resonances when a second equivalent of Co²⁺ is added (Fig. 4C). This requires that the dissociation rate of P, from the bound state be $\sim 10^4$ s⁻¹, increasing from the value of $\sim 10^4$ s⁻¹ estimated for the system at pH 8.0. On the addition of a second equivalent of P, the resonance (still at the chemical shift position of free P)³ increases in intensity and the line width narrows to $\sim 120$ Hz, that observed in Fig. 4B. The area normalized for the number of transients is approximately twice that of the resonance in Fig. 4B. This relationship is expected if the fast exchange condition persists; the resonance in Fig. 4B representing a total of 1 eq of phosphate, 0.52 eq in excess of reconstituted enzyme; the resonance in Fig. 4D representing a total of 2 eq of phosphate, 1.02 eq in excess of reconstituted enzyme. These calculations assume a dissociation constant for E·P of the Co²⁺ enzyme of $5 \times 10^{-8}$ M, a magnitude supported by $^{31}$P[HPO₄]²⁻ binding studies at pH 6.5 ($1 \times 10^{-8}$ M enzyme) which show $\sim 1$ eq of E·P (6). Addition of excess apoenzyme to the final mixture (Fig. 4E) does not alter the line width, suggesting that significant free Co²⁺ does not contribute to the line broadening.

$^{115}$Cd NMR of Metal Ions in Cd²⁺ Alkaline Phosphatase and Phosphorylated Derivative—All data on alkaline phosphatase, including mapping of the tryptic peptides (32), sequencing studies (33), and x-ray diffraction (26, 34), suggest that the subunits of the unliganded dimer are identical. Thus the interpretation of the $^{31}$P NMR spectra given above requires that while the active sites are initially identical, reaction with P, at one site destroys the 2-fold symmetry and the second site can no longer interact with ligand (phosphate). The alteration of the nonliganded site is presumably brought about by conformational changes induced by the ligand and propagated across the monomer-monomer interface. Such changes might take place primarily at the monomer-monomer interface if the active sites themselves are located along the domain of subunit contact.

Demonstration of the existence of negative homotropic interactions between the sites using phosphate or substrate as the reporter group is not completely satisfactory since such methods reflect alterations only at the liganded site. The requisite structural changes at the unliganded site can only be inferred from the observation of half the sites reactivity. A more satisfactory technique would be one in which the environments of both active centers were simultaneously observed during ligand interaction at a single site.

$^{115}$Cd⁴⁺ FT (Fourier Transform) NMR is a technique uniquely satisfying these criteria for alkaline phosphatase. $^{115}$Cd has a nuclear spin of $\frac{1}{2}$ and a chemical shift extremely sensitive to the chemical environment of the Cd²⁺ ion. Chemical shift values for common Cd²⁺ compounds span over 600 ppm (13, 35). The sensitivity of this technique has recently been sufficiently improved to permit observation of $^{137}$Cd⁴⁺ bound to biological macromolecules (14).

The $^{115}$Cd spectrum of phosphate-free alkaline phosphatase dimer shows a single cadmium resonance at 170 ppm (Fig. 5A). Thus the two Cd²⁺ ions, bound at the separate binding sites of the subunits, are in identical environments in the unliganded protein dimer. Therefore, the dimer must have 2-fold symmetry with respect to the immediate environment of the metal ions.

On the addition of 1 eq of phosphate to the protein, the cadmium resonance splits into two resonances, one at 142 ppm and one at 55 ppm, each with an amplitude accounting for half the cadmium initially present. The $^{31}$P NMR spectrum of this sample shows a single resonance at 8.07 ppm corresponding to the Cd²⁺ phosphoryl enzyme (Fig. 2). Addition of 1 eq more of P, does not alter the $^{115}$Cd²⁺ resonances and the $^{31}$P intensity of the additional phosphate appears at 2 ppm, corresponding to free phosphate (Fig. 2C).

Thus, phosphorylation of one active site not only influences the chemical environment of both metal ions, but renders the enzyme of one different from the other as indicated by the 87 ppm difference in the chemical shift for the two $^{115}$Cd²⁺ resonances of the phosphorylated enzyme. Neither environment is the same as that observed in the unliganded enzyme. Thus phosphorylation destroys the 2-fold symmetry of the metal ion sites, a necessary condition of negative cooperativity for this enzyme.

Binding of Cd²⁺ to Apophosphoryl Enzyme—The $^{31}$P and $^{115}$Cd NMR data above indicate that if one of the active sites on the phosphatase dimer is phosphorylated, the immediate environments of the two metal binding sites are no longer identical (Fig. 5). However, it is not possible to determine from these data if the differences in the sites require the presence of metal ions or are a property of the potential metal binding sites of the phosphorylated apoenzyme. Preparation of the apophosphoryl enzyme (30) permits experimental resolution of this question by monitoring the $^{31}$P NMR spectrum of E·P as the binding sites are occupied on titration with metal ion. Regeneration of the Cd²⁺ phosphoryl enzyme using this method is not consistent with a population of metal ion binding sites in a statistical manner (Fig. 6). Although a single resonance, representing a homogeneous species corresponding to the Cd²⁺ phosphoryl enzyme is ultimately observed (Fig. 6B); the intermediate stages of the titration (Fig. 6, B to D) are not consistent with the progressive formation of the final product. Addition of $\leq$1 eq of Cd²⁺ does not alter the chemical shift of the phosphoryl resonance (Fig. 6B), suggesting that the metal either binds preferentially at the unphosphorylized site or is not able to induce the structural changes that result in the 2-ppm downfield chemical shift characteristic of the Cd²⁺ phosphoryl enzyme. Since the presence of two metal ions in one phosphorylated dimer does produce the downfield shift, prior phosphorylation must favor the binding of one metal ion/dimer when the full complement of metal ion is not available. Incremental addition of the second Cd²⁺ ion/dimer results in gradual changes in the spectrum with the appearance of a $^{31}$P resonance at the chemical shift position of the Cd²⁺ phosphoryl enzyme (Fig. 6, C to E). At stoichiometries between one and two Cd²⁺ ions/dimer additional resonances are observed suggesting that binding of the second metal ion to the dimer induces successive conformational changes in the molecule. These conformational changes might take place primarily at the monomer-monomer interface if the active sites themselves are located along the domain of subunit contact.
isomers are sufficiently stable to be detected as discrete resonances in the $^{31}$P NMR spectrum, further evidence that control of metal ion stoichiometry is critical to the appearance and interpretation of the $^{31}$P resonances of phosphate complexes of alkaline phosphatase. Once all molecules contain 2 eq of Cd$^{2+}$, only a single resonance at the chemical shift of the phosphorylated Cd$^{2+}$ enzyme is observed (Fig. 6E). Thus phosphate (or the phosphoseryl moiety) and the metal ion appear to be reciprocally involved in the induction of asymmetric interactions of the subunits of the alkaline phosphatase dimer.

Phosphate Binding to Alkaline Phosphatase Containing Mixture of Co$^{2+}$ and Cd$^{2+}$ – Another indication that the presence of phosphate can induce nonequivalent interactions between the two metal ion sites is provided by following the $^{31}$P resonance of phosphate during the sequential addition of 1 eq of Co$^{2+}$ and 1 eq of Cd$^{2+}$ to a mixture of apoenzyme plus 1 eq of inorganic phosphate (Fig. 7). The first two stages of this experiment (Fig. 7, A and B) parallel and confirm the results of the experiment depicted in Fig. 4. On addition of 1 eq of Cd$^{2+}$ to the sample containing apoenzyme, phosphate and 1 eq of Co$^{2+}$, surprisingly little phosphoryl enzyme is formed (Fig. 7C). In the absence of Co$^{2+}$, addition of 1 eq of Cd$^{2+}$ would be expected to result in formation of 0.5 eq of phosphoryl enzyme (Fig. 2B), generating a low field resonance in the $^{31}$P NMR spectrum of appropriate intensity. Instead, the phosphate resonance is broadened and unshifted. Thus most of the phosphate in the sample must be bound at a Co$^{2+}$ site in a condition of fast exchange with free phosphate (compare Figs. 4C and 7C). As a corollary it may also be concluded that in the presence of phosphate the enzyme must be driven to form a Co$^{2+}$-Cd$^{2+}$ hybrid which prefers to bind phosphate at the Co$^{2+}$ site. Other possible distributions of metalloalkaline phosphatases which could form in this sample would result in the presence of from 0.25 to 0.50 eq of Cd$^{2+}$ alkaline phosphatase. Generation of Cd$^{2+}$ phosphoryl enzyme with the appearance of a $^{31}$P resonance of corresponding intensity at 8 ppm would be the necessary consequence which is not, however, observed. It is difficult, however, in the absence of exact knowledge of the rate constants governing $P_i$ exchange at Co$^{2+}$ and Cd$^{2+}$ sites on the enzyme to rigorously exclude alternative explanations. Preferential association of phosphate at the Co$^{2+}$ site is plausible, since the conformational relationships at the Co$^{2+}$ site promote rapid enzyme turnover while those at the Cd$^{2+}$ site do not (6, 27). The changes at the Cd$^{2+}$ site induced by the asymmetric binding of phosphate to the Co$^{2+}$ site can also be monitored by $^{113}$Cd NMR and are the object of current investigations.

CONCLUSIONS

The $^{113}$Cd NMR and $^{31}$P NMR results described above demonstrate the existence of negative homotropic interactions in the ligand (phosphate) binding of alkaline phosphatase. The chemical environment of the metal ions bound at the two active centers of the enzyme dimer are identical in the absence of phosphate (Fig. 5A). Interaction of phosphate at one site

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**Fig. 5.** $^{113}$Cd$^{2+}$ NMR spectra of Cd$^{2+}$ alkaline phosphatase and Cd$^{2+}$ phosphoryl alkaline phosphatase. Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5. A, $3.65 \times 10^{-3}$ M Cd$^{2+}$ alkaline phosphatase \([Cd^{2+}]_\text{enzyme} = 2.01; B, 4.05 \times 10^{-3}$ M Cd$^{2+}$ alkaline phosphatase, $4.05 \times 10^{-3}$ M K$_2$HPO$_4$, \([Cd^{2+}]_\text{enzyme} = 2.00$. 

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**Fig. 6.** $^{31}$P NMR spectra of the phosphoryl enzyme on Cd$^{2+}$ titration of apophosphoryl alkaline phosphatase (Apo AP-P). Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5. A, $1.20 \times 10^{-3}$ M apophosphoryl alkaline phosphatase; B, $1.19 \times 10^{-3}$ M apophosphoryl alkaline phosphatase, $7.63 \times 10^{-4}$ M Cd$^{2+}$; C, $1.18 \times 10^{-3}$ M apophosphoryl alkaline phosphatase, $1.52 \times 10^{-3}$ M Cd$^{2+}$; D, $1.17 \times 10^{-3}$ M apophosphoryl alkaline phosphatase, $2.25 \times 10^{-3}$ M Cd$^{2+}$; E, $1.16 \times 10^{-3}$ M apophosphoryl alkaline phosphatase, $2.08 \times 10^{-3}$ M Cd$^{2+}$. Data acquisition was begun immediately after metal ion additions for a ~10-h sampling time. Cd$^{2+}$ was added as CdCl$_2$.

differentially alters the environment of both metal ions and therefore the conformational structure of the active centers (Fig. 5b). Covalent (E-P) or noncovalent (E .P) complex formation with phosphate at one site induces structural changes which prevent tight specific binding of ligand at the second site (Figs. 1 to 4). Thus even at relatively high (> 1 mM) ligand concentrations the enzyme displays absolute negative cooperativity. Communication between the subunits giving rise to this phenomenon is apparently mediated by conformational changes propagated across the monomer-monomer interface.

Whether negative cooperativity is a phenomenon incidental to some rearrangement of the enzyme subunits required for phosphorylation or dephosphorylation or serves a functional purpose is unclear at present. If the in vivo function of the enzyme is a selective phosphate transfer rather than nonspecific monoesterase activity, conformational change and negative cooperativity might exert some spatially selective function. There is the possibility that negative cooperativity could aid in the ejection of tightly bound phosphate from the opposite site on interaction of substrate at the nonliganded site. To be effective such a mechanism would require that there be significant affinity of the non-phosphate-containing site for substrate prior to dissociation of the tightly bound phosphate. The NMR data for phosphate binding do not indicate significant affinity for the second site (Figs. 1 to 3). Initial binding of phosphate monoester, however, might be different and this mechanism therefore remains a possibility. Conformational changes associated with the phenomenon of negative cooperativity may affect solvent access to the metal ion site or the phosphoserine. Such changes can be detected by determination of alterations in the spin-lattice relaxation times and nuclear Overhauser enhancements of $^{113}$Cd and $^{31}$P in H$_2$O and D$_2$O and will be the subject of further publication.

**Fig. 7.** $^{31}$P NMR spectra of phosphate binding on sequential additions of Co$^{2+}$ and Cd$^{2+}$ to apoalkaline phosphatase (Apo AP). Conditions: 0.01 M Tris, 0.01 M NaOAc, 0.1 M NaCl, pH 6.5. A, $1.80 \times 10^{-3}$ M apophosphoryl phosphatase, $1.80 \times 10^{-3}$ M K$_2$HPO$_4$; B, $1.76 \times 10^{-3}$ M apophosphoryl phosphatase, $1.77 \times 10^{-3}$ M K$_2$HPO$_4$, $1.73 \times 10^{-3}$ M Co$^{2+}$; C, $1.70 \times 10^{-3}$ M apophosphoryl phosphatase, $1.71 \times 10^{-3}$ M K$_2$HPO$_4$, $1.67 \times 10^{-3}$ M Co$^{2+}$, $1.74 \times 10^{-3}$ M Cd$^{2+}$. Metal ions added as Me$^{3+}$Cl$_2$.

enzyme is a selective phosphate transfer rather than nonspecific monoesterase activity, conformational change and negative cooperativity might exert some spatially selective function. There is the possibility that negative cooperativity could aid in the ejection of tightly bound phosphate from the opposite site on interaction of substrate at the nonliganded site. To be effective such a mechanism would require that there be significant affinity of the non-phosphate-containing site for substrate prior to dissociation of the tightly bound phosphate. The NMR data for phosphate binding do not indicate significant affinity for the second site (Figs. 1 to 3). Initial binding of phosphate monoester, however, might be different and this mechanism therefore remains a possibility. Conformational changes associated with the phenomenon of negative cooperativity may affect solvent access to the metal ion site or the phosphoserine. Such changes can be detected by determination of alterations in the spin-lattice relaxation times and nuclear Overhauser enhancements of $^{113}$Cd and $^{31}$P in H$_2$O and D$_2$O and will be the subject of further publication.

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