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Negative Cooperativity and Half of the Sites Reactivity. Alkaline Phosphatases of *Escherichia coli* with Zn^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, and Cu^{2+} in the Active Sites†

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ABSTRACT: Evidence for the nonequivalence of the active sites in dimeric alkaline phosphatase of *Escherichia coli* has been obtained both at acidic and alkaline pH with Zn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+} enzymes. (1) Reaction of all metallophosphatases with 2,4-dinitrophenyl phosphate proceeds through a transient phase (a burst) at pH 4.1 and 5°. A biphasic burst of about 2 mol of dinitrophenol/mol of dimer was obtained with Zn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+} phosphatases. The biphasicity of the burst indicates that the first site is phosphorylated more rapidly than the second site. The first part of the burst (1 mol of phenol/mol of enzyme) is too fast to be followed by the stopped-flow technique with Co^{2+} and Cu^{2+} phosphatases ($k_{01} > 1000$ sec^{-1}). The second part is much slower and can be followed easily ($k_{02} = 0.8$ and 0.07 sec^{-1} for the Co^{2+} and Cu^{2+} phosphatases, respectively). Both steps can be followed with the Cd^{2+} phosphatase ($k_{01} = 1$ sec^{-1}, $k_{02} = 0.03$ sec^{-1}). Only "native" Zn^{2+} phosphatase contains noncovalent phosphate (one phosphate per mol of enzyme). This endogeneous phosphate can be removed; it affects transient kinetics but neither the total amplitude nor the biphasicity of the burst. (2) The Mn^{2+} alkaline phosphatase gives a monophasic burst of 2 mol of dinitrophenol/mol of enzyme at pH 4.1, 5°. The two sites exhibit independent catalytic behavior in this metalloenzyme of low catalytic activity. (3) Half of the sites reactivity was demonstrated near pH 8.0, 5°, for the Zn^{2+}, Co^{2+}, and Cu^{2+} enzymes. Only one of the two active sites can be phosphorylated at any given time in all these enzymes. (4) Half of the sites reactivity was also demonstrated in the Co^{2+} enzyme. Stopped-flow measurement of the spectral changes at the Co^{2+} chromophore, induced by inorganic phosphate and β-glycerophosphate binding to the Co^{2+} phosphatase, was followed at 640 nm. These measurements demonstrated the exclusive formation of the 1:1 complex, namely the binding of only one substrate molecule per dimer at a time. All these results clearly confirm that site-site interactions are involved in the alkaline phosphatase mechanism. This enzyme is an interesting model for the analysis of "catalytic" cooperativity between subunits.

Considerable work has been devoted in recent years to the analysis of the role of subunit interactions in enzyme catalysis. It has been found that both positive and negative cooperativity may occur for substrate or effector binding and that sometimes both occur simultaneously in the same enzyme.

In the particular case where absolute negative cooperativity is observed at the catalytic level of substrate transformation (*i.e.*, when only one of the sites of a functional dimer functions at a given time) the enzyme is said to display a half of the sites reactivity. This half of the sites reactivity has now been found in a number of enzymes (Lazdunski *et al.*, 1971; Levitzki *et al.*, 1971; Lazdunski, 1972). Particularly good models of this behavior include dehydrogenases such as alcohol dehydrogenases and glyderaldehyde-3-phosphatedehydrogenase and alkaline phosphatases. The alkaline phosphatase of *Escherichia coli* is a dimer comprising identical subunits. It is a metalloenzyme with zinc in its active site (for a review see Lazdunski, 1972). We present in this work a stopped-flow analysis of intersubunit catalytic cooperativity in a series of alkaline phosphatases which contain different metals in the active site: Zn^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, and Cd^{2+}.

Methods

Preparation of Metallophosphatase. The zinc alkaline phosphatase was prepared from *Escherichia coli* CW 3747 as previously described (Lazdunski and Lazdunski, 1967). The stock solution of the enzyme is stored at pH 8.0 (Tris-Cl buffer) and −20°. The maximal activity of the enzyme is 37 μmol min^{-1} mg^{-1} at pH 8.5, 0.4 M NaCl, 25°.

The apoenzyme was obtained by two different techniques. (1) One method was by overnight incubation of a Zn^{2+} phosphatase solution (1 mg/ml) with 50 mM EDTA at pH 6.5 and 25°, followed by chromatography on a Sephadex G-25 column (18 × 1.5 cm) equilibrated with 50 mM EDTA at pH 6.5 and 25°. Pooled fractions were concentrated to 30–40 mg/ml and then passed through a column of Sephadex G-25 equilibrated

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with 10 mM TES[1] (pH 7.5). (2) The second method was by treatment with Chelex 100 (200–400 mesh) in a 1 M Tris buffer (pH 8.0) as described by Csopak (1969).

The apoenzyme is zinc-free, completely inactive. Metallophosphatases were obtained by reconstitution on addition of a five-fold molar excess of metal ion to the apoenzyme at pH 7.8 (10 mM TES–Cl), 25°. The metals used were spectrographically pure (Johnson and Mathey Co., Ltd.). All experiments were carried out in demineralized, twice distilled water. The method of preparation of apophosphatase does not influence the pre-steady-state and the steady-state kinetics of reconstituted metallophosphatases.

Protein concentrations were determined from absorbance at 278 nm, $A_{1cm}^{0.1\%} = 0.77$ (Schlesinger and Barret, 1965). Steady-state kinetics and Co^{2+} phosphatase spectra were carried out with a Cary (Model 15) spectrophotometer equipped to expand the scale to 0.1 optical density (OD) unit full scale.

Assay for Inorganic Phosphate in Metallophosphatase Preparations. The inorganic phosphate content of the enzymes was determined by modification of the method of Ames and Dubin (1960). Alkaline phosphatase (0–10 nmol) was dissolved in 0.3 ml of 1% sodium dodecyl sulfate before addition of 0.7 ml of a solution of ascorbic acid (1.4%) and ammonium molybdate (0.36%) in H_2SO_4 (0.86 M). After incubation at 45° for 20 min the samples were read at 820 nm. Sodium dodecyl sulfate prevents protein precipitation in acidic medium. Absorbance at 820 nm is not influenced by sodium dodecyl sulfate and is linear with phosphate concentrations between 0 and 30 μM. The inorganic phosphate content was also evaluated after hydrolysis of the phosphatase samples in 6 N HCl at 120° for 24 hr. The phosphate contents of sodium dodecyl sulfate phosphatases and the phosphatase hydrolysates were found to be identical.

Stopped-Flow Experiments. The mechanism of action of *E. coli* alkaline phosphatase involves the intermediate phosphorylation of a serine residue in the active site of the enzyme (Schwartz and Lipmann, 1961; Engström, 1962)

$$E + S \rightleftarrows ES \longrightarrow E\text{–}P \longrightarrow E + P_i + ROH$$

ES is the Michaelis complex and E–P the phosphoenzyme. Stopped-flow kinetics are followed by measuring the release of ROH. The number of moles of ROH released in the pre-steady state is a direct measure of the number of phosphates covalently incorporated per mole of enzyme.

Experiments were run at 5 ± 0.2° in a modified commercial Durrum-Gibson spectrophotometer with a calibrated light path of 1.8 cm and a dead time of 2.5 msec. The original Durrum monochromator was replaced by a more precise and larger grating monochromator (Jobin-Yvon HR 2). This modification gave a much better signal-to-noise ratio in particular in the near-ultraviolet region (300–400 nm). The stopped-flow apparatus was equipped with three interchangeable light sources, *i.e.* Xenon (450 W, Orram), deuterium (40 W, Jobin-Yvon), and tungsten quartz iodine (50 W, Mazda) in order to work at high sensitivity between 200 and 800 nm. The direct recording in absorbance was obtained by placing a logarithmic amplifier (Philbrick-Nexus Research 4551), which transforms the transmission values, in the circuit between the photomultiplier (EMI 9558 QA) and the oscilloscope (Tektronix 564 R). Other modifications have been described elsewhere (Di Franco and Iwatsubo, 1972). The slit was never greater than 1.5 mm which corresponds to a band pass of 2.5 nm. Phosphatase-catalyzed hydrolysis of 2,4-dinitrophenyl phosphate or *p*-nitrophenyl phosphate was followed at 360 or 410 nm. In experiments at 360 nm a supplement band pass MTO filter (H 325 a) was inserted between the monochromator and the photomultiplier to eliminate residual stray light. Similarly a Kodak wratten 98 filter was used at 410 nm. Since p*K* values of nitrophenols vary with temperature, molecular extinction coefficients were determined at 5° under our experimental conditions of pH with a Cary 15 spectrophotometer. Identical values of molecular extinction coefficients were found with the optical system previously described for the stopped-flow apparatus. They are $\epsilon_M = 0.7 \times 10^4\ M^{-1}\ cm^{-1}$ at pH 4.1 and $\epsilon_M = 1.45 \times 10^4\ M^{-1}\ cm^{-1}$ at pH 8.0 for 2,4-dinitrophenol and $\epsilon_M = 1.40 \times 10^4\ M^{-1}\ cm^{-1}$ at pH 8.0 for *p*-nitrophenol. Beer's law is rigorously obeyed for each chromophore studied in the stopped-flow apparatus. Zero absorbance was checked before each experiment by mixing the substrate solution with the enzyme buffer (10 mM TES, pH 7.8). Burst size and rate constants for the transient phase were evaluated as described by Bender *et al.* (1967). 2,4-Dinitrophenyl phosphate was synthesized according to Kirby and Varvoglis (1966). 2,4-Dinitrophenyl phosphate was preferentially used at acidic pH; the pK_a of the leaving group, 2,4-dinitrophenol, is 4.15 under our experimental conditions (5°). *p*-Nitrophenyl phosphate was used only at alkaline pH; the pK_a of *p*-nitrophenol is 7.45 under the same conditions (5°). 2,4-Dinitrophenyl phosphate and *p*-nitrophenyl phosphate can be used indifferently in stopped-flow experiments. Metalloalkaline phosphatases are not specific; they do not differentiate between chemically distinct leaving groups (Lazdunski *et al.*, 1971). The steady-state kinetics for the hydrolysis of 2,4-dinitrophenyl phosphate and *p*-nitrophenyl phosphate by *E. coli* alkaline phosphatase are identical at both acidic and alkaline pH (Trentham and Gutfreund, 1968). In all our stopped-flow experiments the reacting solutions were diluted by a factor of 2 after mixing. Throughout this paper, all concentrations are expressed as final concentrations *after* mixing. Similarly pH values reported in this text (pH 4.1 and 8.0) are measured values *after* mixing (identical with a 1:1 mixture of the enzyme buffer and substrate buffer).

All pH values were determined at 5 ± 0.1° with a Radiometer (pH 26) pH meter. The registered oscilloscopic tracings were photographed on 36 × 24 mm film with a Nikon camera. The negatives were projected and enlarged on graph paper (160 × 200 mm) giving absorbance changes with time.

Results

Evidence for the Nonequivalence of the Active Sites in Metallophosphatases at Acidic pH

Phosphate Content of "Native" Zn^{2+} Phosphatase, Apophosphatase, and Other Metallophosphatases. It had been demonstrated in several laboratories that Zn^{2+} alkaline phosphatase binds tightly 1 mol of inorganic phosphate on one of its two sites at alkaline pH. Because the dissociation constant of the noncovalent 1:1 phosphate–phosphatase complex is about 2 μM in the pH 8.0 region, it appeared to us that the so-called native alkaline phosphatase, obtained after a purification procedure in which high concentrations of phosphatase are manipulated, could be in fact the 1:1 Zn^{2+} phosphatase–phosphate complex. This turned out to be true for a "native" enzyme solution (our stock solution) at a concentration of 1.1 mg/ml, *i.e.* 12 μM. Assay for inorganic phosphate revealed the presence of 1.1 ± 0.1 mol of phosphate noncovalently bound per mol of "native" Zn^{2+} alkaline phosphatase. It was observed several years ago that at very high concentrations of the Zn^{2+} enzyme

[1] Abbreviation used is: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

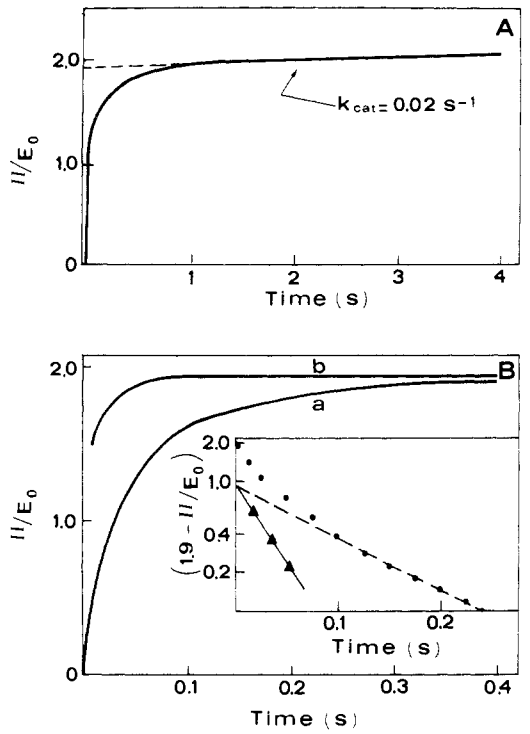

FIGURE 1: Stopped-flow oscilloscope tracings obtained by mixing *E. coli* Zn^{2+} alkaline phosphatase with 2,4-dinitrophenyl phosphate. Conditions after mixing: (A) 0.5 mM substrate and 5 μM "native" enzyme in 50 mM acetate buffer (pH 4.1), 5°; time scale 1 sec/division; (B) 0.5 mM substrate, time scale 0.1 sec/division; (a) 5 μM "native" enzyme; (b) 5 μM "reconstituted" Zn^{2+} phosphatase. Insert: log plot of pre-steady-state liberation of 2,4-dinitrophenol *vs.* time from oscilloscope tracing a. π/E_0 represents the number of moles of 2,4-dinitrophenol (π) liberated per mole of dimeric enzyme (E_0): (▲) points obtained by subtracting the slower reaction ($t_{1/2}$ = 77 msec) from the observed data (●).

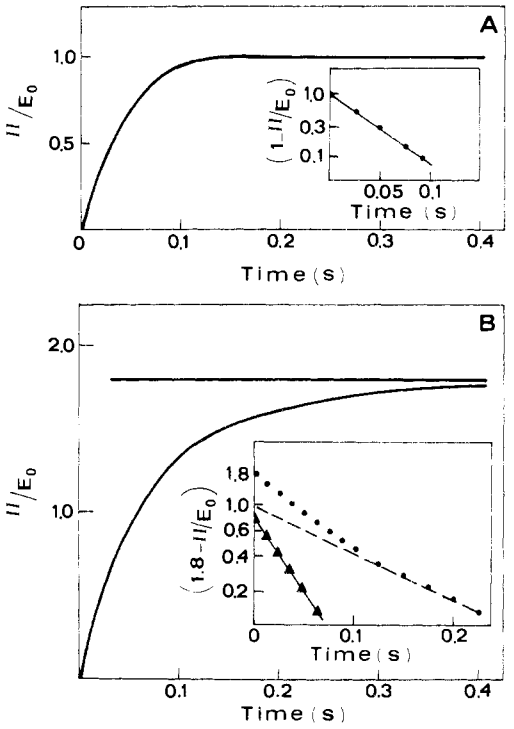

FIGURE 2: Stopped-flow oscilloscope tracings for reaction of "native" Zn^{2+} phosphatase with stoichiometric amounts of 2,4-dinitrophenyl phosphate. (A) 3.5 μM 2,4-dinitrophenyl phosphate-3.4 μM "native" Zn^{2+} phosphatase (pH 4.1), 5°. Insert: log plot indicating first-order reaction in the pre-steady state. (B) 10 μM 2,4-dinitrophenyl phosphate-5 μM "native" enzyme (pH 4.1), 5°. The horizontal line is the continuation of the lower tracing obtained between 0.65 and 0.9 sec after flow stop. Insert: log plot indicating a two-step process; (▲) points obtained as described in the legend of Figure 1 (insert).

it is possible to isolate the noncovalent 2:1 phosphate-phosphatase complex (Lazdunski *et al.*, 1969). Such an observation probably explains recent findings by Bloch and Schlesinger (1973) that endogeneous phosphate content of their "native" Zn^{2+} phosphatase amounts to 1.5-2 mol of phosphate/mol of enzyme. This phosphate is associated with the active site of the purified enzyme since inactive apophosphatase is totally free of inorganic phosphate. Zn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, and Mn^{2+} alkaline phosphatases obtained by reconstitution, that is, by addition of the appropriate metal to the apoenzyme, are also devoid of inorganic phosphate. All metallophosphatases samples (at concentrations ranging from 20 to 40 mg/ml) contained less than 0.05 mol of phosphate noncovalently bound per mol of enzyme. No reintroduction from eventual trace phosphate contamination of glassware occurred. It has been checked that the extremely low phosphate content of Co^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, and "reconstituted" Zn^{2+} phosphatase remained invariant after 3 months.

As will be seen later, the fact that "native" Zn^{2+} alkaline phosphatase contain noncovalently bound inorganic phosphate affects its pre-steady-state kinetic properties. For that reason, mechanistic conclusions previously drawn from stopped-flow or temperature jump analysis of this enzyme should probably be reexamined.

Transient Kinetics of 2,4-Dinitrophenyl Phosphate Hydrolysis by Zn^{2+} Alkaline Phosphatase at pH 4.1. Figure 1A shows that reaction of 2,4-dinitrophenyl phosphate with "native" Zn^{2+} alkaline phosphatase (the noncovalent 1:1 orthophosphate-phosphatase complex) proceeds through a transient phase before a steady state is reached. The magnitude of the burst corresponds to the release of 2 mol of phenol/mol of enzyme dimer. The logarithmic plot of curve a in Figure 1B shows that the burst is not first order. The initial part of the burst corresponds to a rate of about 29 sec^{-1} but the rate gradually slows down to about 9 sec^{-1} and finally to a steady-state rate constant of 0.02 sec^{-1}.

The profile of the transient phase was studied at different substrate concentrations ranging from 10 μM to 0.5 mM using an enzyme concentration of 5 μM. The amplitude of the burst was found to be constant at a value of 1.9 ± 0.1 mol of dinitrophenol/mol of dimeric phosphatase. Transient kinetics remained unchanged between 10 μM and 0.5 mM substrate concentration giving the same rate constants, 28 and 9 sec^{-1}, as were previously determined at 0.5 mM substrate concentration.

A typical tracing obtained using a substrate concentration (10 μM) equal to the active-site concentration of "native" Zn^{2+} phosphatase, that is, twice the dimeric enzyme concentration, is presented in Figure 2B.

Figure 2A presents a situation in which the substrate concentration (3.5 μM) is only half of the active-site concentration, that is, equal to the enzyme concentration (3.4 μM). In that case and as expected, only 1 mol of phenol is released per mol of dimeric enzyme. The interest of Figure 2A is that it shows that the logarithmic transformation of the transient phase is perfectly linear. Presteady state in this case follows a pure first-order process with a rate constant of 26 sec^{-1}. The biphasic kinetics in the transients obviously appear only when substrate concentration is high enough to permit saturation of both enzyme active sites.

Figure 1B shows that the pre-steady-state behaviors of "na-

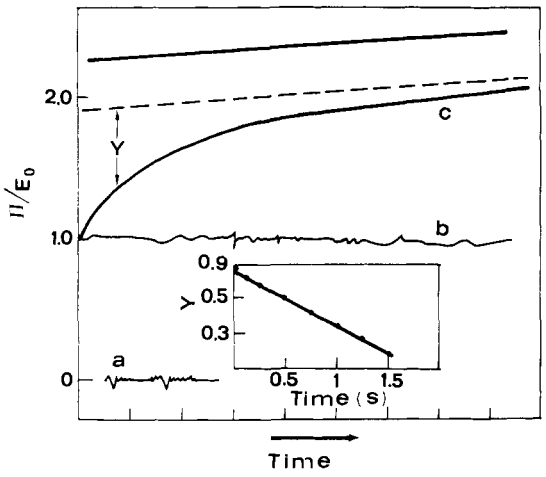

FIGURE 3: Oscilloscope tracings for reaction of Co^{2+} alkaline phosphatase (4 μM) with 2,4-dinitrophenyl phosphate (0.5 mM) (pH 4.1), 5°: (a) oscilloscope tracing obtained after mixing 4 μM of apoenzyme with 0.5 mM 2,4-dinitrophenyl phosphate; time scale 5 msec/division; (b) Co^{2+} phosphatase–2,4-dinitrophenyl phosphate system; time scale 5 msec/division; (c) same system, time scale 0.5 sec/division. The upper tracing, taken in the steady state, is the continuation of tracing c obtained between 7 and 11 sec after flow stop. The dotted line is the extrapolation of the steady state over the time period corresponding to tracing c. *Y* is the difference between the dotted line and tracing c. Insert: log plot demonstrating first-order kinetics for the second part of the burst.

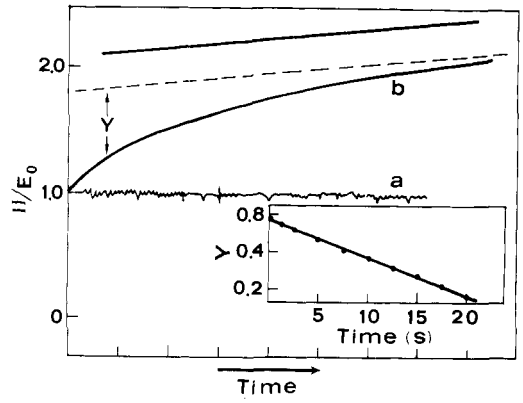

FIGURE 4: Oscilloscope tracings for reaction of Cu^{2+} phosphatase (4 μM) with 2,4-dinitrophenyl phosphate (0.5 mM) at pH 4.1: (a) time scale 5 msec/division; (b) time scale 5 sec/division; upper tracing (between 40 and 70 sec) and dotted line have same significance as in Figure 3. Insert: log plot demonstrating first-order kinetics for the second part of the burst (trace b).

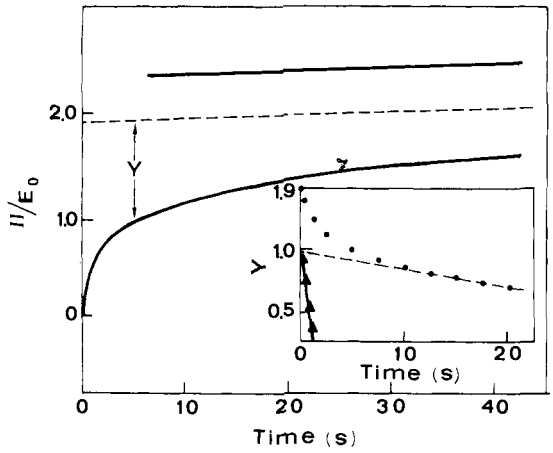

FIGURE 5: Oscilloscope tracings for reaction of the Cd^{2+} phosphatase (4 μM) with 2,4-dinitrophenyl phosphate (0.5 mM) (pH 4.1), 5°; upper tracing (between 115 and 160 sec) and dotted line are as in Figure 3. Insert: log plot indicating a two-step process; (▲) points obtained as described in the legend of Figure 1 (insert).

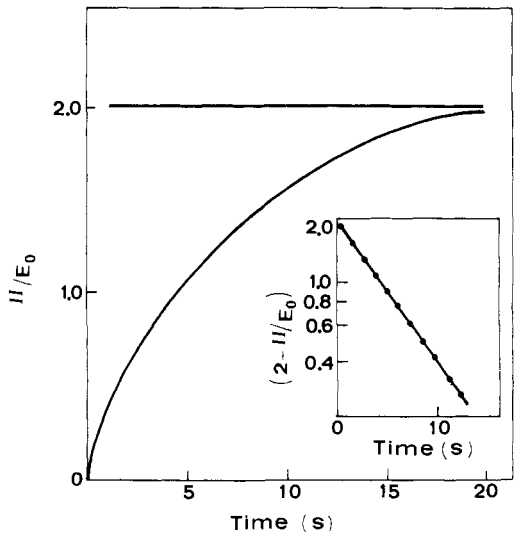

FIGURE 6: Oscilloscope tracings for reaction of Mn^{2+} phosphatase (4 μM) with 2,4-dinitrophenyl phosphate (0.5 mM) (pH 4.1), 5°; upper tracing starts 20 sec after the flow stop. Insert: log plot demonstrating first-order reaction in the pre-steady state.

tive" Zn^{2+} phosphatase, which contains one noncovalent phosphate/mol, and of "reconstituted" Zn^{2+} phosphatase with no bound phosphate, are not identical. In both cases, the burst amplitude is 2 mol of dinitrophenol, but pre-steady-state kinetics with "reconstituted" Zn^{2+} phosphatase are much faster. The burst profile again indicates that there is more than one step in the transient phase; however, rate constants could not be determined with accuracy.

Substitution of Zn^{2+} by Co^{2+}, Cu^{2+}, Cd^{2+}, and Mn^{2+} in the Active Site of the Enzyme; Influence on Transient Kinetics. Oscilloscope tracings obtained by mixing Co^{2+}, Cu^{2+}, Cd^{2+}, or Mn^{2+} phosphatases with 2,4-dinitrophenyl phosphate at pH 4.1 in the stopped-flow apparatus are presented in Figures 3–6. A transient phase was observed before onset of the steady state with all metallophosphatases. In all cases stoichiometry of the total burst amplitude is 2 mol of dinitrophenol/mol of phosphatase.

Figure 3 shows a biphasic burst of 1.8 mol of dinitrophenol/mol of phosphatase for the Co^{2+} phosphatase at pH 4.1 and 5°. An initial burst of 1.0 mol of dinitrophenol/mol of phosphatase is too fast to be followed and occurs with a rate constant higher than 1000 sec^{-1}. The second part of the total burst occurs with a rate constant of only 0.9 sec^{-1}.

A similar situation is found in Figure 4 for the Cu^{2+} phosphatase. Again, although values of the kinetic constants are quite different from those of the Co^{2+} phosphatase, a total burst of 1.8 mol of dinitrophenol/mol of enzyme is obtained and this burst is biphasic.

The kinetics of both steps of the transient phase can be easily calculated with the Cd^{2+} phosphatase. Figure 5 shows that the burst of 2,4-dinitrophenol liberated in the pre-steady-state period is 2.0 mol/mol of phosphatase. The rapid burst of 1 mol of phenol occurs with a first-order rate constant of 1 sec^{-1}; the slower burst amplitude is also 1 mol of phenol but this step occurs with a first-order rate constant of only 0.03 sec^{-1}. A simi-

TABLE I: Burst Size, Pre-Steady-State, and Steady-State Kinetic Characteristics of Zn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, and Mn^{2+} Phosphatases.

Metallophosphatases	pH	Burst Size	k_{01} (sec^{-1})	k_{02} (sec^{-1})	k_{cat} (sec^{-1})
Zn^{2+} phosphatase (native)	4.1[a]	2	29	9	0.02
Zn^{2+} phosphatase		2	$\geq$1000	30–40	0.02
Co^{2+} phosphatase		1.8	$\geq$1000	0.8	0.07
Cu^{2+} phosphatase		1.8	$\geq$1000	0.07	0.008
Cd^{2+} phosphatase		2	1	0.03	0.004
Mn^{2+} phosphatase		2	0.2		0.001
Zn^{2+} phosphatase (native)	8.0[b]	0.8	$\geq$1000		
Zn^{2+} phosphatase		1	$\geq$1000		8
Co^{2+} phosphatase		1	$\geq$1000		1
Cu^{2+} phosphatase		0.4	0.5–0.6		0.13
Mn^{2+} phosphatase		0.05			0.05

[a] Substrate, 2,4-dinitrophenyl phosphate, 0.5 mM. [b] Substrate, *p*-nitrophenyl phosphate, 10 mM.

lar type of biphasic transient kinetics in which the rate of each of the two steps can be calculated was obtained at alkaline pH with Zn^{2+} phosphatase acting on "bad" substrates: phosphorothioates and thiophosphates (Chlebowski and Coleman, 1972; Fernley, 1973).

The stoichiometry of the burst observed with the Mn^{2+} phosphatase at pH 4.1 is 2 mol of dinitrophenol/mol of enzyme. However, burst kinetics with the Mn^{2+} phosphatase are very different from those observed with the other metallophosphatases. In this case, the transient phase follows a pure first-order process with a rate constant of 0.2 sec^{-1}. Therefore, incorporation of Mn^{2+} into the active site of the enzyme suppresses the biphasic profile observed in the pre-steady state with other metallophosphatases. Table I summarizes burst sizes and kinetic constants observed at pH 4.1 for the different metallophosphatases.

We chose Co^{2+} phosphatase to evaluate the influence on the transient phase of preincubation with inorganic phosphate of a metallophosphatase distinct from the Zn^{2+} phosphatase. Stopped-flow kinetic experiments with 2,4-dinitrophenyl phosphate (25 mM) involving the 1:1 Co^{2+} phosphatase–inorganic phosphate complex (3.7–5 μM), in place of the Co^{2+} phosphatase free of inorganic phosphate, were carried out and compared to data in Figure 3. Preincubation at pH 7.8 (30–60 min) of the Co^{2+} phosphatase with inorganic phosphate, before mixing with 2,4-dinitrophenyl phosphate, was found to have no effect on the total magnitude of the burst at pH 4.1 and 5° (π/E_0 = 1.8) and no effect on the biphasicity of the burst. The first part of the total burst (π/E_0 = 1) remains too rapid to be followed; the rate of the slower part (π/E_0 = 0.8) is not significantly affected ($k_{02} \simeq$ 0.6 sec^{-1} instead of 0.8 sec^{-1} for the Co^{2+} phosphatase free of phosphate). Identical results were obtained after preincubation of 2 mol of inorganic phosphate (20 μM)/mol of Co^{2+} phosphatase (10 μM) before mixing with 2,4-dinitrophenyl phosphate in the stopped-flow apparatus.

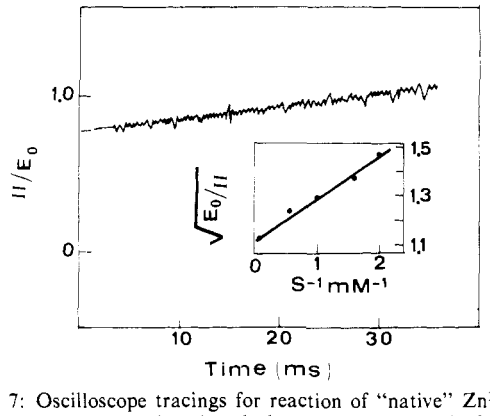

FIGURE 7: Oscilloscope tracings for reaction of "native" Zn^{2+} phosphatase (4 μM) with *p*-nitrophenyl phosphate (10 mM) (pH 8.0) (50 mM; Tris-Cl buffer), 5°. Insert: burst amplitude as function of initial substrate concentration (plot of $(\pi/E_0)^{-1/2}$ *vs.* $1/S$). This profile permits determination of an apparent "K_m" constant of 0.1 mM.

Half of the Sites Reactivity of Metallophosphatases Near pH 8.0

Figure 7 shows that catalyzed hydrolysis of a saturating concentration of *p*-nitrophenyl phosphate (10 mM) by the "native" alkaline phosphatase (the 1:1 phosphatase–inorganic phosphate complex) involves a very rapid release of *p*-nitrophenol. The pre-steady-state period occurs within the dead time of the apparatus. Burst amplitude indicates a stoichiometry of only 0.8 mol of *p*-nitrophenol liberated per dimer in the transient phase. This is an indication that only one of the two sites is phosphorylated at a given time in steady-state conditions.

The only difference between "native" Zn^{2+} alkaline phosphatase and "reconstituted" Zn^{2+} alkaline phosphatase is observed for the substrate concentration dependence of the burst amplitude (compare inserts of Figures 7 and 9). Some workers (Fernley and Walker, 1969; Halford, 1971; Halford *et al.*, 1972; Bloch and Schlesinger, 1973) have used low substrate concentrations (5 μM, 50 μM, 0.2 mM) in stopped-flow kinetics with the "native" Zn^{2+} phosphatase and, as a result, they have been unable to see any burst of the leaving group in the alkaline pH region.

The pre-steady-state period which is too fast to be seen at pH 8.0 can be easily followed at pH 6.2 with the "native" Zn^{2+} phosphatase (Figure 8a). Half of the sites reactivity is also observed at this more acidic pH value. The transient phase follows pure first-order kinetics indicating that the rate constant for the formation of the monophosphorylated derivative is 3.0 sec^{-1}, ten times faster than the rate constant (k_{cat}) for the dephosphorylation process. A burst of only 1 mol of 2,4-dinitrophenol/mol of protein dimer is also observed with the "reconstituted" Zn^{2+} phosphatase at pH 6.2 (Figure 8b) but in contrast with the "native" Zn^{2+} phosphatase the pre-steady-state period is very short; steady state is attained after 2.5 msec.

The conclusion from all these results is that there is no significant difference in the burst size between "native" and "re-

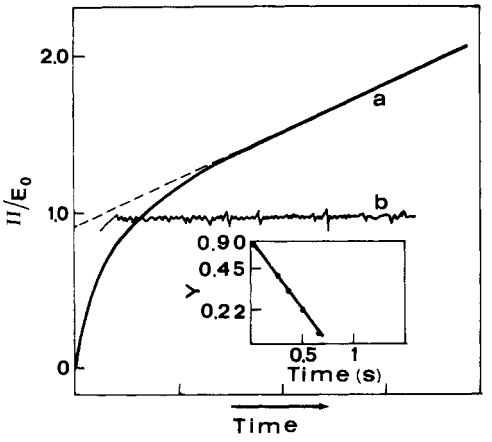

FIGURE 8: Stopped-flow oscilloscope tracings for reaction of Zn^{2+} alkaline phosphatase with 2,4-dinitrophenyl phosphate at pH 6.2: (a) 4 μM "native" enzyme–0.5 mM substrate; time scale 1 sec/division; (b) 2 μM "reconstituted" Zn^{2+} phosphatase–10 μM substrate; time scale 5 msec/division. Insert: log plot for tracing a.

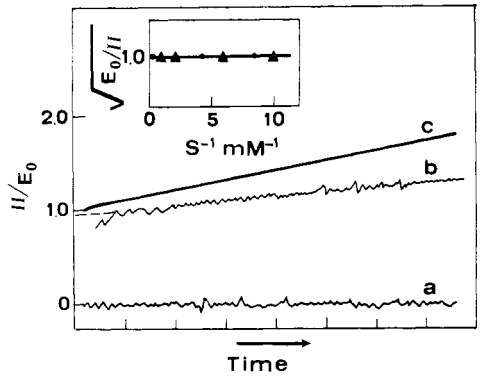

FIGURE 9: Oscilloscope tracings for reaction of Zn^{2+} and Co^{2+} phosphatases with *p*-nitrophenyl phosphate (10 mM) (pH 8.0), 5°. (a) 4 μM apoenzyme, time scale 5 msec/division; (b) 4 μM "reconstituted" Zn^{2+} phosphatase, time scale 5 msec/division; (c) 4 μM Co^{2+} phosphatase; time scale 0.1 sec/division. Insert: same significance as in Figure 7; (▲) Zn^{2+} phosphatase; (●) Co^{2+} phosphatase.

constituted" Zn^{2+} phosphatase. They both display half of the sites reactivity, both at pH 6.2 and at 8.0 at 5°. The only difference between "native" and "reconstituted" Zn^{2+} alkaline phosphatases resides in the rate of the transient phase. Noncovalently bound inorganic phosphate in the "native" enzyme obviously increases the length of the pre-steady-state period under all experimental conditions.

The analysis of the stopped-flow kinetics obtained at pH 8.0, 5°, with the Co^{2+} phosphatase is compared in Figure 9 with that of the "reconstituted" Zn^{2+} phosphatase. Again 1 mol of *p*-nitrophenol/mol of metallophosphatase is liberated in the pre-steady-state period. Both for the Co^{2+} and the Zn^{2+} alkaline phosphatases only one of the two active sites is phosphorylated at any given time under steady-state conditions.

An analysis of stopped-flow data was also carried out with the Co^{2+} phosphatase using 2,4-dinitrophenyl phosphate (0.5 mM) instead of *p*-nitrophenyl phosphate. The results obtained were identical with those presented in Figure 9 for the Co^{2+} phosphatase–*p*-nitrophenyl phosphate system.

Half of the sites reactivity was also observed with the Cu^{2+} phosphatase at pH 6.5 (Figure 10a). However, the behavior of the Cu^{2+} phosphatase is clearly different from that of the Zn^{2+} and Co^{2+} phosphatases at pH 8.0 (Figure 10b). A fractional burst of 0.4 mol of *p*-nitrophenol/mol of enzyme is obtained at

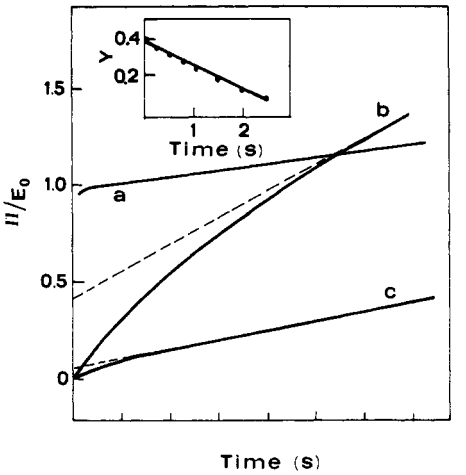

FIGURE 10: Oscilloscope tracings for reaction of Cu^{2+} and Mn^{2+} phosphatases with 2,4-dinitrophenyl (0.2 mM) or *p*-nitrophenyl (10 mM) phosphates: (a) Cu^{2+} phosphatase (2 μM)–2,4-dinitrophenyl phosphate system (pH 6.5), 5°, time scale 2 sec/division; (b) Cu^{2+} phosphatase (4 μM)–*p*-nitrophenyl phosphate system (pH 8.0), 5°, time scale 1 sec/division; (c) Mn^{2+} phosphatase (4 μM)–*p*-nitrophenyl phosphate system (pH 8.0), 5°, time scale 1 sec/division. Insert: log plot from tracing b.

pH 8.0, 5° (Figure 10b). The pre-steady-state period can be followed easily in this case; the first-order rate constant of the burst period is 0.55 sec^{-1}, whereas the first-order rate constant corresponding to the maximal rate under steady-state conditions (k_{cat}) is 0.13 sec^{-1}. The fractional burst is explained in that case by the fact that the phosphorylation and dephosphorylation steps have similar rates.

Calculations using classical equations for a two-step process involving a covalent intermediate[2] and k_0 and k_{cat} values presented in Table I, assuming that the two active sites of the Cu^{2+} phosphatase are functional and independent (*i.e.*, absence of half of the sites reactivity), give a value of 1.2 mol of *p*-nitrophenol/mol of dimeric phosphatase for the burst amplitude. This calculated burst is not consistent with experimental results which indicate a burst of only 0.4 mol of *p*-nitrophenol. The same calculations with the assumption that *only one* of the two active sites of the Cu^{2+} phosphatase can be phosphorylated at any instant under steady-state conditions give a theoretical burst of 0.36 mol of *p*-nitrophenol/mol of dimeric phosphatase. Calculated and experimental data are in very good agreement with the latter assumption. Cu^{2+} phosphatase also behaves as a half of the sites enzyme at pH 8.0. The calculated rate constant for the phosphorylation of the active site, 0.33 sec^{-1} (k_2), is of the same order of magnitude as the dephosphorylation rate constant, 0.22 sec^{-1} (k_3).

A burst of only 0.1 mol of *p*-nitrophenol/mol of Cu^{2+} phosphatase was found at pH 9.3, 5°. This low value of the burst demonstrates that the phosphorylated derivative of the Cu^{2+} enzyme does not accumulate significantly at very alkaline pH. This is a confirmation of what has been previously found by other techniques (Lazdunski *et al.*, 1971).

[2] A classical equation for a two-step process is

$$E + S \rightleftharpoons E\text{–}S \xrightarrow{k_2} E\text{–}P \xrightarrow{k_3} E + P_i + ROH$$

k_2 and k_3 are the first-order rate constants of the phosphorylation and dephosphorylation steps, respectively. $k_{cat} = [k_2k_3/(k_2 + k_3)]$; $k_0 = k_2 + k_3$ when $[S] > K_m$. k_0 is the first-order rate constant observed for the transient phase. $\pi/E_0 = [k_2/(k_2 + k_3)]^2$ is the burst amplitude when $[S] > K_m$.

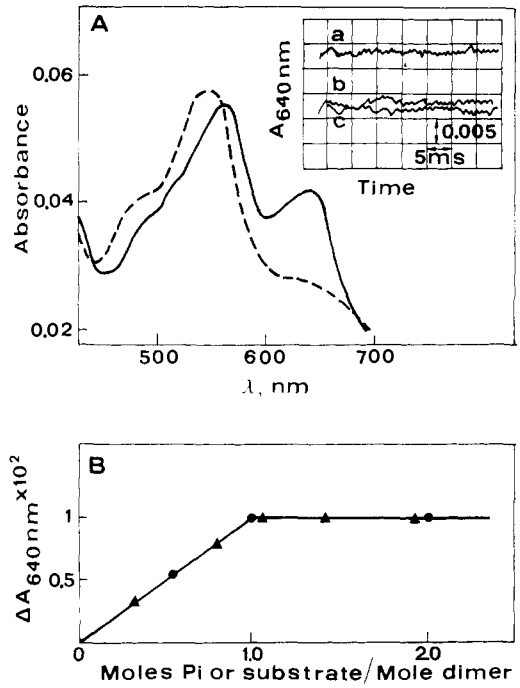

FIGURE 11: (A) Visible absorption spectra of Co^{2+} phosphatase and of Co^{2+} phosphatase–orthophosphate complex. Conditions: (—) 125 μM Co^{2+} phosphatase (5 g-atoms of Co^{2+}/mol of dimeric enzyme), pH 8.0, 10°; (- - -) same as (—) plus 2 mol of P_i/mol of enzyme. Insert: oscilloscope tracings obtained at 640 nm by mixing in the stopped-flow apparatus Co^{2+} phosphatase (65 μM) with: (a) 10 mM Tris-Cl buffer (pH 8.0) (base line); (b) 65 μM inorganic phosphate in Tris-Cl buffer (pH 8.0); (c) 65 μM β-glycerophosphate in 10 mM Tris-Cl buffer (pH 8.0). Experiments were carried out at 5°. (B) Stopped-flow titration of Co^{2+} phosphatase with inorganic phosphate (▲) or β-glycerophosphate (●), pH 8.0, 5°.

The Mn^{2+} phosphatase gives no significant burst of *p*-nitrophenol at pH 8.0, 5° (Figure 10c). This is an indication that no phosphorylated intermediate of this metallophosphatase accumulates under steady-state conditions; phosphorylation of the active site is the rate-limiting step. The Cd^{2+} phosphatase also behaves as a half of the sites enzyme at pH 8.0. It gives a burst of 1.0. mol of *p*-nitrophenol/mol of enzyme.

Co^{2+} phosphatase was again chosen to evaluate the influence on the transient phase of preincubation with inorganic phosphate of a metallophosphatase distinct from the Zn^{2+} phosphatase. Stopped-flow experiments at pH 8.0, 5°, with *p*-nitrophenyl phosphate (10 mM) involving the 1:1 Co^{2+} phosphatase–inorganic phosphate complex (preincubation of a 1:1 mixture of each partner at 10μM) gave a burst of 0.1 mol of *p*-nitrophenol/mol of enzyme in place of the burst of 1.0 mol of phenol observed with Co^{2+} phosphatase free of inorganic phosphate (Figure 9).

A last indication of the half of the sites reactivity of the Co^{2+} phosphatase is presented in Figure 11. The association of inorganic phosphate induces a large variation of the visible absorption spectrum of the Co^{2+} phosphatase (Simpson and Vallee, 1968; Applebury and Coleman, 1969; Taylor *et al.*, 1973). Typical spectra for Co^{2+} phosphatase and for 1:1 and 1:2 mixtures are presented in Figure 11A. The perturbation at 640 nm has been used to follow the kinetics of inorganic phosphate binding to the Co^{2+} phosphatase (Figure 11A, insert b). The spectral perturbation is completed in less than 2.5 msec. The inorganic phosphate concentration dependence of the amplitude of the spectral change indicates a 1:1 stoichiometry of binding of orthophosphate to Co^{2+} alkaline phosphatase (Figure 11B). This result confirms the strong negative cooperativity already observed by other techniques for the binding of inorganic phosphate to this metalloenzyme (Lazdunski *et al.*, 1969; Petitclerc *et al.*, 1970; Simpson and Vallee, 1970; Reid and Wilson, 1971).

A spectral change at 640 nm is also induced by substrates at pH 8.0 and 5°. A stopped-flow study of the spectral change at 640 nm for the Co^{2+} phosphatase–β-glycerophosphate system shows (Figure 11A, insert c) that the amplitude of the variation at 640 nm is identical with that observed for the binding of inorganic phosphate to the Co^{2+} phosphatase. The stopped-flow analysis of the stoichiometry of association of β-glycerophosphate with the Co^{2+} phosphatase indicates that the maximal spectral perturbation occurs after association of 1 mol of β-glycerophosphate with 1 mol of dimeric enzyme. This technique of spectral perturbation, in which Co^{2+} plays the role of a reporter group in the active site, has demonstrated an absolute negative cooperativity, *i.e.* half of the sites reactivity, both for inorganic and organic phosphate.

Discussion

Previous studies carried out with the Zn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+} phosphatases have shown that inorganic phosphate, the product of the reaction, was able, under saturating concentration conditions, to phosphorylate the two sites of the enzyme, at acidic pH (Lazdunski *et al.*, 1969, 1970, 1971). The characteristic of this covalent phosphorylation is negative cooperativity. The first site is phosphorylated at a low concentration of inorganic phosphate; much higher concentrations are necessary to phosphorylate the second one. Negative cooperativity increases from low to high pH. For example, the interaction energy between the two subunits for the phosphorylation process is 2.15 kcal mol^{-1} at pH 4.2 and 3.85 kcal mol^{-1} at pH 5.0 for the Zn^{2+} enzyme (Lazdunski *et al.*, 1971). Negative cooperativity for the phosphorylation of the two active sites by inorganic phosphate at acidic pH has also been demonstrated by kinetic techniques. The exchange of unlabeled phosphate for labeled phosphate in the Zn^{2+} diphosphophosphatase at pH 4.2 displays biphasic kinetics. It indicates that one of the two covalent phosphates is exchanged very rapidly whereas the second one is exchanged much more slowly. The same experiment with the Zn^{2+} monophosphophosphatase indicated a pure first-order process for the exchange.

Quenching experiments[3] have also been carried out to isolate the phosphorylated complex formed in the steady state, when

[3] Quenching experiments to isolate the phosphorylated complex formed with inorganic phosphate or with ^{32}P-labeled substrates in the steady-state have been criticized by Schlesinger and coworkers (Halford *et al.*, 1972; Bloch and Schlesinger, 1973). The typical criticism has been formulated as follows in the last paper by Bloch and Schlesinger (1973). "The theoretical foundation for such experiments is somewhat dubious because they involve "fixing" covalent phospho-enzyme by rapid irreversible acid denaturation that transforms phospho-phosphatase into a stable phospho-protein. It is assumed that denaturation is complete and faster than any linked processes such as phosphorylation or dephosphorylation." Such a criticism, although it has been repeated several times by this group of workers, is quite incorrect (see also Lazdunski, 1972). It is true that it is important to be sure neither phosphorylation nor dephosphorylation occurs during quenching. However, since 1969 controls have been made in this laboratory and reported to prove that such a situation does not occur (see, for example, Chappelet-Tordo *et al.*, 1974). Dephosphorylation cannot occur since acidic pH conditions inhibit this type of transformation. Moreover, no phosphorylation occurred during quenching; both addition of *E. coli* phosphatase to a quench solution (10 N HCl in 8 M urea) containing the labeled phosphate and addition of labeled phosphate to a quench solution containing phosphatase failed to produce ^{32}P incorporation into the active site. On the other hand the phosphate concentration de-

Zn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+} phosphatases act on ^{32}P-labeled AMP, pyrophosphate, and ATP. This work has shown that both active sites can be phosphorylated at acidic pH in all metallophosphatases. The present work is in excellent agreement with these previous results. Formation of diphosphorylated derivatives of metallophosphatases at acidic pH implies observation of a burst of 2 mol of dinitrophenol/mol of protein dimer in the pre-steady-state period of hydrolysis of 2,4-dinitrophenyl phosphate. This is exactly what has been demonstrated in this paper at pH 4.1, 5°. The present data give complementary information concerning the reactivity of each one of the active sites. With the Zn^{2+}, the Cu^{2+}, the Co^{2+}, and the Cd^{2+} phosphatases, phosphorylation of one of the sites is more rapid than phosphorylation of the second one. The difference in rates of phosphorylation of each site is strongly accentuated for the Co^{2+}, Cu^{2+}, and "reconstituted" Zn^{2+} phosphatases; it is still important but less marked for the Cd^{2+} phosphatase (Table I). In the latter case the ratio of first-order rate constants for the phosphorylation of each of the sites is 30 at pH 4.1, 5°.

At alkaline pH, near pH 8.0, inorganic phosphate forms noncovalent complexes with the Zn^{2+}, Co^{2+}, and Cu^{2+} phosphatases whereas substrates such as AMP, ATP, or pyrophosphate form phosphorylated derivatives of these enzymes, in the steady state, which can be isolated by quenching techniques (Lazdunski *et al.*, 1969, 1970, 1971). Noncovalent complexes formed with the Zn^{2+}, Co^{2+}, and Cu^{2+} phosphatases have been studied in a variety of ways. For example, equilibrium dialysis (Lazdunski *et al.*, 1969; Reid and Wilson, 1971) or spectral perturbations of visible spectra, circular dichroic (CD) spectra, or magnetic CD spectra of the Co^{2+} and/or Cu^{2+} phosphatases have shown once again that the characteristic property of the binding is negative cooperativity (Applebury and Coleman, 1969; Taylor and Coleman, 1972; Taylor *et al.*, 1973). The 1:1 complexes formed with inorganic phosphate have even been isolated with the Zn^{2+}, the Co^{2+} (Petitclerc *et al.*, 1970), and the Cu^{2+} phosphatases (Lazdunski *et al.*, 1970).

The stopped-flow analysis presented in this paper for the Zn^{2+} or Co^{2+} phosphatases indicates a burst of only 1 mol of phenol/mol of protein dimer in the pre-steady-state period of hydrolysis of *p*-nitrophenyl phosphate. The data are again in good agreement with previous results obtained in this laboratory, using a quenching technique to isolate phosphorylated complexes formed in the course of the action of Zn^{2+} or Co^{2+} phosphatases on ^{32}P-labeled substrates. A monophosphophosphatase was also found to accumulate in the steady state around pH 8.0.

All these results demonstrate that only one of the two active sites of the alkaline phosphatase is phosphorylated at any instant at alkaline pH; that is, under conditions of high catalytic activity. Half of the sites reactivity was observed not only for the Zn^{2+} and the Co^{2+} phosphatases but also for the Cu^{2+} enzyme.

The half of the sites reactivity of alkaline phosphatase is demonstrated not only by labeling with ^{32}P-labeled substrates or by using stopped-flow techniques to measure the amplitude of the pre-steady-state burst of the leaving group, but also by measuring the perturbation of the visible spectrum of Co^{2+} phosphatase which occurs during complex formation with β-glycerophosphate (Figure 11).

The tentative conclusions of a recent paper describing much less exhaustive stopped-flow kinetic studies with the sole Zn^{2+} phosphatase–2,4-dinitrophenyl phosphate system was that the active sites on the phosphatase molecule may be equivalent and independent (Bloch and Schlesinger, 1973). Such a result is obviously contradicted by all the data presented in this paper which were obtained with a variety of metallophosphatases and in a variety of pH conditions.

It has already been demonstrated in recent years that Zn^{2+}, Co^{2+}, and Cu^{2+} phosphatases have different steady-state activities (Lazdunski and Lazdunski, 1968; Lazdunski *et al.*, 1970). The order of efficiency of these enzymes, at pH 8.0, is Zn^{2+} phosphatase > Co^{2+} phosphatase > Cu^{2+} phosphatase. At acidic pH, activities are lower and the order of efficiency is changed: Co^{2+} phosphatase > Zn^{2+} phosphatase > Cu^{2+} phosphatase (Table I). The Co^{2+} phosphatase which is one-eighth as active as the Zn^{2+} phosphatase at pH 8.0 is four times more active at pH 4.1. The Cu^{2+} phosphatase which is about one-tenth as active as the Co^{2+} phosphatase is one-third as active as the Zn^{2+} phosphatase at pH 4.1. The Cu^{2+} enzyme is one-sixtieth as active as the Zn^{2+} phosphatase at pH 8.0.

Both at alkaline and at acidic pH the Cd^{2+} and Mn^{2+} alkaline phosphatases have very low activity. This activity is so low that it cannot be given with accuracy due to the possibility of contamination by traces of Zn^{2+} phosphatase. These two enzymes must, however, be considered as catalytically active phosphatases since phosphorylation of the active site occurs in both cases. The Cd^{2+} phosphatase has preserved the property of negative cooperativity for substrate transformation between pH 4.0 and 8.0. Of all the phosphatases the Mn^{2+} phosphatase has a very characteristic behavior. It is the only metallophosphatase for which there is not, up to now, the slightest indication of cooperativity between sites. It behaves as if its two sites were independent sites. A 2:1 noncovalent complex is formed between inorganic phosphate and Mn^{2+} phosphatase at alkaline pH, under conditions in which Zn^{2+}, Co^{2+}, and Cu^{2+} phosphatases form 1:1 complexes (Chappelet *et al.*, 1970); the two sites behave as if they were identical and independent for the phosphorylation by [^{32}P]pyrophosphate at acidic pH (Chappelet *et al.*, 1970) and the stopped-flow data presented here, at pH 4.1, show very clearly that the Mn^{2+} phosphatase is the only one to follow a monophasic kinetic behavior in the pre-steady state (Figure 6). Replacement of Zn^{2+} by Mn^{2+} apparently abolishes site–site interactions in the dimeric enzyme; it also produces a dramatic decrease in catalytic efficiency. It is tempting to propose that these two facts are related. Cooperativity between sites in the alkaline phosphatase would then be required for a high specific activity.

Half of the sites reactivity exists not only for the *Escherichia coli* alkaline phosphatase but also for the intestinal enzyme (Chappelet-Tordo *et al.*, 1974). Flip-flop type mechanisms have already been proposed to reconcile two apparently antinomic properties of these phosphatases; *i.e.*, negative cooperativity for both substrate binding and transformation (half of the sites reactivity) and Michaelis–Menten kinetics (Lazdunski *et al.*, 1971; Chappelet-Tordo *et al.*, 1974). Because Michaelis–Menten kinetics are characteristic of monomeric enzymes it is often believed that observation of this type of behavior with oli-

pendence of the covalent phosphorylation of Zn^{2+} and Co^{2+} phosphatase at acidic pH, the fact that no covalent phosphorylation occurs by quenching at acidic pH of the noncovalent orthophosphate–phosphatase complex formed at pH 8 or higher (Lazdunski *et al.*, 1971), and many other results described in our papers since 1969 also demonstrate clearly that the quenching technique is excellent to measure the amount of phosphoryl-enzyme (Lazdunski, 1972). Finally, as reported in this Discussion section, there is an excellent agreement between ^{32}P labeling and stopped-flow data with all metallophosphatases and at all pH values. In fact, the two techniques are complementary and should always be used together by workers interested in studying the mechanism of alkaline phosphatases.

gomeric enzymes indicates functional independence of the subunits. Such an interpretation is obviously not true in the case of alkaline phosphatases. Half of the sites reactivity is in fact a widespread phenomenon among oligomeric enzymes (Bernhard *et al.*, 1970; Lazdunski *et al.*, 1971; Levitzki *et al.*, 1971; Lazdunski, 1972, 1974; Stallcup and Koshland, 1973). It is observed not only for Michaelian enzymes but also for allosteric enzymes with sigmoidal kinetics (Lazdunski, 1972). The possible functional and evolutionary significance of this property has been discussed elsewhere (Lazdunski, 1972, 1974; Stallcup and Koshland, 1973).

Acknowledgments

We thank Dr. Paul Tordo for the synthesis of 2,4-dinitrophenyl phosphate and Dr. D. Grossman for careful reading of the manuscript.

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