Kinetic Properties of Cobalt Alkaline Phosphatase*

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ABSTRACT: The kinetics of cobalt alkaline phosphatase of *Escherichia coli* have been studied in an effort to elucidate the role of the metal atoms in the function of this enzyme. When assayed at pH 8.0, the enzymatic activity of the cobalt enzyme is about 12% of that of the native enzyme. In many respects, the native zinc and cobalt enzymes are similar. They hydrolyze a variety of phosphomonoesters at essentially similar rates. High ionic strength activates both metalloenzymes and phosphate, arsenate, and phenylphosphonate inhibit them competitively. However, the two metalloenzymes differ in their pH dependence, phosphotransferase activity, and rate-limiting step. For the cobalt enzyme, V_{max} increases with pK values of 6.8 and 10.5. K_M increases with a pK of 9.6. This pH dependence differs qualitatively and quantitatively from that observed for the zinc enzyme, suggesting that the metal atom at the active site of alkaline phosphatase may function both in substrate binding and in catalysis. In contrast to the native enzyme, the cobalt enzyme lacks phosphotransferase activity. Assuming the currently accepted formation of a phosphoryl-enzyme intermediate, studies of the transient phase of the hydrolysis of phosphomonoesters suggest that dephosphorylation of the phosphoryl-enzyme is the rate-limiting step for cobalt phosphatase over the pH range from 4.5 to 11.0. This step is rate limiting for the native enzyme only at pH values less than 7. Dephosphorylation is slower for the cobalt enzyme than for native phosphatase, while the apparent rate for phosphorylation of the enzyme by substrate appears to be faster for the cobalt enzyme.

Alkaline phosphatase of *Escherichia coli* is a zinc metalloenzyme (Plocke *et al.*, 1962; Simpson *et al.*, 1968). Suitable chelating agents remove the metal from the protein with concurrent loss of catalytic function. Addition of cobalt(II) in stoichiometric amounts to the resultant apoenzyme generates a catalytically active cobalt enzyme (Plocke and Vallee, 1962) which exhibits an optically active absorption spectrum in the visible wavelength region (Simpson and Vallee, 1968). In contrast to the native enzyme which is both a hydrolase and a phosphotransferase (Wilson *et al.*, 1964), cobalt phosphatase catalyzes the hydrolysis of various phosphomonoesters, but not the transfer of phosphate to Tris or other suitable acceptors (Tait and Vallee, 1966).

The kinetics of cobalt alkaline phosphatase have been studied to elucidate further the role of the metal in this enzyme. The cobalt enzyme is similar to the native zinc enzyme in substrate specificity, activation energy, inhibition characteristics, and many features of its steady-state kinetics. It differs from native alkaline phosphatase in pH dependence and in the rate-limiting catalytic step.

Experimental Section

Alkaline phosphatase from *E. coli* was prepared as previously described (Simpson *et al.*, 1968). Zinc was removed by dialysis against 8-hydroxyquinoline-5-sulfonic acid (Simpson and Vallee, 1968), resulting in an inactive apoenzyme. This procedure was followed by extensive dialysis against metal-free distilled water, and finally against 0.05 M Tris (pH 8.0). The apoenzyme was stored at a high concentration (~20 mg/ml) to avoid extraneous metal contamination and remained metal free and fully stable for more than 3 months. It contained less than 0.1 g-atom of zinc/mol wt 89,000. During the first 30 sec of assay of the apoenzyme, residual activity was less than 1% of that of the native protein. Thereafter, activity increased gradually over several minutes to from 10 to 30% of that of the native enzyme, reflecting scavenging and binding of metal ions, presumably zinc, from the "metal-free" assay mixture. It should be noted that less than 10^{-8} M zinc would be sufficient to restore a full complement of zinc to the apoenzyme at the concentration usually present in the assay mixture and to regenerate the activity characteristic of the native enzyme.

The zinc or cobalt enzymes were reconstituted by addition of 0.01 M cobalt sulfate in 0.003 M HCl or 0.01 M zinc sulfate in water, both spectroscopically pure grade (Johnson Matthey and Co., Ltd.), to a 10^{-5} M solution of apoenzyme in 0.01 M Tris (pH 8.0). Buffers and glassware were rendered metal free as previously described (Thiers, 1957; Himmelhoch *et al.*, 1966).

Addition of 4 g-atoms of cobalt(II)/mole of apoenzyme generates a cobalt enzyme whose activity is stable from 30 sec to more than 24 hr after reconstitution. During the 30–60 sec required for assays, activity was unchanged in the presence of 10^{-4} M zinc sulfate, 10^{-5} M *o*-phenanthroline, or 1×10^{-6} M dithionite over the pH range 5–12.[1] The activities of enzyme

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[1] *o*-Phenanthroline was added to bind excess cobalt, thus testing the hypothesis that the reduced level of activity of the cobalt enzyme, when compared to the native protein, might possibly be due to inhibition by free cobalt(II). Dithionite produced a reducing atmosphere to prevent the oxidation of cobalt(II).

preparations fluctuated widely when less than 4 g-atoms of cobalt/mole was added to the apoenzyme, probably due to variable contamination with zinc. In contrast, the hydrolytic activity of enzymes to which more than 4 g-atoms of cobalt had been added, though stable, was decreased when compared to protein containing a complement of four cobalt atoms per mole. All kinetic studies detailed here were performed with enzyme formed by addition of 4 g-atoms of metal/mole of apoprotein.

The cyclohexylamine salt of DNPP[2] was prepared as described by Bunton *et al.* (1967). The melting point of the product was 135–140° (literature value 145°), and the sample was 99% pure as determined spectrophotometrically at 400 mμ after complete hydrolysis by alkaline phosphatase, assuming ϵ 1.11 × 10⁴ M^{-1} cm^{-1} for the product (Ko and Kézdy, 1967). Cysteamine *S*-phosphate was prepared from phosphorothioate as described by Åkerfeldt (1959) and Yasuda and Lambert (1954). The sample used for the experiments described contained fewer than 1% of free sulfhydryl groups and was 95% pure as determined spectrophotometrically after complete hydrolysis with native alkaline phosphatase. NPP was obtained from Sigma Chemical Co. and BGP from Eastman Organic Chemicals.

Concentrations of enzyme were determined assuming $A_{278}^{1\%}$ 7.2 and mol wt 89,000 (Plocke *et al.*, 1962; R. T. Simpson, J. L. Bethune, and B. L. Vallee, in preparation).

Spectrophotometric assays of steady-state rates with chromogenic substrates were conducted in a total volume of 3 ml in a Unicam SP 800 recording spectrophotometer. Temperature was controlled to ±0.1°. The reaction was initiated by addition of the enzyme. Routine assays were conducted at a substrate concentration of 1×10^{-3} M in 0.01 M Tris-Cl–1.0 M NaCl (pH 8.0) and 25°. Generally, enzyme concentrations of 1–10 μg/ml were employed. These conditions were varied appropriately for the various kinetic determinations detailed. In some cases, initial steady-state rates were determined with a Durrum-Gibson stopped-flow apparatus. The appearance of product, either 4-nitrophenol or 2,4-dinitrophenol, was measured by increase in absorbance at 400 mμ. Presteady-state kinetics were determined with the Durrum-Gibson instrument. The hydrolysis of cysteamine *S*-phosphate was also assayed spectrophotometrically. Appearance of the product was measured by reaction with 4×10^{-4} M dithionitrobenzoate (Ellman, 1959). Thionitrobenzoate was quantitated by its absorbance at 412 mμ using ϵ 1.36 × 10⁴ M^{-1} cm^{-1}.

The hydrolysis of nonchromogenic substrates was assayed by measurement of the rate of formation of inorganic phosphate, using either the method of Fiske and SubbaRow (1925) or Chen *et al.* (1956). In either case, the reaction was stopped at intervals by the addition of a suitable aliquot of the reaction mixture to the acidic reagent.

Employing the same buffers as used for assay, 1 M NaCl, the p*K* of 4-nitrophenol was 7.05 at 25°, while it was 6.80 at 45°. These values are in good agreement with those reported when 0.4 M NaCl was employed (Lazdunski and Lazdunski, 1966). The p*K* of 2,4-dinitrophenol at 25° was assumed to be 3.76 (Trentham and Gutfreund, 1968).

Tris-chloride, acetate, MES, and glycine buffers were used as detailed in the legends to the figures. pH values of the solutions were measured before and after assay in each cuvet with a Radiometer type PHM 26 pH meter, calibrated with at least two standard buffers of known temperature dependence, adjusted to the temperature of the assay. At alkaline pH values correction was made for the high sodium content of the assay mixtures.

Results

Like the native enzyme, cobalt alkaline phosphatase catalyzes the hydrolysis of a variety of phosphate esters with almost equal facility (Table I), although the acidities of the leaving groups of these substrates vary widely. In addition, it hydrolyzes CSP, in which a phosphorus–sulfur bond is hydrolyzed (Herrington *et al.*, 1967; Neumann *et al.*, 1967) and pyrophosphate, in which a phosphoanhydride bond is cleaved.

TABLE I: Activity of Cobalt Alkaline Phosphatase.[a]

Substrate	Activity (μmoles/min mg)
4-Nitrophenylphosphate	3.45
2,4-Dinitrophenylphosphate	4.10
β-Glyceryl phosphate	3.85
5′-Adenosine monophosphate	2.85
Pyrophosphate	3.25
Cysteamine *S*-phosphate	2.66

[a] All assays were performed as in the Experimental Section at 25° in 0.01 M Tris-Cl–1 M NaCl at a substrate concentration of 1×10^{-3} M.

Due to the facility of their assay, NPP and DNPP were selected as substrates for detailed kinetic studies of the cobalt enzyme. Figure 1 details the substrate concentration dependence of the hydrolytic activity of cobalt alkaline phosphatase over a wide range (10^{-5}–2×10^{-2} M) of concentrations of NPP and over a narrower range (10^{-5}–10^{-4} M) of concentrations of DNPP at pH 8.0, and at two ionic strengths, $\Gamma/2 = 1.0$ and $\Gamma/2 = 0.05$. The Lineweaver–Burk plot at the high ionic strength is linear over a wide substrate range (10^{-5}–10^{-2} M). In contrast, at the lower ionic strength, this plot is linear over a narrower substrate range (10^{-5}–10^{-4} M), and there is substrate activation at concentrations greater than 10^{-4} M. At both ionic strengths substrate inhibition is apparent when substrate concentrations exceed 10^{-2} M. To avoid the nonlinearity of substrate concentration dependence at low ionic strengths, all succeeding investigations were performed in 1.0 M NaCl, where substrate activation was not apparent.

Table II summarizes the kinetic parameters for the hydrolysis of NPP, DNPP, and CSP by the cobalt and zinc enzymes. The maximal velocities observed for cobalt phosphatase are about 15% of those of the native zinc enzyme, irrespective of the substrate employed. Viewing either the zinc or the cobalt

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[2] Abbreviations employed are: NPP, 4-nitrophenylphosphate; DNPP, 2,4-dinitrophenylphosphate; βGP, β-glycerylphosphate; CSP, cysteamine *S*-phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid.

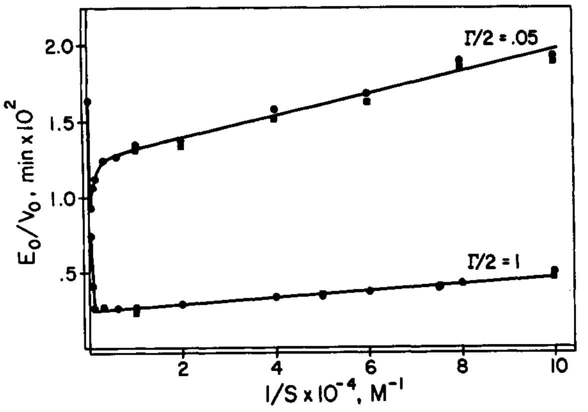

FIGURE 1: Substrate concentration dependence of the hydrolysis of DNPP (■) and NPP (●) by cobalt alkaline phosphatase. Assays were carried out as in the Experimental Section, at ionic strength 0.05 (upper curve), 0.01 M Tris-Cl, 0.045 M NaCl; or at ionic strength 1.0 (lower curve), 0.01 M Tris-Cl, 1.0 M NaCl. All assays were performed at pH 8.0 at 25°.

TABLE II: Kinetic Parameters for Cobalt and Zinc Alkaline Phosphatase.[a]

	Cobalt Phosphatase		Zinc Phosphatase	
Substrate	K_M (M × 10^6)	V_{max}	K_M (M × 10^6)	V_{max}
4-Nitrophenylphosphate	6.0	3.8	7.0	24.4
2,4-Dinitrophenylphosphate	6.0	4.2	8.0	26.2
Cysteamine *S*-phosphate	7.0	2.7	7.0	24.0

[a] Assays were performed as in the Experimental Section at 25°, pH 8.0, in 0.01 M Tris-Cl–1.0 M NaCl. Velocities are expressed as micromoles of substrate hydrolyzed per milligram of enzyme per minute.

enzyme, the maximal velocities are nearly identical for all three substrates. The K_M values are similar for both enzymes with all three substrates.

Native alkaline phosphatase exhibits phosphotransferase activity in the presence of 1 M Tris, since Tris acts as an acceptor for the phosphate group released by substrate hydrolysis (Wilson *et al.*, 1964). When the native enzyme is assayed in 1 M Tris, this phosphotransferase activity manifests either as an increase in nitrophenol release, compared with that observed in 0.01 M Tris, 1 M NaCl, or as a difference between nitrophenol formed and P_i released. In the presence or absence of either acceptor (1 M Tris or 0.9 M glycerol) at pH 8.0, cobalt phosphatase releases phosphate from NPP at a rate which is identical with that of the formation of nitrophenol. Assay of cobalt phosphatase in 1 M Tris and 10^{-4} M Co(II)[3] does not increase the release of nitrophenol at pH 4.5, 8.0, or 11, when compared with assay in the absence of acceptor.

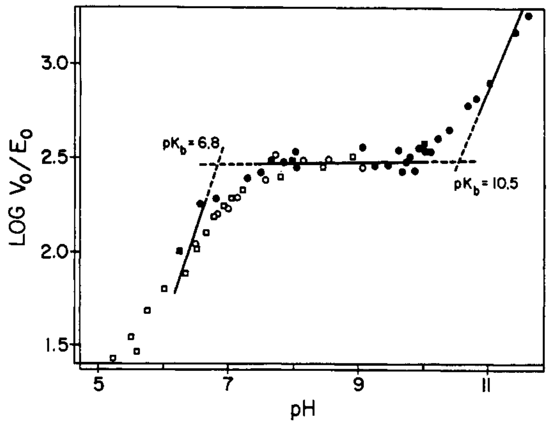

FIGURE 2: pH dependence of the rate of hydrolysis of DNPP (■, □) and NPP (●, ○) by cobalt alkaline phosphatase. Assays were carried out at 25° and pH values indicated at an ionic strength of 1.0. Buffers employed were: pH 5.2–7.4, MES; pH 5.5–7.7, Tris-acetate; pH 7.1–9.0, Tris-Cl, pH 8.7–11.7 glycine, all at concentrations of 0.01 M. Closed symbols indicate maximal velocities determined from Lineweaver–Burk plots. Open symbols indicate single velocities determined in the region where K_M is invariant at a substrate concentration 30-fold greater than the K_M.

In contrast to the similarities of substrate specificity between the cobalt and zinc phosphatases, the pH dependence of their activities differs.[4] Figure 2 shows the effect of pH on log V_{max} and Figure 3 relates pK_M to pH for the hydrolysis of both NPP and DNPP. K_M's and V_{max}'s were calculated from the linear segments of Lineweaver–Burk plots, over the substrate range 10^{-5}–10^{-3} M. At all pH values studied up to pH 11.7, substrate inhibition was observed at concentrations above 10^{-2} M. Despite the increase in K_M at alkaline pH values, the enzyme was never assayed at substrate concentrations high enough to produce substrate inhibition.

Even though the p*K*'s of the leaving groups of DNPP and NPP differ by more than 3 pH units, their hydrolysis is nevertheless indistinguishable over the pH range from 5 to 11.5.

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[3] Since cobalt might be expected to complex with Tris, attempts to assay cobalt alkaline phosphatase for transferase activity must take into account the dual role of Tris, as a metal binding agent and, therefore, both as a potential inhibitor of cobalt phosphatase and as a potential acceptor of phosphate. For this reason, when attempting to determine cobalt phosphate transferase activity in 1 M Tris, it is necessary to add cobalt(II) in amounts sufficient to prevent inhibition by Tris due to metal chelation, yet in low enough concentration so that cobalt itself is not inhibitory to the enzyme. Cobalt(II) (10^{-4} M) was found to be a suitable concentration for this purpose.

[4] Above pH 9.5 rates fell off rapidly after the initiation of the reaction, and care was taken to use only initial rates. Loss of activity occurred after approximately 30 sec and showed some substrate dependence, being faster at lower substrate concentrations and slower at higher concentrations. Loss of activity at pH 9.5 and above paralleled the loss of activity when the enzyme was preincubated at high pH and assayed at pH 8.0. Denaturation was assumed to be irreversible and not due to loss of metal since activity could not be regenerated at pH 8.0, by either long preincubation at this pH or addition of 10^{-4} M cobalt(II) or 10^{-4} M zinc to the assay mixture. These observations are in consonance with the previously demonstrated dissociation of native alkaline phosphatase into subunits above pH 11 (Sussman and Gottlieb, 1968).

TABLE III: pK's and Heats of Ionization.

Enzyme	Parameter	Temp (°C)	pK	ΔH_i (kcal/ mole)
Cobalt	V_{max}	10	6.90	6.4
		25	6.80	
		35	6.45	
		45	6.35	
	K_M	25	9.55	14.2
		45	8.90	
	V_{max}	25	10.50	13.1
		45	9.90	
Native[a]	V_{max}	25	7.10	6.8
		45	6.80	
	K_M	25	8.55	17.5
		45	7.80	

[a] Data of Lazdunski and Lazdunski (1966). pK's were determined graphically to within 0.05 pH unit as shown in Figures 2 and 3. At least ten points were used for each determination. The error in each pK from the graphic determination alone is ±0.1 pH unit.

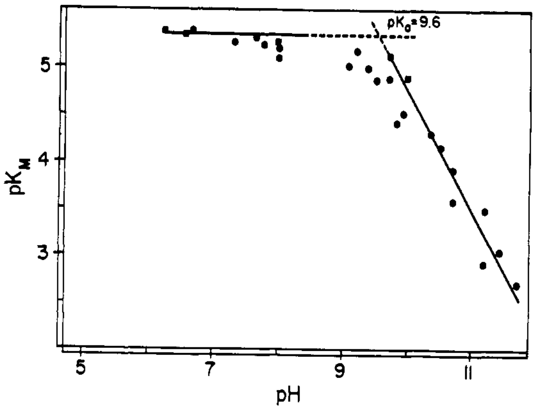

FIGURE 3: pH dependence of the K_M for hydrolysis of DNPP (■, □) and NPP (●, ○) by cobalt alkaline phosphatase. Conditions, buffers, and symbols as in Figure 2.

As for zinc phosphatase, V_{max} for cobalt phosphatase increases with a pK near 7. The slope of the log V_{max} *vs.* pH plot is not unity below pH 7 (Figure 2), suggesting that at these pH values more than one moiety could be involved in activity. The pK of the major group involved can be approximated to be 6.8 at 25° by assuming a slope of 1 (Figure 2). In addition, above pH 10, the log of the activity of the cobalt enzyme increases linearly with a slope close to 1 and extrapolating to a pK of 10.5 for this increase in catalytic rate (Figure 2).

Like the K_M for zinc phosphatase, which increases with a pK of 8.6, that for cobalt phosphatase also increases at alkaline pH values, with a pK of 9.6 (Figure 3). Activities assayed in an unbuffered solution brought to pH 11.0 with NaOH (as a control) were similar. Further, cobalt sulfate alone, at concentrations ranging from 10^{-8} to 10^{-4} M, did not affect the nonenzymatic hydrolysis of NPP over the pH range from 5 to 12. This nonenzymatic rate was less than 1% of the observed enzymatic rates, and no correction was made for its occurrence.

Heats of ionization were determined in order to correlate each of these three pK values with specific protein moieties. With DNPP as substrate, a ΔH_i of 6.4 kcal/mole was calculated for the acidic pK of the enzyme–substrate complex (Table III). Similarly, a ΔH_i of 14.2 kcal/mole for the pK related to the increase in K_M, and a ΔH_i of 13.1 kcal/mole for the pK associated with the increase in activity at alkaline pH values, were found (Table III).

Orthophosphate and phenylphosphonate, and the phosphate analog, arsenate, are competitive inhibitors of native alkaline phosphatase. They also inhibit the cobalt enzyme competitively. Table IV compares the K_I's for the reconstituted zinc and cobalt enzymes at pH 8.0. The K_I for P_i is nearly the same as the K_M for hydrolysis of phosphate esters, just as for the native enzyme.

The activation energy for hydrolysis of DNPP by cobalt phosphatase was determined to be 9.8 kcal/mole, also similar to that reported for native alkaline phosphatase acting on NPP (Lazdunski and Lazdunski, 1966).

Native alkaline phosphatase exhibits a "burst" of presteady-state enzymatic activity at and below pH 6 (Fernley and Walker, 1965; Fife, 1967; Ko and Kézdy, 1967; Trentham and Gutfreund, 1968; *cf.* also Figure 4). Unlike the native enzyme, at 25° and pH 5.7, cobalt phosphatase shows no detectable presteady-state phase over the time period from 10 to 1000 msec after the initiation of the reaction (Figure 4). This observation was confirmed by stopped-flow measurements at several pH values from 4.8 to 12. In all cases, however, the extrapolation of the steady-state rate to zero time yields an intercept different from that of the absorbance due to substrate alone. This suggested that the rate of the transient kinetic phase might be greater than that measurable in the stopped-flow instrument. On lowering the temperature of the assay to 5°, this postulated "burst" becomes measurable even at pH 8.0 (Figure 5). Even at this lowered temperature, the half-time for the transient phase is about 15 msec. The stoichiometry of the "burst" is nearly exactly 1 mole of product/mole of enzyme, similar to the results obtained with the native enzyme (Fernley and Walker, 1965; Ko and Kézdy, 1967; Trentham and Gutfreund, 1968).

To evaluate solely the step of metal removal and replacement and its possible effects on the kinetic properties of al-

TABLE IV: Inhibition of Cobalt and Zinc Phosphatases.[a]

	K_I (M)	
Inhibitor	Cobalt Phosphatase	Zinc Phosphatase
Phosphate	1×10^{-5}	1×10^{-5}
Phenylphosphonate	5×10^{-3}	6×10^{-3}
Arsenate	2×10^{-5}	3×10^{-5}

[a] Inhibition constants were determined from plots of $1/V$ *vs.* [I] at two different concentrations of the substrate, NPP. Assays were carried out in 0.01 M Tris-Cl–1.0 M NaCl, pH 8.0, 25°.

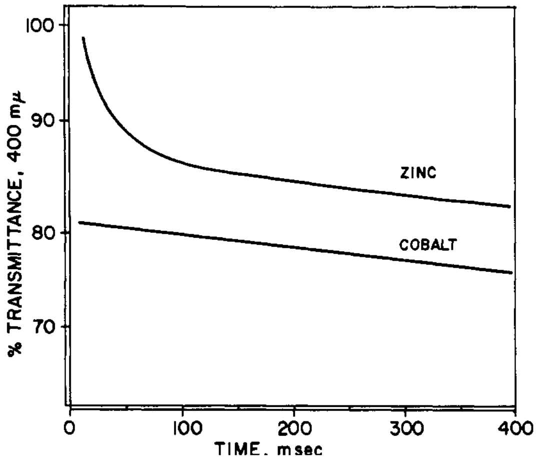

FIGURE 4: Stopped-flow kinetics of zinc and cobalt alkaline phosphatase, as indicated. The figure is an enlargement of oscilloscopic traces obtained using equal volumes of enzyme solution and 2 × 10^{-4} M DNPP in the Durrum-Gibson stopped-flow instrument. Final concentrations in the 2-cm path-length cuvet were 0.01 M Tris-acetate (pH 5.75), 0.1 M NaCl, 1 × 10^{-4} M DNPP, and 0.23 mg/ml of zinc phosphatase or 0.46 mg/ml of cobalt phosphatase. The assay was conducted at 25° and 100% transmittance was established with substrate alone in the cuvet.

kaline phosphatase, the kinetic parameters of the native and the reconstituted zinc enzyme were compared. The properties of the two enzymes were found to be virtually indistinguishable (Table V).

Discussion

Alkaline phosphatase of *E. coli* has served as a prototype system for investigation of the mechanism of hydrolysis of phosphomonoesters. A unique seryl residue has been implicated in the enzymatic mechanism (Schwartz and Lipmann, 1961; Engstrom, 1962a,b; Schwartz, 1962; Pigretti and Milstein, 1965). Investigations of both the steady-state and transient kinetics of hydrolysis have helped to define the rate-limiting steps in the catalytic mechanism and the possible groups involved in hydrolysis (Fernley and Walker, 1965; Lazdunski and Lazdunski, 1966; Ko and Kėzdy, 1967; Trentham and Gutfreund, 1968). Further, the zinc of this metalloenzyme is requisite for enzymatic activity (Plocke *et al.*, 1962). The present investigations have utilized this latter feature of the protein as a probe for investigation of the mechanistic features of hydrolysis of phosphate esters by this enzyme.

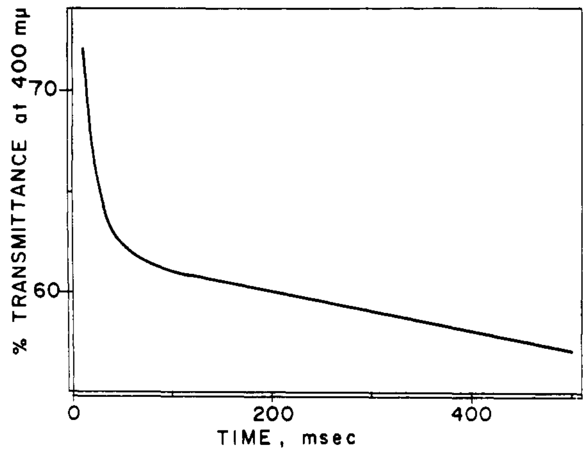

FIGURE 5: Stopped-flow kinetics of cobalt alkaline phosphatase. The final concentrations of reactants in the cuvet were 0.01 M Tris-Cl, 1 M NaCl, 1 × 10^{-4} M NPP, and 0.46 mg/ml of cobalt phosphatase. The assay was conducted at 5° and pH 8.0; 100% transmittance was established with distilled water in the cuvet.

TABLE V: Comparison of Native and Reconstituted Zinc Phosphatases.[a]

Parameter	Native (M)	Reconstituted (M)
K_M NPP	7 × 10^{-6}	11 × 10^{-6}
K_M DNPP	8 × 10^{-6}	
K_M CSP	7 × 10^{-6}	7 × 10^{-6}
K_I phosphate	1 × 10^{-5}	1 × 10^{-5}
K_I phenylphosphonate	6 × 10^{-3}	6 × 10^{-3}
K_I arsenate		3 × 10^{-5}

[a] Assays were carried out as in the Experimental Section in 1 M NaCl–0.01 M Tris, pH 8.0, 25°. K_M's and V_{max}'s were calculated as described in Table II. Inhibition constants were determined with NPP as substrate as described in Table III.

Due to the differing effectiveness of the group IIb and transition metals in ligand shell transfer reactions and as Lewis acids (Dennard and Williams, 1966), investigation of the kinetic characteristics of different functional metal derivatives of a metalloenzyme might generate significant information about the role of the metal atom in substrate binding and catalysis. Substitution of the functional metal atoms at the active site of metalloenzymes may be considered analogous to a specific chemical modification of a catalytically essential amino acid residue which would alter its properties, *e.g.*, its acid–base characteristics, while enzymatic activity is retained. For organic active-site species such modifications have proven difficult to achieve in practice. In contrast, in several metalloenzymes, functional metals have been substituted with relative facility while preserving enzymatic activity (*cf.* Vallee and Wacker, 1968). A comprehensive kinetic investigation of such metalloenzyme derivative has apparently not been performed. Thus, among those metalloenzymes where different metals have been shown to form functional species, only the substrate concentration dependence of the several metallocarboxypeptidases (Davies *et al.*, 1968) and certain features of the kinetics of cobalt carbonic anhydrase (Lindskog, 1966) have been studied previously.

Cobalt alkaline phosphatase is enzymatically active and similar to the native enzyme in certain of its kinetic properties.[5]

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[5] Since the cobalt-substituted alkaline phosphatase under discussion has many similarities to the native zinc enzyme, but only 10–15% of its activity, it would appear necessary to prove beyond doubt that it is not merely a native or zinc-substituted phosphatase which is 90% denatured or 90% apoenzyme. Evidence that this is *not* the case is as follows. (1) The apoenzyme, when assayed under conditions identical with those used for assaying the cobalt-substituted enzyme, has virtually no activity and (2) addition of high concentrations of zinc (1 × 10^{-4} M) to the assay mixture at all pH values studied does not change the activity of the cobalt enzyme.

However, the cobalt and zinc enzymes differ kinetically in three broad areas, and jointly these provide strong evidence implicating the metal atom at the active site of alkaline phosphatase in both substrate binding and catalysis. These areas are: (1) the pH and temperature dependence of enzymatic activity for the two enzymes, (2) the rate-limiting step for the hydrolytic reaction, and (3) the lack of phosphotransferase activity for the cobalt enzyme. These will be considered in turn.

pH Dependence. The investigations of Lazdunski and Lazdunski (1966) have defined the characteristics of the pH dependence of activity of native alkaline phosphatase. Between pH 5 and 10, the native enzyme is assumed to exist in three forms, two active and one inactive. The ionization of a group with a pK of 7.1 at 25° (ΔH_i = 6.8 kcal/mole), presumed to be imidazole, produces an active enzyme. The ionization of a second group with pK = 8.6 at 25° (ΔH_i = 17.5 kcal/mole) does not affect activity but decreases the affinity of the enzyme for substrate, as indicated by an increase in K_M.

Cobalt alkaline phosphatase can be considered in these terms. The acidic pK found for cobalt phosphatase with both NPP and DNPP as substrate is 6.8. Together with a ΔH_i of 6.4 kcal/mole, this is consistent with the ionization of an imidazole moiety, as previously suggested for the native enzyme. Since the log V_{max} *vs.* pH plot below pH 7 does not have a slope of 1, this pK must be considered to be approximate. The presence of a nonintegral slope suggests that more than one ionizable group in the enzyme–substrate complex may be involved in activity at pH values below neutrality.[6]

The absorption spectrum of cobalt phosphatase is altered at pH values less than 8, possibly reflecting a change in geometry of metal atoms at the active site (Simpson and Vallee, 1968). The pK for the change in the absorption spectrum of the catalytically essential cobalt ions is apparently identical with that for the increase in V_{max}, suggesting that the interactions involved in forming the catalytically optimal active-site conformation must be complex, and could readily involve both the metal and a histidyl residue.

The most salient differences in steady-state kinetic properties between native and cobalt alkaline phosphatase become apparent from their pH dependence above neutrality. K_M increases with pH for the native zinc enzyme according to a theoretical titration curve with a pK of 8.6 at 25° (Lazdunski and Lazdunski, 1966). K_M of the cobalt enzyme also increases above neutrality; however, the pK is displaced to 9.6 (Figure 3), suggesting either a direct or an indirect role of the metal in substrate binding. The magnitude of the kinetic dissociation constant supports the view that this binding might be directly to the metal, as do the results of phosphate binding studies to the native and metal-free enzymes (R. T. Simpson and B. L. Vallee, unpublished observations), and the spectral shift observed for the cobalt enzyme upon the addition of phosphate (Simpson and Vallee, 1968). Ionization of a molecule of water within the hydration sphere of the metal could be one possible ionization intimately connected with a metal atom and which might change affinity for substrate at alkaline pH. A search for model complexes with which to test this hypothesis is currently under way.

Above pH 10, the maximal activity of cobalt alkaline phosphatase markedly increases (Figure 2). Whether the pK for this increase is related to the pK for the increase in K_M at alkaline pH has not been resolved, although both pK values show a large temperature dependence, suggesting that the two changes are related closely. There are different views regarding the occurrence of slight increases in maximal activity at high pH for the native enzyme. Both gradually increasing maximal activity up to pH 10 (Fernley and Walker, 1965) and no change above pH 8.5 (Lazdunski and Lazdunski, 1966) have been observed. Differences of this nature might arise due to the anomalous kinetics of alkaline phosphatase, especially substrate inhibition. At high pH values, measurement of maximal hydrolytic activities requires the presence of higher substrate concentrations, due to increases in K_M. Substrate inhibition for both enzymes, zinc and cobalt, is not pH dependent in the pH range 8–11; hence inhibition may supervene to prevent the detection of higher enzymatic activities. The difference in the pH dependence of inhibition and K_M suggest that inhibition involves the binding of substrate to groups different from those which bind substrate to form the enzymatically productive E·S complex. In any case, above pH 10 the maximal activities of the zinc and cobalt enzymes differ strikingly in their pH dependence.

Nature of the Rate-Limiting Step. The over-all reaction catalyzed by alkaline phosphatase has been expressed as

$$\mathrm{E} + \mathrm{ROP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E\cdot ROP} \xrightarrow{k_2} \underset{\substack{+\\ \mathrm{ROH}}}{\mathrm{E{-}P}} \xrightarrow{k_3} \mathrm{E} + \mathrm{P_i} \qquad (1)$$

where E·ROP is the Michaelis enzyme–substrate complex, E–P represents the phosphoryl-enzyme intermediate (Schwartz and Lipmann, 1961), k_2 is the rate constant for cleavage of the phosphoester bond, and k_3 is the rate constant for dephosphorylation of the phosphoryl-enzyme. At pH values below about 7, k_3 is rate limiting for the native enzyme (Fernley and Walker, 1965; Ko and Kézdy, 1967; Trentham and Gutfreund, 1968). The over-all catalytic rate constant is $k_0 = k_2 \times k_3/(k_2 + k_3)$.

Attempts to analyze the steady-state and presteady-state kinetics of the native zinc enzyme within the context of this scheme have not been totally successful. The experimental data (Trentham and Gutfreund, 1968) suggest that between phosphorylation and dephosphorylation of the native enzyme an additional rate-limiting step must be postulated. This step could be a conformational change in the enzyme, as has been suggested (Trentham and Gutfreund, 1968). The kinetics of the cobalt enzyme are consistent with eq 1, though they do not indicate, of course, whether or not a more complicated scheme may actually obtain. If eq 1 pertains, the data suggest that dephosphorylation of the cobalt phosphoryl-enzyme is the rate-limiting step throughout the pH range studied.

Both zinc (Garen and Levinthal, 1960; Heppel *et al.*, 1962) and cobalt alkaline phosphatase (Table I) hydrolyze a variety of phosphate esters at essentially similar rates. Even though NPP and DNPP differ markedly in the acidity of their leaving

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[6] Although Lazdunski and Lazdunski (1966) report a good fit to a theoretical titration curve with a pK of 7.1 for the activity of native alkaline phosphatase, Trentham and Gutfreund (1968) and Fernley and Walker (1965) have found curves which did not have this theroetical slope, as is the case for the cobalt enzyme. Further, a more recent report suggested that the pK for the increase in V_{max} for zinc phosphatase is much higher, *i.e.*, 7.7 at 45° in 1 M NaCl (Lazdunski and Lazdunski, 1967).

groups, their rates of hydrolysis are quite similar (Table II and Figure 2). This lack of dependence of the hydrolytic rate on the nature of the leaving group suggests that the rate-limiting step must occur subsequent to the actual bond-breaking process. Hence, for cobalt phosphatase k_2 would not be rate limiting. Instead it would appear that k_3 is rate limiting for the cobalt enzyme, a suggestion generated by the observation of a very rapid, presteady-state burst of activity between pH 5 and 12. At 25° this burst is seen only indirectly as a shift in baseline, but at 5°, a rapid burst can be observed directly (Figures 4 and 5). Thus, for the cobalt enzyme k_3 is decreased while k_2 is increased, compared with the native enzyme. The mechanistic implications of this finding will be discussed in a future communication. We wish to emphasize that this interpretation is based on eq 1 which represents the simplest kinetic scheme for the enzyme. Should a different scheme pertain, the conclusions would obviously require revision in the light of such alternatives.

Phosphotransferase Activity. The phosphotransferase activity of alkaline phosphatase has been thought to arise from competition of the acceptor with water in dephosphorylation of the phosphoryl-enzyme intermediate (Wilson *et al.*, 1964). It has been noted previously that cobalt phosphatase lacks measurable phosphotransferase activity at pH 8.0 (Tait and Vallee, 1966). We have extended these studies for the pH range from 4.5 to 11 and find no transferase activity over the whole range. In contrast, 1 M Tris nearly doubles native alkaline phosphatase activity at pH 8.0 and increases it three to four times at pH 5.90 (Trentham and Gutfreund, 1968). These changes in k_0 for the zinc enzyme in the presence of acceptor are those expected due to the relative magnitudes of k_2 and k_3 at the different pH values. Thus, the most marked effect of the acceptor species is seen when $k_3 \ll k_2$ (Trentham and Gutfreund 1968). In contrast to these studies of zinc phosphatase, the cobalt enzyme does not exhibit phosphotransferase activity, although the stopped-flow data suggest that dephosphorylation is rate limiting for cobalt phosphatase, *i.e.*, $k_3 \ll k_2$ (Figure 5).

Transferase activity has been thought to arise through competition of acceptor and water in dephosphorylation of the phosphoryl-enzyme. These findings suggest modification of this concept, particularly, that transferase activity may require specific binding of acceptor molecules to the enzyme. In this regard, it has been shown that the degree of phosphotransferase activity associated with an acceptor molecule is a function of the stereochemistry of amino and hydroxyl groups on the acceptor, rather than its nucleophilicity (Wilson *et al.*, 1964). A similar requirement for a β-amino or alcohol substituent in the alcohol portion of phosphate esters in order to obtain maximal rates was observed by Butcher and Westheimer (1955) in investigation of the lanthanum hydroxide gel catalysis of phosphoester hydrolysis. Jointly these two observations suggest that the nature of the microscopic interaction of metal atom and acceptor molecule may well be critical in generating phosphotransferase activity. The substitution of cobalt for zinc might interfere with correct binding of the acceptor molecule to the enzyme, thereby preventing the expression of transferase activity.

These investigations of the kinetics of cobalt alkaline phosphatase have generated conclusions which are pertinent to any consideration of the mechanism for the hydrolysis of phosphomonoesters by the nonspecific phosphomonesterases. The present studies suggest that the substitution of cobalt for zinc in alkaline phosphatase markedly reduces k_3, the rate constant for dephosphorylation of the phosphoryl enzyme, and greatly increases k_2, the apparent rate constant for cleavage of the oxygen–phosphorus bond in the mechanism as depicted in eq 1. In addition, the active-site metal atoms of alkaline phosphatase have been implicated in substrate binding and metal–acceptor interactions suggested as a critical feature in phosphotransferase activity. These investigations, and others in progress, should help in the further elucidation of the role played by metal atoms in the catalytic mechanism of alkaline phosphatase. Further, they suggest the suitability of kinetic studies of different metal-substituted species as a general approach to detect mechanistic features of metalloenzymes.

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Considerations in Evaluating the Zinc Content of Horse Liver Alcohol Dehydrogenase Preparations*

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ABSTRACT: Since horse liver alcohol dehydrogenase was first reported to contain 2 g-atoms of Zn/mol wt 73 × 10^3, the methods for purification of the enzyme and its molecular weight have been revised repeatedly. The specific activity of the product has increased substantially, resulting in uncertainties concerning the molar stoichiometry of metal and protein. The present paper reviews the results of zinc analyses of horse liver alcohol dehydrogenase preparations in this laboratory since the inception of work on this enzyme. The observed variations in zinc content are outside the limits of confidence of the methods employed for metal analysis. However, improvements in horse liver alcohol dehydrogenase isolation and characterization, including molecular weight determinations and recognition of the subunit structure and isoenzymes of horse liver alcohol dehydrogenase, all appear to bear on the variations in molar stoichiometry observed over the years. The zinc contents of recently obtained and uniformly treated samples of horse liver alcohol dehydrogenase vary from 3.1 to 4.3 g-atoms of Zn per mole of horse liver alcohol dehydrogenase, based on a molecular weight of 80 × 10^3. A variety of conditions alter both the zinc content and the catalytic activity of the enzyme. Only 2 g-atoms of Zn/mole of horse liver alcohol dehydrogenase appear to be directly related to enzymatic activity, as shown by selective removal of zinc from horse liver alcohol dehydrogenase, confirming the existence of two active-site zinc atoms.

Recent advances in protein chemistry have resulted in increased precision and sensitivity of methods employed in the determination of the physical, chemical, and functional properties of enzymes. Concurrently, procedures of high resolution have been developed for enzyme isolation and purification. When obtained by means of such improved methods, the numerical values for some of the properties of enzymes isolated by these refined procedures differ from those obtained on earlier preparations. Unfortunately, such reexaminations are often performed on some but not all of the pertinent parameters. This leaves open the question whether the changes observed originate from intrinsic differences of the materials examined, improved methodology, or both.

These evolving problems in analytical protein chemistry are well illustrated by LADH[1] first crystallized in 1948 (Bonnichsen and Wassén) and later shown to contain zinc (Theorell *et al.*, 1955; Vallee and Hoch, 1957). Since these early studies, procedures for the purification of LADH have been modified (Bonnichsen and Brink, 1955; Dalziel, 1958, 1960). Studies on LADH prepared by any of the procedures consistently show that it binds two molecules of reduced coenzyme, NADH (Theorell and Bonnichsen, 1951; Ehrenberg and Dalziel, 1958; Ulmer *et al.*, 1961), and requires zinc for catalytic function (Vallee and Hoch, 1957; Plane and Theorell, 1961; Yonetani, 1963). However, values for its total zinc content (Theorell *et al.*, 1955; Vallee and Hoch, 1957; Åkeson, 1964; Oppenheimer *et al.*, 1967), molecular weight (Ehrenberg and Dalziel, 1958; Hamburg, 1966; Drum *et al.*, 1967), and specific absorptivity at 280 mμ (Dalziel, 1958; Sigman, 1965; Hamburg, 1966; Taniguchi *et al.*, 1967) have varied, apparently depending upon the mode of preparation. Moreover, LADH has recently been shown to exist in several isoenzyme forms (Pietruszko *et al.*, 1966, 1969) and to have subunit structure (Drum *et al.*, 1967).

As a general convention, metal content of enzymes is usually expressed as a molar ratio, *i.e.*, gram-atoms of metal per mole of protein, rather than the ratio of mass of metal per gram of protein. Hence, alterations of the values of either metal content or molecular weight can also affect the molar stoichiometry. In order to delineate the role of zinc in LADH further, a more definitive identification of the factors affecting the zinc content of the molecule seemed necessary. Toward this end, this paper reviews the data on the zinc content

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[1] Abbreviation: LADH, horse liver alcohol dehydrogenase.