Phosphate Binding to Alkaline Phosphatase

METAL ION DEPENDENCE*

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SUMMARY

The metal ion dependence of $^{32}$P binding to Escherichia coli alkaline phosphatase has been studied by means of equilibrium dialysis. Whereas the apoenzyme does not bind phosphate (no sites with $K < 5 \times 10^{-3} \text{M}$), the addition of 2 Zn(II) cations per molecule induces the tight binding of 1 phosphate dianion ($K = 6 \times 10^7 \text{M}$). The magnitude of this binding constant is affected by ionic strength, pH, and protein concentration. Of the first transition and IIB metal ions, Mn(II), Co(II), Zn(II), and Cd(II) all induce the tight binding of one phosphate per dimer, but only Zn(II) and Co(II) induce enzymatic activity. Cu(II) is less effective, while Ni(II) and Hg(II) are ineffective in inducing phosphate binding.

The formation of the phosphoryl enzyme, as measured by treatment of the $^{32}$P-protein mixture with perchloric acid, is also metal ion-dependent. The Zn(II) enzyme forms the phosphoryl enzyme only at low pH, a maximum of 0.6 mole per mole of dimer being observed at pH 5. The apoenzyme forms no phosphoryl enzyme at any pH. In marked contrast, Cd(II) induces the formation of significant equilibrium concentrations of phosphoryl enzyme in the alkaline pH range, reaching a maximum stoichiometry of 1 mole per mole of dimer at pH 6.5. Isolation of a phosphorylated peptide from the Cd(II) enzyme shows that the same seryl residue is phosphorylated in the Cd(II) protein as in the native Zn(II) enzyme. Mn(II) and Co(II) also induce the formation of significant amounts of phosphoryl enzyme in the alkaline pH region. Phosphorylation of the Cd(II) enzyme is relatively slow, $k = \sim 3 \times 10^{-3} \text{sec}^{-1}$, but once formed the Cd(II) phosphoryl enzyme does not break down as shown by its failure to catalyze the exchange of $^3$O from H$_2$O into inorganic phosphate. The data suggest that the metal ion plays an important role in formation and breakdown of the phosphoryl enzyme as well as in the binding of phosphate.

Studies of phosphate binding to Escherichia coli alkaline phosphatase have been of major interest in elucidating the mechanism of action of this metalloenzyme. A scheme for the reaction

$$E + S \leftrightarrow E.S \leftrightarrow E.P \rightarrow ROH \leftrightarrow E.P + E + P,$$

pathway of this enzyme which includes a minimal number of intermediates is given by Equation 1 where $E.S$ and $E.P$ are noncovalent Michaelis complexes and $E.P$ is the phosphoryl enzyme. One of the major features of the active center is a metal ion-dependent. A great number of substrates, whose leaving groups differ widely, have the same $K_m$ and $V_{max}$. The $K_m$ of these esters differ little from the $K_i$ for the phosphate itself (1-5). The value for $K_m$ and $K_i$ is of the order of $10^{-4} \text{M}$. Substantial evidence also exists for the formation of a phosphoryl enzyme intermediate ($E.P$). At low pH this covalent intermediate can be isolated and has been shown to be a phosphorylated serine side chain (6-11). Kinetic and thermodynamic evidence also strongly supports the presence of this intermediate in the reaction pathway (12-15). Orthophosphate is not only a competitive inhibitor of the hydrolytic reaction but can be considered a virtual substrate since the enzyme catalyzes $^3$O exchange from water into phosphate. Significant equilibrium concentrations of the phosphoryl enzyme intermediate are formed at low pH in the presence of orthophosphate (6, 8, 9, 11, 13).

Alkaline phosphatase specifically requires the presence of 2 tightly bound divalent zinc ions to catalyze the hydrolysis of phosphate monoesters (1, 16-18). Previous studies have been carried out to investigate the role of metal ions in the structure of the protein (19, 20). We are now interested in determining what role the metal ions play in the catalytic action and whether the metal ion is directly involved at the active site. The set of studies presented here was undertaken to determine whether the metal ions affect any of the intermediates in the hydrolytic reaction pathway and to investigate further the nature of these intermediates. A preliminary account of this work has been given (21).

EXPERIMENTAL PROCEDURE

Reagents—All chemicals were reagent grade. Buffer solutions, HCl, and NaOH were prepared free of metal as previously described (19, 22). Metal solutions were prepared from spectrographically pure metal chlorides or sulfates (Johnson Matthey Company, Ltd., London). $^{32}$P was purchased from New England Nuclear as phosphoric acid $^{32}$P in 0.02 N HCl.

Preparation of Alkaline Phosphatase—The enzyme was isolated by...
from E. coli C90 by osmotic shock according to the procedure of Neu and Heppel (23). The purification has been described previously (20) except for a modification made in all steps involving precipitation of the enzyme with (NH$_4$)$_2$SO$_4$. In these steps ZnSO$_4$ was added such that the concentration was 10 times the equivalent moles of alkaline phosphatase. This prevented loss of zinc from the enzyme. The protein was crystallized according to the method of Jakoby (24). Prior to use, the crystalline protein was dissolved in a small volume of 0.01 M Tris-0.01 M sodium acetate, pH 8, and passed through a Sephadex G-100 column (2 x 100 cm). The gel filtration separated a small peak at the void volume, constituting about 0.5 to 1% of the total protein applied. The remaining protein was clutched just after the void volume as one symmetrical peak and had a constant specific activity across the peak. The dissolved crystalline protein has a specific activity of 3000 ± 500 mmoles of substrate hydrolyzed per hour per mg of protein when assayed at 25° and contains an average of 3 g atoms of zinc per mole of alkaline phosphatase dimer at pH 8.

Preparation of Apo- and Me(II)-Alkaline Phosphatases—The apoenzyme was prepared by treating the enzyme with a suspension of Chelex 100 (200 to 400 mesh) (Bio-Rad) in 1.0 M Tris buffer, pH 8.0, as originally suggested by Cohn and also described by Csopak (25). In 2 to 3 hours the zinc content is less than 0.1 g atom per mole. The apoenzyme was then routinely dialyzed against metal-free buffer, 0.01 M Tris-0.01 M sodium acetate, pH 8.0, prior to use for the equilibrium dialysis experiments. Alkaline phosphatases containing metal ions other than zinc were prepared from the apoenzyme by two methods. The apoenzyme was titrated with the required stoichiometric amounts of Me(II) or dialyzed against equimolar Me(II) contained in 0.01 M Tris-0.01 M sodium acetate, pH 8.0. The physicochemical properties of this preparation of the native enzyme, the apoenzyme, and the enzyme reconstituted with other metal ions have been previously published (19, 20).

Metal Analyses for Zn(II), Mn(II), Co(II), Ni(II), Cu(II), and Hg(II)—These analyses were performed by atomic absorption spectroscopy with the use of a Jarrell-Ash spectrometer.

Enzymatic Activity—Alkaline phosphatase activity was assayed by measuring the release of p-nitrophenol from p-nitrophenylphosphate. The enzyme was diluted in 1 M Tris-HCl, pH 8, and added to the cuvette containing assay solution, millimolar p-nitrophenylphosphate (Sigma) in 1 M Tris-HCl, pH 8. The release of p-nitrophenol was followed at 410 nm with $E_{1%}^{1cm} = 0.72$ (26). For molar calculations, a molecular weight of 80,000 was used (20).

Equilibrium Dialysis—This was performed as previously described (27) with dialysis bags of 1-ml capacity made from cellulose tubing. Protein solutions containing ~10$^{-4}$ M alkaline phosphatase dimer were dialyzed against 50-mU volumes of $^{32}$P at the concentrations and pH values indicated in the text contained in 0.01 M Tris-0.01 M sodium acetate, 4°. After equilibration for 48 hours, 0.50- or 0.25-ml aliquots were removed by syringe and injected rapidly into the scintillation mixture. Blanks were determined by taking similar 0.50- or 0.25-ml aliquots from the dialysate. For experiments in which the apoenzyme or metalloenzymes other than zinc were used, the dialysis tubing was soaked in 5 × 10$^{-4}$ M o-phenanthroline or EDTA, pH 7.0, for several days and then washed exhaustively with metal-free water. Vessels used were acid-washed (22) and buffers were metal-free. Following dialysis procedures, optical density of the recovered material was checked to ensure that no protein was lost. The apoenzyme preparations used could be reactivated to 90 to 95% of the original activity by the readdition of Zn(II) ions.

No corrections were made for Donnan effects. At pH 8 there is a net negative charge of 19 per dimer (28). With 0.01 M Tris-chloride buffer and protein concentrations of 10$^{-4}$ M there would be a maximum 4% error. The equilibrium dialysis data were plotted as titration curves and then further analyzed according to the general form of Scatchard’s equation (29),

$$\frac{v_i}{c} = n_iK_i - v_iK_i$$

where $c$ is the concentration of free phosphate, $n_i$ is the number of identical independent sites having a binding constant of $K_i$, and $v_i$ is the average number of phosphates bound per mole of protein. A plot of $(v_i/c)$ against $v_i$ should yield a straight line with the ordinate intercept equal to $n_iK_i$ and the abscissa intercept equal to $n_i$. If more than one class of binding sites is present, a Scatchard plot may show curvature. In this case the simplest (but not only) interpretation is to assume that there are $i$ sets of sites which are noninteracting such that the following equation holds:

$$\frac{v}{c} = \sum_i \frac{v_i}{c} = \sum_i \frac{n_iK_i}{1 + K_i c}$$

Where applicable, the data were fitted by an appropriate choice of constants by Equation 3.

Determination of Acid-precipitable Enzyme-bound Phosphate—A 0.25-ml aliquot of enzyme solution (~10$^{-5}$ M in alkaline phosphatase dimer) was extracted by syringe from the dialysis bag used in the equilibrium dialysis procedure described above. This aliquot was injected rapidly into 0.5 ml of 70% perchloric acid and vigorously mixed. After 5 to 10 min, a 0.1-ml aliquot of this solution was placed on a 2.4-cm disc of Whatman No. 3 filter paper. The disc was then washed three times with cold 5% trichloracetic acid and once with cold 95% ethanol. The disc was then air-dried and put into a previously cooled scintillation vial containing 15 ml of a dioxane solution of 2,5-diphenylazoazole and 1,4-bis(5-phenylazo)xylbenzene (30) and counted immediately in a Tri-Carb liquid scintillation counter (Packard). A separate 0.25-ml aliquot of the dialysate was treated identically to determine the blank counts on the disc. After correction for the blank, the moles of phosphate bound per mole of protein were determined from the initial specific activity of the phosphate and the molarity of the protein.

For measurements of the phosphorylation rate of the Cd(II) enzyme, the first step of the procedure was altered in that equilibrium dialysis was not performed. The Cd(II) enzyme (1 × 10$^{-5}$ M) was incubated with $^{32}$P-phosphate (1 × 10$^{-4}$ M) contained in 0.01 M Tris-0.01 M sodium acetate in small test tubes at 25°. Aliquots were removed at time intervals of 10 sec to 24 hours and treated with perchloric acid as described above.

We thank Dr. Mildred Cohn of the University of Pennsylvania for suggesting the Chelex treatment.
Phosphate Binding to Alkaline Phosphatase

FIG. 1. Scatchard plots of \(^{32}P\) binding to native alkaline phosphatase. A, with 0.01 M Tris, pH 8: ——, 1 \times 10^{-5} \text{ M} alkaline phosphatase; ○, 1.5 \times 10^{-5} \text{ M} alkaline phosphatase; □, 2.78 \times 10^{-5} \text{ M} alkaline phosphatase; ○, 1.66 \times 10^{-5} \text{ M} alkaline phosphatase dialyzed against equimolar Zn(II). The data are fitted as described under "Experimental Procedure" with the following constants: ——: \(n_1 = 1.0, K_1 = 1.1 \times 10^{-6} \text{ M}; n_2 = 1.0, K_1 = 1.0 \times 10^{-5} \text{ M} \); ○: \(n_1 = 1.0, K_1 = 1.2 \times 10^{-6} \text{ M}; n_2 = 2.0, K_2 = 2.5 \times 10^{-5} \text{ M} \); which is indistinguishable from ——: \(n_1 = 1.0, K_1 = 1.2 \times 10^{-6} \text{ M}; n_2 = 1.0, K_2 = 2.6 \times 10^{-5} \text{ M} \). B, with 0.01 M Tris-0.01 M sodium acetate, pH 5.5: ——, 1.6 \times 10^{-5} \text{ M} alkaline phosphatase with \(n_3 = 1.0, K_3 = 2.5 \times 10^{-5} \text{ M} \); ○, 1.3 to 1.6 \times 10^{-5} \text{ M} alkaline phosphatase. Data are fitted with ——: \(n_1 = 1.0, K_1 = 3.1 \times 10^{-2} \text{ M}; n_2 = 1.0, K_2 = 1 \times 10^{-4} \text{ M} \); ○: \(n_1 = 1.0, K_1 = 3.1 \times 10^{-2} \text{ M} \) and \(n_2 = 2.0, K_2 = 2.5 \times 10^{-4} \text{ M} \) which is indistinguishable from ——: \(n_1 = 1.0, K_1 = 3.1 \times 10^{-2} \text{ M}; n_2 = 2.0, K_2 = 2.5 \times 10^{-4} \text{ M} \). A slightly better fit for the data at high phosphate is obtained if it is assumed that two loose sites are present, \(K_2 = 2.5 \times 10^{-5} \text{ M} \). The data show well that there is one tight binding site. In the region of high phosphate the experimental error is large (32, 33) and except in one case the protein concentrations are such that saturation of the higher order sites cannot be achieved (see "Discussion"). The native alkaline phosphatase used in this experiment contained 3 g at K(II) per mole of dimer; no extra precautions were taken to control the low levels of metal contamination. With the presence of excess zinc in solution, the results of phosphate binding are consistent with the general bulk of data and require no change in acceptable constants for the binding sites.

When the pH is decreased to 5.5, the Scatchard plot shows that alkaline phosphatase increases its affinity for phosphate at the tighter binding site (Fig. 1A). Higher order binding sites still exist at this pH, but their affinity seems little changed from that at pH 8; \(K_3 = 6.8 \times 10^{-5} \text{ M} \) for the tight binding site at pH 5.5.

Phosphate binding in the presence of salt shows that ionic strength significantly affects the dissociation constant of the tight binding site. In 0.1 M KCl, \(K_1 = 3.1 \times 10^{-7} \text{ M} \); in 0.5 M KCl, \(K_1 = 6.6 \times 10^{-7} \text{ M} \). Higher order sites with dissociation constants comparable to those present in the absence of salt are present as well in both cases (Fig. 1B).

Binding of Phosphate by Apoalkaline phosphatase and Metalloalkaline phosphatases—Apoalkaline phosphatase binds no phosphate specifically (Fig. 2). Binding of phosphate, however, with a dissociation constant greater than \(5 \times 10^{-5} \text{ M} \) cannot be
detected in these experiments. Upon adding stoichiometric amounts of Zn(II) to the apoenzyme the presence of a high affinity binding site can be restored. Addition of 1 eq of Zn(II) per mole of dimer restores a tight binding site with $K_1 = 5.9 \times 10^{-7} \text{ M}$. At least one loose site, $K_2 \approx 5 \times 10^{-4} \text{ M}$, is also present which has a similar affinity to that present on the apoenzyme. Addition of 2 eq of Zn(II) per mole of dimer restores one tight binding site with $K_1 = 5.9 \times 10^{-7} \text{ M}$ in addition to the site with $K_2 = 5 \times 10^{-4} \text{ M}$ (Fig. 2). Two sites are necessary for the formation of the high affinity phosphate binding site.

The binding of phosphate to alkaline phosphatase is also induced by Mn(II), Co(II), Cd(II), and Cu(II) in addition to Zn(II), even though only the Zn(II) and Co(II) phosphatases show significant enzymatic activity (Table I). Ni(II) and Hg(II) are not effective in inducing phosphate binding. The titration curve for phosphate binding by the Co(II) and Cd(II) proteins is given in Fig. 3. There are insufficient data for a representative Scatchard plot, but the titration curve again indicates there is one tight binding site and one or more loose binding sites. The binding constants for the high affinity binding site of the various metalloalkaline phosphatases are given in Table II.

**Covalent and Noncovalent Phosphate Binding as Functions of pH**—Alkaline phosphatase binds 1 phosphate anion specifically and one or more others at least an order of magnitude less tightly. In order to determine how many of these phosphates can be covalently bound, the total phosphate binding was determined as a function of pH at a fixed phosphate concentration ($10^{-5} \text{ M}$) and the fraction covalently bound was determined by treatment with perchloric acid (Fig. 4). The total phosphate bound remains constant at about 1.5 moles per dimer between pH 8 and 6 and then decreases to zero over the region pH 6 to 3 (Fig. 4A). At pH 8 none of the total phosphate is covalently bound (Fig. 4B). As the pH is lowered covalently bound phosphate is stabilized and the amount detectable increases to a maximum of 0.6 mole per mole of dimer at pH 5. Below pH 5 the amount of covalently bound phosphate decreases in the same manner as the total bound phosphate decreases (Fig. 4, A and B).

The apoenzyme binds between 0.2 and 0.5 mole of phosphate per dimer over the pH range 3 to 8 (0.2 mole is expected to bind on the basis of a dissociation constant of $5 \times 10^{-4} \text{ M}$). None of this phosphate is covalently bound since no phosphoryl enzyme can be isolated from the apoenzyme at any pH (Fig. 5). At $10^{-4} \text{ M}$ phosphate the amount of total phosphate bound to the Mn(II), Co(II), and Cd(II) alkaline phosphatase as a function of pH is very similar to that bound by the Zn(II) enzyme (Fig. 5A). The decrease in total binding to these metalloenzymes takes place at higher pH values than for the Zn(II) enzyme. For all three of these metallophosphatases, the pH dependence of formation of the phosphoryl enzyme is radically different from that of the Zn(II) enzyme. Between pH 6 and 8, the Cd(II) enzyme forms a large amount of phosphoryl enzyme.

A maximum of 1 mole per mole of dimer is observed at pH 6.5 (Fig. 5B). The Mn(II) enzyme also forms significant amounts of phosphoryl enzyme throughout the alkaline pH region. A maximum of 0.4 mole per mole of dimer is formed at pH 7.0. The Co(II) enzyme forms only a small amount of phosphoryl enzyme with a maximum of 0.2 mole per mole of dimer at pH 6. In all three cases the decrease in phosphoryl enzyme at low pH parallels the decrease in total phosphate binding (Fig. 5).

2 Phosphate binding to Cu(II) alkaline phosphatase is difficult to interpret. While Cu(II) induces binding of phosphate significantly above that shown by the apoenzyme, a study of the dependence of phosphate binding on phosphate concentration shows that binding is less tight than in the case of the manganese, cobalt, zinc, and cadmium enzymes. Changes in the state of aggregation of the enzyme induced by Cu(II) have also been observed (M. L. Applebury, unpublished observation).

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**Table I**

| Enzyme | $^{32}P$ bound | No. of determinations | Phosphatase activity |
|--------|----------------|-----------------------|----------------------|
| ZnAP*  | 1.36 ± 0.19    | (20)                  | 3000                 |
| ApoAP  | 0.22 ± 0.15    | (10)                  | 100                  |
| MnAP*  | 1.23 ± 0.06    | (4)                   | 200                  |
| CoAP   | 1.20 ± 0.19    | (4)                   | 600                  |
| NiAP   | 0.30 ± 0.12    | (4)                   | 100                  |
| CuAP   | 0.5-0.8*       | (6)                   | 100                  |
| CdAP   | 1.31 ± 0.07    | (6)                   | 100                  |
| HgAP   | 0.06 ± 0.04    | (4)                   | 100                  |

* The abbreviation used is: AP, alkaline phosphatase with prefix indicating the metal ion species present.

$^*$ Phosphate binding determined at $1 \times 10^{-5} \text{ M}$ free phosphate.

$^c$ Assay as described under "Experimental Procedure." 1 M Tris, pH 8.0.

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**Fig. 3.** Titration curve of $^{32}P$ binding to Co(II) (■), Cd(II) (▲), and apoalkaline phosphatase (○). 1.0 $\times 10^{-5} \text{ M}$ enzyme, 0.01 M Tris, pH 8. Co(II) and Cd(II) were added to the dialysate at a concentration equimolar to the enzyme.

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$^2$ Partial hydrolysis of the $^{32}P$-labeled Cd(II) enzyme in 0.2 N HCl followed by chromatography of the hydrolysate on Dowex 50 yields large amounts of $^{32}P$-phosphoserine and a more slowly eluting labeled peptide containing aspartic acid and alanine in the ratio 1:2 in addition to phosphoserine. These findings are compatible with the previous report of the amino acid sequence surrounding the active serine isolated from the native Zn(II) enzyme, Asp-SerP-Ala-Ala (8).
TABLE II
Binding of phosphate to metalloalkaline phosphatases

The following dissociation constants are given for the high affinity phosphate binding site of alkaline phosphatase. The values of $n_1$ and $K_1$ were calculated by linear least squares analysis from the data in Figs. 1 and 2 after correcting for the higher order sites described under "Results."

| Me(II) phosphatase | Conditions | pH   | $n_1$    | $K_1 \times 10^{6}$ M |
|---------------------|------------|------|----------|----------------------|
| Native alkaline phosphatase | 0.01 M Tris, $4^\circ$ | 8.0 | 1.0 ± 0.2 | 1.2 ± 0.2 |
| Native alkaline phosphatase | 0.01 M Tris, $4^\circ$ | 8.0 | 1.0 ± 0.2 | 1.2 ± 0.2 |
| Native alkaline phosphatase | 0.1 M KCl-0.01 M Tris, $4^\circ$ | 8.0 | 1.0 ± 0.3 | 0.31 ± 0.06 |
| Native alkaline phosphatase | 0.5 M KCl-0.01 M Tris, $4^\circ$ | 8.0 | 1.1 ± 0.2 | 0.66 ± 0.08 |
| Native alkaline phosphatase | 0.01 M Tris-0.01 M sodium acetate, $4^\circ$ | 5.5 | 1.0 ± 0.2 | 0.68 ± 0.09 |
| Apo alkaline phosphatase | 0.01 M Tris, $4^\circ$ | 8.0 | 0.5 | 0.6 |
| Apo alkaline phosphatase | 0.01 M Tris, $4^\circ$ | 8.0 | 1.0 ± 0.1 | 0.59 ± 0.07 |
| Co(II) alkaline phosphatase | 0.01 M Tris, $4^\circ$ | 8.0 | 1 | 0.5-1.0 |
| Cd(II) alkaline phosphatase | 0.01 M Tris, $4^\circ$ | 8.0 | 1 | 0.5-1.0 |

* This value is consistent with the data; however, least square analysis gives a deviation of 100% in the value of $K_1$ for the one-zinc enzyme.

Rate of Phosphorylation of Cd(II) Alkaline Phosphatase by Inorganic Phosphate and Catalysis of $^{32}$O Exchange from $H_2^{18}O$ into Inorganic Phosphate by Zn(II) and Cd(II) Alkaline Phosphatases—The remarkable stability of the phosphoryl enzyme formed by Cd(II) phosphatase suggested a more detailed study of the rate of phosphorylation of the enzyme by inorganic phosphate and a determination of whether once formed the Cd(II) phosphoryl enzyme continues to turn over. If the latter occurs, $^{32}$O from $H_2^{18}O$ should be incorporated into inorganic phosphate as has previously been shown for the Zn(II) enzyme (8, 34).

Since both protein concentration and phosphate concentration can potentially affect the amount of phosphoryl enzyme formed, we examined the phosphorylation of the Cd(II) enzyme over a 10-fold range of protein concentration ($10^{-5}$ M to $10^{-4}$ M) and a 50-fold range of phosphate concentration ($10^{-6}$ M to $5 \times 10^{-4}$ M). The results are shown in Table III. Throughout this range of concentrations only a single site per dimer can be phosphorylated in the Cd(II) enzyme.

The time dependence of phosphorylation of this site by the Cd(II) enzyme at pH 6.5 is shown in Fig. 6 with $10^{-5}$ M enzyme and $10^{-4}$ M phosphate. Under these conditions phosphorylation is relatively slow, with a half-time of approximately 3 min. The pseudo-first order rate constant for phosphorylation of the Cd(II) enzyme calculated from this data is $\sim 3 \times 10^{-3}$ sec$^{-1}$. This is the rate constant for phosphorylation, since as shown below formation of the Cd(II) phosphoryl enzyme is essentially an irreversible process.

The ability of Zn(II) and Cd(II) phosphatases to catalyze the exchange of $^{32}$O from $H_2^{18}O$ into inorganic phosphate is shown in Fig. 7 as a function of pH. A detailed study of the pH dependence of this reaction for the Zn(II) enzyme has not been previously available. The Zn(II) enzyme catalyzes this ex-
Fig. 5. Noncovalent (A) and covalent (B) $^{32}$P binding to Mn(II) (◇), Co(II) (▽), and Cd(II) (▼) alkaline phosphatases as a function of pH. 0.01 M Tris-0.01 M sodium acetate, 4°, 1 × 10$^{-5}$ M $^{32}$P, 1 × 10$^{-2}$ M protein. Co(II), Mn(II), and Cd(II) salts were added to the dialysate at a concentration equimolar to the apo-enzyme.

**TABLE III**

Formation of phosphoryl enzyme by Cd(II) alkaline phosphatase as function of enzyme and phosphate concentrations at pH 6.5

Conditions as described under “Experimental Procedure.”

| Enzyme concentration $M$ | Phosphate concentration $M$ | Phosphoryl enzyme formed moles/mole dimer |
|------------------------|----------------------------|------------------------------------------|
| $1 \times 10^{-4}$    | $1 \times 10^{-4}$        | 1.05                                     |
| $1 \times 10^{-4}$    | $1 \times 10^{-4}$        | 0.95                                     |
| $1 \times 10^{-4}$    | $5 \times 10^{-4}$        | 1.09                                     |

change at a constant rate between pH 5.6 and 7.5. The rate of exchange decreases slowly but not dramatically between pH 7.5 and 9. In marked contrast Cd(II) phosphatase does not catalyze detectable $^{18}$O exchange at any pH (Fig. 7). The rate of $^{18}$O exchange observed for the Zn(II) enzyme (Fig. 7) shows that the over-all process leading to $^{18}$O exchange is a relatively slow one. In the presence of 1.5 atom per cent excess $H_{2}^{18}O$, increase of $^{18}O$ in the phosphate oxygens by 0.4 atom per cent excess requires 48 hours. Calculation of the $P_{i} \rightarrow HOH$ exchange according to Boyer and Bryan (31) shows that the rate constant for exchange by the Zn(II) enzyme under the conditions of Fig. 7 is ~0.2 sec$^{-1}$ at pH 6.5.

Fig. 6. Time rate of phosphorylation of Cd(II) alkaline phosphatase by inorganic $^{32}$P. Cd(II) alkaline phosphatase, $1 \times 10^{-4}$ M, was incubated with $10^{-4}$ M inorganic $^{32}$P contained in 0.01 M Tris-0.01 M acetate, pH 6.5, 25°. Samples were withdrawn at the time intervals indicated and quenched immediately in perchloric acid. Acid-stable protein-bound phosphate, moles per mole of dimer (●), was determined as described under “Experimental Procedure.”

Fig. 7. Exchange of $^{18}$O from $H_{2}^{18}O$ into inorganic phosphate catalyzed by Zn(II) and Cd(II) alkaline phosphatases as a function of pH. 0.05 M phosphate, $1.7 \times 10^{-6}$ M enzyme, 25°. The ordinate indicates the atom per cent excess $^{18}$O contained in phosphate oxygen isolated after 48 hr in the presence of the Zn(II) enzyme (●); Cd(II) enzyme (■); no enzyme (○). Inset, time course of $^{18}$O exchange. ●, Zn (II) enzyme, pH 8.0; ■, Cd (II) enzyme, pH 6.5. Conditions as described above.
Apocatalytic phosphate binds no phosphate with a measurable dissociation constant smaller than $5 \times 10^{-4} \text{ m}$ (Fig. 2 and Table II). A high affinity binding site may be restored upon the addition of metal ions to the apoenzyme. The presence of 2 metal ions is required for the tight binding of one phosphate to alkaline phosphatase with a dissociation constant of $6 \times 10^{-7} \text{ m}$. Readaddition of 1 eq of zinc generates a binding site with a dissociation constant of $6 \times 10^{-3} \text{ m}$, but with only 0.5 mole of $^{32}\text{P}$ bound per dimer (Fig. 2 and Table II). Since a stoichiometry of 0.5 cannot exist at the molecular level, an acceptable interpretation requires that, in the presence of phosphate and 1 metal ion per dimer, 50% of the molecules are dimers containing 2 Zn(II) cations and 1 phosphate anion, while the remainder of the protein is present as zinc-free apocatalytic phosphate. Such a model suggests that the binding of 2 zinc ions to the apoenzyme will be cooperative in the presence of phosphate. Measurements of the zinc dissociation constants in the presence of phosphate have not been reported, but some evidence exists that the zinc-protein complex is stabilized in presence of phosphate (11).

The interpretation of binding data for the native three-zinc enzyme and for the enzyme in presence of excess zinc is more difficult. The binding data indicate that more than one phosphate binding site is present and Scatchard plots are distinctly nonlinear (Fig. 1). When nonlinear Scatchard plots are observed there are several interpretations possible (32, 35, 36). Nonlinearity may be ascribed to: (a) the presence of independent noninteracting homogeneous sites each having a single dissociation constant; (b) the presence of identical sites with interaction, in this case anticooperativity, between the two sites; or (c) the presence of nonidentical sites with interaction between them, e.g., the formation of ternary complexes of protein-Me(II)-phosphate which affect the tertiary or quaternary protein structure and thus affect the specific binding site. For ease of presentation, the first case was used for curve fitting of the nonlinear data. There are many problems in distinguishing these possibilities. At high protein concentrations and excess metal ion alkaline phosphatase undergoes self-association (37). Reynolds and Schlesinger have reported that the tetramer species binds three or more phosphates per mole of dimer (37). Our collection of data was carried out with $10^{-5}$ to $10^{-7} \text{ m}$ protein which should exist primarily as a dimer. The equilibrium dialysis data for the native enzyme can be adequately described by the assumption of one tight binding site, $K_1 = 1.2 \times 10^{-4} \text{ m}$, and one or more other loose sites (see “Results” and Fig. 1). The kinetic $k_m$ for $p$-nitrophenylphosphate and $K_a$ for phosphate under these same conditions are $K_m = K_1 = [1.0 \pm 0.5] \times 10^{-4} \text{ m}$.

This tight binding site is also the one which appears to be affected by ionic strength and pH (Table II, Figure 1). Ionic strength and changes in pH are known to affect the amount of aggregation of alkaline phosphatase (20). Thus quaternary changes in the protein structure may account for changes in the dissociation constant for phosphate binding.

The pH dependence of phosphate binding is a complex function which involves the equilibrium between at least two species, the Michaelis complex and the covalent phosphoryl enzyme, as shown in Equation 4. The method of equilibrium dialysis measures the total bound phosphate and does not distinguish its chemical form. At alkaline pH the predominant species in the case of the Zn(II) enzyme is the noncovalent complex (Fig. 4). At pH 5, the stable covalent intermediate is the prominent species (Fig. 4B). These observations agree well with previous kinetic (13, 14) and phosphate labeling studies (6, 8–11).

Below pH 5 both the total bound phosphate and the amount of covalently bound phosphate decrease (Fig. 4). Previous studies have shown that below pH 5 the enzyme undergoes significant changes in tertiary and quaternary structure. The Zn(II) ions dissociate from the dimer and below pH 4 the dimer dissociates into monomers which unfold to random coiled polymer chains (20, 39, 40). Thus it is not surprising that the phosphate binding to alkaline phosphatase decreases in this region. Higher concentrations of phosphate or zinc may retard the loss of zinc, as indicated in studies by Piguetti and Milstein (11), but below pH 4 there is little indication that the enzyme does not dissociate to monomers after 24 to 48 hours as previously described (20).

Substitution of the first transition and 11B metal ions for Zn(II) in alkaline phosphatase produces dramatic changes in enzymatic activity (Table I) as well as in the pH stability of the phosphoryl enzyme relative to the noncovalent complex. Binding studies by several methods show that the metals Co(II), Mn(II), Cd(II), and Ni(II) compete with zinc for binding sites of alkaline phosphatase (18, 19). In the case of the Cd(II) and Mn(II) proteins, the absence of enzyme activity cannot be attributed to the lack of a phosphate binding site (Fig. 3 and Table I). In addition to binding phosphate, both the Cd(II) and Mn(II) enzymes form the phosphoryl enzyme (Fig. 5) which is implicated as an important step in the catalytic reaction pathway (8, 12–15). The distinguishing feature of these proteins compared to the active metallophosphatases is the high stability of these phosphoryl enzymes at the higher pH values. This stability is so great for Cd(II) that 1 mole of covalently linked phosphate per mole of enzyme can be isolated at pH 6.5 and 0.75 mole at pH 8.0 (Fig. 5).

In order to form observable equilibrium concentrations of the phosphoryl enzyme at any pH, $k_2$ must be greater than $k_1$ ($k_2/k_1 \ll 1$). This condition is met by the Cd(II) enzyme. While phosphorylation of the Cd(II) enzyme is relatively slow, $k_2 = 3 \times 10^{-3} \text{ sec}^{-1}$ at pH 6.5 (Fig. 6), dephosphorylation simply does not occur over a measurable time (Fig. 7). The failure to form the Cd(II) phosphoryl enzyme completely at pH 8 may relate to the presence of an even more stable Michaelis complex as postulated in the case of the Zn(II) enzyme (14). At pH 5 to 5.5 the Zn(II) enzyme not only forms significant equilibrium concentrations of the phosphoryl enzyme, but catalyzes its turnover with a rate constant of $~0.2 \text{ sec}^{-1}$ (Fig. 7). This suggests that phosphorylation of the Zn(II) enzyme must occur faster than for the Cd(II) enzyme pictured in Fig. 6. This is confirmed by our attempts to measure the phosphorylation rate of the Zn(II) enzyme. By 10 sec (the shortest time conveniently measured by our techniques), the phosphorylation of Zn(II) enzyme at pH 5.5 is complete. Thus Cd(II) must also have an
effect on the phosphorylation rate in addition to its dramatic effect on dephosphorylation.

Since the maximum catalytic constant observed for the catalysis of $^{32}$P exchange into phosphate by the Zn(II) enzyme is 0.2 sec$^{-1}$, considerably slower than catalysis of phosphate ester hydrolysis, phosphorylation of the enzyme from HOP must be at least an order of magnitude slower than phosphorylation from ROP. The rate constant for phosphorylation of the enzyme by 4-methylumbelliferyl phosphate has been determined to be 6.1 sec$^{-1}$ at pH 6.34 (41).

The lack of activity or low activity* of the Cd(II) and Mn(II) enzymes may be explained by their failure to catalyze the breakdown of the phosphoryl enzyme at significant rates. These changes in the rates of phosphorylation and dephosphorylation induced by different metal ions may relate either to intrinsic chemical properties of the cation or to slight changes induced in the conformation of protein side chains located at the active center. The decrease in total bound phosphate and phosphoryl enzyme for all of the metallophosphatases at the lower pH values (Figs. 4 and 5) may be adequately accounted for by the loss of metal ion and dissociation of the protein into subunits (20, 39, 40).

The observed stoichiometric features of this protein raise interesting questions about the necessary structural organization of the active protein. Hydrodynamic studies show that the protein exists predominately as a dimer at neutral pH (20, 39, 40). The dimer consists of two identical subunits (42, 43) (see "Appendix"). The dimer binds between 2 and 4 metal atoms at neutral pH (18–20, 44, 45) and in the presence of excess metal ions or higher pH it will bind even more (20). Spectrophotometric titration of the apoenzyme with Co(II) shows only two

* Mn(II) alkaline phosphatase shows very low activity, but it is significantly greater than the activity shown by the zinc-free enzyme (Table 1). This may represent significant activity since the phosphoryl enzyme formed at neutral pH by the Mn(II) enzyme does not appear as stable as that formed by the Cd(II) enzyme (Fig. 5).

APPENDIX

X-RAY STUDIES ON SINGLE CRYSTALS OF ESCHERICHIA COLI ALKALINE PHOSPHATASE

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The crystalline preparations used in the present studies contained large single crystals of *Escherichia coli* alkaline phosphatase suitable for x-ray diffraction studies (Fig. 8). Initial x-ray studies show the crystals to be of the space group P2$_1$2$_1$2, each unit cell containing three dimers. The monomer is the asymmetrical unit. The unit cell dimensions are: $a = 70.5$ Å, $b = 70.5$ Å, $c = 155.6$ Å, $\alpha = 90^\circ$, $\beta = 90^\circ$. The 3 fold screw axis is along the c axis; the 2-fold rotation axes relate pairs of identical monomers. These data tend to support the conclusion that the dimer rather than an even multiple of the dimer is the unit favored by the forces between subunits. Reflections from the presently available crystals indicate that it should be possible to achieve a resolution of $\sim 2.5$ Å.

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Phosphate Binding to Alkaline Phosphatase: METAL ION DEPENDENCE
Meredith L. Applebury, Barbara P. Johnson and Joseph E. Coleman

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