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Intestinal Alkaline Phosphatase. Catalytic Properties and Half of the Sites Reactivity

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ABSTRACT: Typical catalytic properties of the intestinal alkaline phosphatase have been determined. They include the analysis of variations of K_m and V_m with pH, the analysis of the ionic strength dependence and of nucleophile effects, and the analysis of the enzyme specificity. The work was carried out both with the free enzyme in the pure state and with the bound enzyme in the purified intestinal membrane vesicles. The kinetic properties of the intestinal phosphatase are not markedly altered by association with the membrane matrix. The nonequivalence of the two actives sites in the dimeric enzyme has been established in a variety of ways. Equilibrium dialysis has shown the existence of one "tight" and one "loose" binding site for the noncovalent association of inorganic phosphate at pH 8.0. Two different sites have also been demonstrated from the analysis of the inorganic phosphate concentration dependence of the phosphorylation of the essential serine residues at acidic pH. Stopped-flow analysis of the catalyzed hydrolysis of 2,4-dinitrophenyl phosphate at acidic pH indicated that one of the two active sites was phosphorylated very rapidly ($k_1 > 1000$ sec^{-1}) whereas the other one was phosphorylated much more slowly ($k_2 = 100$ sec^{-1}). Half of the sites reactivity was demonstrated at pH 7.0–8.5. Quenching of the phosphoenzyme formed, under steady-state conditions, with [^{32}P]AMP as a substrate, has shown that only one of the two sites is phosphorylated at any instant. This result was confirmed by the stopped-flow observation of a burst of only 1 mol of 2,4-dinitrophenol/mole of dimeric phosphatase in the presteady state of the catalyzed hydrolysis of 2,4-dinitrophenyl phosphate. These results have been interpreted as a demonstration of the occurrence of "kinetic cooperativity" between subunits in the catalytic mechanism of the intestinal alkaline phosphatase.

Intestinal alkaline phosphatase is a dimeric enzyme comprising two apparently identical subunits. It is a metalloenzyme with four zinc atoms/mol of protein. Intestinal alkaline phosphatase is an integral component of the continuous phase of the outer membrane of the microvillii (Holt and Miller, 1962; Hugon and Borgers, 1966). It is tightly associated with duodenum, jejunum, and ileum membranes (Louvard *et al.*, 1973b). Alkaline phosphatase may be engaged in the uptake of phosphate into the intestine (Moog and Glazier, 1972).

Kinetic properties of intestinal alkaline phosphatases have already been studied in several laboratories using partially purified preparations (Morton, 1955; Lazdunski and Ouellet, 1962; Fernley and Walker, 1967; Fernley and Bisaz, 1968; Fernley, 1971).

The first purpose of this paper is to analyze the kinetic properties both of the pure enzyme in the free state and of the phosphatase integrated into membrane vesicles purified from brush borders of duodenum, jejunum, and ileum.

The second purpose of the paper is to demonstrate by equilibrium dialysis, by quenching of the phosphoenzyme formed in the course of catalysis, and by stopped-flow technique that the two sites of the enzyme are not independent in catalysis. It will be shown that intestinal alkaline phosphatase can be classified as a half-site enzyme.

Materials and Methods

(a) *Materials.* Purified calf intestine alkaline phosphatase was obtained as described in the previous paper (Fosset *et al.*, 1974). Calf and pig intestinal membranes containing alkaline phosphatase were prepared from duodenum, jejunum, and ileum, according to Louvard *et al.* (1973a). Electron microscope analysis of the brush border membranes showed that they form closed vesicles. The vesicle preparation appeared to be free of any visible contaminant.

The free enzyme was routinely stored at pH 8.0 (0.1 M Tris-Cl buffer) at −20°. Its maximal specific activity was 250 (μmol min^{-1} mg^{-1} in 0.4 M NaCl at pH 8.5 and 25°). Intestinal membranes were stored at −80° in a 10 mM Tris-Cl buffer at pH 7.30 containing 0.17 M NaCl and 10 mM Mg^{2+}. [^{32}P]AMP and [^{32}P]orthophosphate were obtained from the Commissariat à l'Energie Atomique. All other reagents were of the highest grade commercially available.

(b) *Methods.* Phosphatase concentrations were determined from absorbance at 278 nm, $A_{1\,cm}^{0.1\%} = 0.76$ (Fosset *et al.*, (1974).

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The hydrolysis of *p*-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate was followed at 410 nm with a Gilford 2400 spectrophotometer equipped with a thermostated cell holder. Rates of hydrolysis of other phosphates were measured by the release of inorganic phosphate (Fiske and Subbarow, 1925). Michaelis constants (K_m) and maximal rates (V_m) for the hydrolysis of *p*-nitrophenyl phosphate or 2,4-dinitrophenyl phosphate were obtained from Lineweaver–Burk plots. K_m values for other phosphates were determined by competition with *p*-nitrophenyl phosphate (Lazdunski and Lazdunski, 1969).

Covalent phosphorylation of the active site of calf intestine alkaline phosphatase was measured as previously described for *E. coli* alkaline phosphatase (Lazdunski *et al.*, 1969, 1971). Isolation of the phosphophosphatase involved quenching of the derivative at very acidic pH. It is obviously important to be sure that in such a procedure neither phosphorylation nor dephosphorylation occurs during quenching. Dephosphorylation cannot occur since acidic pH conditions inhibit this type of transformation. As in the case of *Escherichia coli* enzyme no covalent phosphorylation occurred during quenching: both addition of intestinal 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. Furthermore no covalent phosphorylation occurs by quenching of the noncovalent orthophosphate–phosphatase complex (Figure 6 and Lazdunski *et al.*, 1969). Finally the covalent phosphorylation data found for the Zn^{2+}, Co^{2+}, Cd^{2+}, and Mn^{2+} phosphatases of *E. coli* using the quenching procedure have been fully confirmed by the stopped-flow analysis data of the presteady state at different pH (Lazdunski, 1973; D. Chappelet-Tordo, M. Iwatsubo, and M. Lazdunski, manuscript in preparation). It will be seen later in the text that an identical situation is found here.

Noncovalent binding of orthophosphate to the intestinal alkaline phosphatase was measured by the gel filtration procedure described by Hummel and Dryer (1962) under conditions where no substantial covalent phosphorylation occurs.

Gel filtration was performed on (10 × 1 cm) columns of Sephadex G-25. The columns were thoroughly washed with the appropriate buffer (10 mM Tris-Cl (pH 8.0)–0.4 M NaCl) and then equilibrated with the radioactive ligand solution in the same buffer (20–50 μCi/ml). Enzyme solutions (0.5 ml containing 0.17–2.5 mg of phosphatase) were prepared in the equilibration mixture. Fractions of 0.6 ml were collected and assayed for protein content and radioactivity (Packard liquid scintillator). Three fractions separated the excluded phosphatase from the ligand depletion trough.

Rapid kinetic measurements were carried out in a modified commercial stopped-flow apparatus (type Durrum–Gibson) equipped to measure variations in absorbance. The direct recording in absorbance was obtained by placing in the circuit, between the photomultiplier (EMI 9558 QA) and the oscilloscope (Tektronix 564 R), a logarithmic amplifier (Philbrick-Nexus Research 4551) which transforms the transmission values. Other modifications have been described elsewhere (Di Franco and Iwatsubo, 1972). Phosphatase-catalyzed hydrolysis of 2,4-dinitrophenyl phosphate was followed either at 360 nm (ϵ_M for dinitrophenolate anion, 1.45×10^4 M^{-1} cm^{-1}) or at 410 nm (ϵ_M for dinitrophenolate anion, 0.95×10^4 M^{-1} cm^{-1}).

Results

(A) *Catalytic Properties of the Free and Membrane-Bound Intestinal Alkaline Phosphatase.* Free intestinal alkaline phosphatase obeys Michaelis–Menten kinetics between pH 7.5 and pH 10.0 and over a very wide range of substrate concentrations as shown by the typical results presented in the insert of Figure 1.

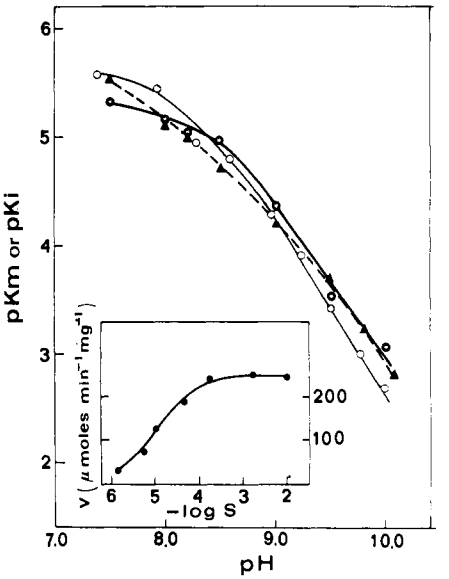

FIGURE 1: pK_m (or pK_I) *vs.* pH profiles at 25° in 0.4 M NaCl: (○) enzyme, free alkaline phosphatase; substrate, *p*-nitrophenyl phosphate; K_I, dissociation constant of the free phosphatase–inorganic phosphate complex; (◘) enzyme, free alkaline phosphatase; competitive inhibitor for *p*-nitrophenyl phosphate, inorganic phosphate; (▲) enzyme, ileum membrane-associated alkaline phosphatase; substrate, *p*-nitrophenyl phosphate. The following buffers (0.01 M) were employed from high to low pH: ethanolamine-Cl, Tris-Cl, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate-Cl, 3-(*N*-morpholino)ethanesulfonate-Cl. Under our experimental conditions, the nature of the buffer has no influence upon K_m or K_I values. Insert: substrate concentration dependence of the rate of hydrolysis of *p*-nitrophenyl phosphate by free intestinal phosphatase, 10 mM Tris-Cl (pH 8.5)–0.4 M NaCl, 25°. An excellent fit is observed between experimental points and the theoretical curve calculated from the classical Michaelis–Menten equation. $v = V_m[S]/(K_m + [S])$ with $K_m = 12$ μM, $V_m = 250$ μmol min^{-1} mg^{-1}.

The pH dependence of K_m is given in Figure 1 using *p*-nitrophenyl phosphate as a substrate. K_m values increased considerably from about 2.5 μM to about 2 mM between pH 7.5 and pH 10.0. The pH dependence of K_i, the true dissociation constant of the enzyme–inorganic phosphate complex, is also presented in Figure 1. The pK_i–pH profile for inorganic phosphate is very similar to the pK_m–pH profile observed for *p*-nitrophenyl phosphate. Very similar pK_m–pH profiles have been previously obtained for the *E. coli* alkaline phosphatase (Lazdunski and Lazdunski, 1966).

The pH dependence of the maximal rate for *p*-nitrophenyl phosphate hydrolysis is given in Figure 2. As has already been demonstrated in the past with partially purified intestinal phosphatase preparations, the highest V_m values are obtained at pH's higher than 10.5 (Fernley and Walker, 1967). Moreover, V_m–pH profiles are dependent upon ionic strength. Activation energies were determined at pH 6.0, 8.0, and 10.0. Arrhenius plots were found to be linear between 15 and 50°. The lowest activation energy, 6.7 kcal mol^{-1}, was obtained at pH 8.0; much higher values, 14.2 and 13.4 kcal mol^{-1}, were obtained at pH 6.0 and 10.0, respectively.

The specificity of the pure intestinal alkaline phosphatase was studied at pH 8.0 and 10.0 (Table I). Data obtained with *p*-chloroanilidophosphonate indicate that the enzyme hydrolyzes this P–N bond only very slowly (if at all) as compared to P–O bonds. Other phosphates, although chosen for their

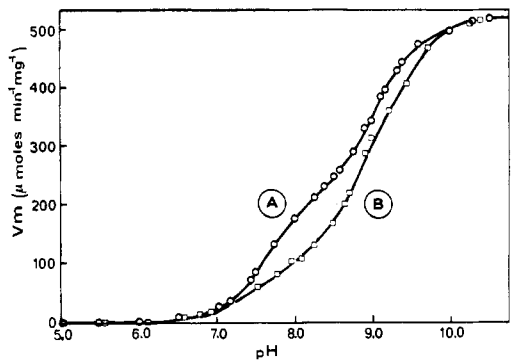

FIGURE 2: The effect of pH on the maximal rate V_m for the hydrolysis of *p*-nitrophenyl phosphate at 25°. Buffers contain 0.4 M NaCl (A) or 0.04 M NaCl (B). The chemical nature of buffers has no influence upon maximal rates.

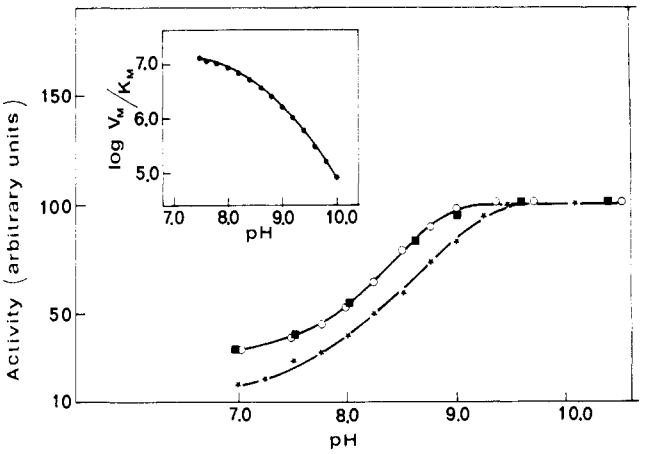

FIGURE 3: The effect of pH on the maximal rate, V_m, for the hydrolysis of *p*-nitrophenyl phosphate by membrane-bound intestinal alkaline phosphatase: (■) pig jejunum membrane; (○) pig ileum membrane; (★) pig duodenum membrane; 25°, 0.4 M NaCl. Same buffers as in Figure 1. Rates are given in arbitrary units. Insert: log V_m/K_m for ileum membrane-associated alkaline phosphatase. Alkaline phosphatase remains tightly associated with intestinal membranes between pH 7.0 and 10.5. No solubilization of the enzyme has been observed in this pH range

considerable differences in leaving group (*i.e.*, reactivity), are hydrolyzed at pH 8.0 at similar maximal rates (144 μmol min^{-1} mg^{-1} < V_m < 220 μmol min^{-1} mg^{-1}) in the presence of the enzyme. More important differences are observed at pH 10.0, the best substrates being AMP and phosphoenolpyruvate. Pyrophosphate is hydrolyzed only very slowly at very alkaline pH although the K_m value for this substrate is similar to the K_m values for AMP, phosphoenolpyruvate, and ATP. K_m values for the different substrates do not vary enormously at either pH 8.0 or 10.0.

The pK_m–pH profile for the membrane-bound phosphatase is nearly identical with that of the soluble enzyme (Figure 1). The V_m–pH profiles of pig membrane-bound alkaline phosphatase from duodenum, jejunum, and ileum membranes are presented in Figure 3; they are superimposable for the jejunum and ileum membranes and slightly different for the duodenum membranes. Nearly identical results were obtained with the membrane-bound calf intestine alkaline phosphatase. The variation of V_m with pH occurred over a range of 4 pH units (6.5–10.5) for the free enzyme; for the membrane-bound enzyme this pH range is limited to about 2.5 units (pH 7–9.5). Maximal V_m values are obtained near pH 9.5 for the membrane-bound enzyme instead of 10.5 for the free phosphatase.

Alkaline phosphatases from different sources are known to show phosphotransferase activity in the presence of nucleophiles (Fernley, 1971; Reid and Wilson, 1971). This property has been extensively studied in the case of *E. coli* alkaline phosphatase (Wilson *et al.*, 1964; Tait and Vallee, 1966; Lazdunski and Lazdunski, 1969).

Figure 4A indicates that the presence of Tris considerably increases the maximal activity of free alkaline phosphatase from calf intestine at pH 8.0. A more limited increase in activity is observed at pH 10.0. In contrast with Tris, ethanolamine did not affect the maximal activity at pH 8.0 and had an inhibitory effect at high concentrations, at pH 10.

The stimulation of the enzymatic activity by Tris persists both at pH 8.0 and 9.0 when alkaline phosphatase is in its natural environment, that is, bound to the intestinal membrane.

The ionic strength dependence of V_m at different pH's is presented in Figure 5. High ionic strength conditions increase the activity of the free phosphatase at pH 8.0 by a factor of about 2, whereas they appreciably decrease the activity of the membrane-bound enzyme. The sensitivity to changes of ionic strength is abolished at pH 10.0 for both forms of alkaline phosphatase.

TABLE I: Specificity of the Pure Intestinal Alkaline Phosphatase.

Substrates	pH 8.0[a]		pH 10.0[b]	
	V_m (μmol min^{-1} mg^{-1})	K_m (10^{-6} M)	V_m (μmol min^{-1} mg^{-1})	K_m (10^{-3} M)
p-Nitrophenyl phosphate	180	3.6	500	1.5
2,4-Dinitrophenyl phosphate	170	2.3	400	2
α-Naphthyl phosphate	220	2.7	770	0.9
β-Glycerophosphate	210	15	910	30
AMP	210	18	1130	21
ATP	165	16	550	30
Phosphoenolpyruvate	144	5.5	1190	19
Pyrophosphate	144	16	<10	30
Inorganic phosphate		7.2		1.9
p-Chloroanilidophosphonate	<5	230		

[a] 0.1 M Tris-Cl–0.4 M NaCl, 25°. [b] 0.16 M ethanolamine–0.4 M NaCl, 25°.

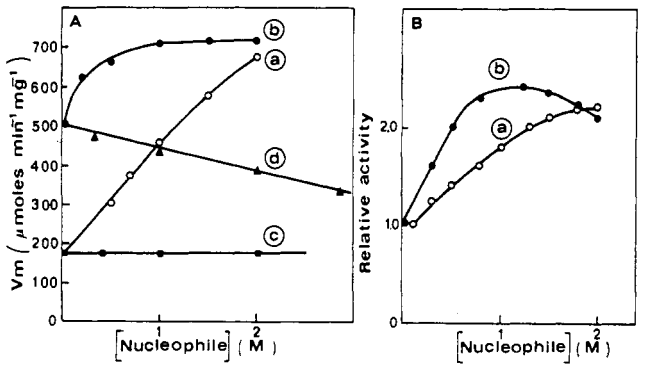

FIGURE 4: Influence of nucleophilic reagents on the maximal rate of hydrolysis of *p*-nitrophenyl phosphate by calf intestine alkaline phosphatase. (A) Free enzyme. Influence of increasing concentrations of Tris: (a) pH 8.0, 25°, ionic strength 1.5; (b) pH 10.0 (0.01 M ethanolamine-Cl buffer), 25°, ionic strength 1.5. Influence of increasing concentrations of ethanolamine: (c) pH <8.0, 25°, ionic strength 1.5; (d) pH 10.0, 25°, ionic strength 1.5. In all cases, ionic strength was adjusted with NaCl. (B) Membrane-bound enzyme. Influence of increasing concentrations of Tris: (a) pH 8.0, 25°, ionic strength 1.0, adjusted with NaCl. Activity is expressed relative to that observed in 0.1 M Tris-Cl buffer (pH 8.0). (b) pH 9.0, 25°, 0.1 M ethanolamine-Cl buffer, ionic strength 0.4. Activity is expressed relative to that observed in 0.1 M ethanolamine buffer (pH 9.0).

(B) *Properties of the Complexes Formed by Calf Intestine Alkaline Phosphatase with Organic and Inorganic Phosphates.* Previous work by Engström (1961, 1962) has shown that catalysis by intestinal alkaline phosphatase proceeded through the intermediate formation of a phosphoenzyme. Phosphorylation of the active site occurring both with ^{32}P inorganic phosphate, the reaction product, and with [^{32}P]glucose 6-phosphate, took place on a serine residue (Engström, 1959).

The simplest catalytic mechanism of the enzyme can be schematized as

$$E + ROPO_3^{2-} \rightleftharpoons E \cdots ROPO_3^{2-} \longrightarrow \begin{matrix} PO_3^{2-} \\ | \\ O \\ | \\ E \end{matrix} + ROH \longrightarrow E + PO_4H^{2-}$$

where E represents the active site; $E \cdots ROPO_3^{2-}$, the noncovalent complex formed with the substrate; $E\text{–}OPO_3^{2-}$, the enzyme phosphorylated on the essential serine residue.

Knowledge of the molecular weight and of the subunit structure of the intestinal alkaline phosphatase (Fosset *et al.*, 1974) now permits a detailed study of the titration properties of the active sites. Figure 6 shows the extent of covalent phosphorylation of phosphatase by saturating concentrations of [^{32}P]orthophosphate at different pH's. Phosphorylation of the active site by inorganic phosphate can be represented as

$$E + P_i \rightleftharpoons E \cdots P_i \rightleftharpoons E\text{–}OPO_3^{2-}$$

where P_i is the inorganic phosphate and $E \cdots P_i$ the noncovalent complex which is formed prior to phosphorylation of the active site.

The extent of covalent phosphorylation is higher at acidic than at alkaline pH. This result confirms previous data obtained by Engström (1962). Both active sites present in the dimer can be phosphorylated at pH's about 4. In contrast, only very low levels of [^{32}P]phosphate could be incorporated into the enzyme at pH's higher than 8. Acidic conditions, that is, conditions in which dephosphorylation of the enzyme is the rate-limiting step, favor the formation of $E\text{–}OPO_3^{2-}$. In contrast, alkaline conditions favor the formation of the noncomplex $E \cdots P_i$; phosphorylation is the slowest step in that case. Consequently noncovalent binding of inorganic phosphate can be conveniently studied about pH 8. It was shown in the previous paper (Fosset *et al.*, 1974) that the intestinal phosphatase remains in a dimeric state at pH 7.5 in the pres-

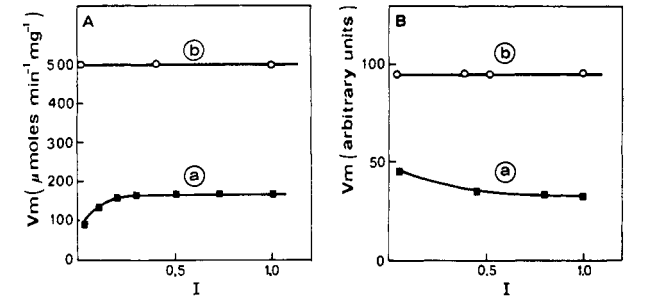

FIGURE 5: Influence of the ionic strength on the maximal rate of hydrolysis of *p*-nitrophenyl phosphate by calf intestine alkaline phosphatase. (A) Free enzyme: (a) pH 8.0, 0.1 M Tris-Cl buffer, 25°; (b) pH 10.0, 0.1 M ethanolamine-Cl buffer, 25°. (B) Membrane-bound enzyme: (a) pH 8.0, 0.1 M Tris-Cl buffer, 25°; (b) pH 9.0, 0.1 M ethanolamine-Cl buffer, 25°. The ionic strength was increased with NaCl.

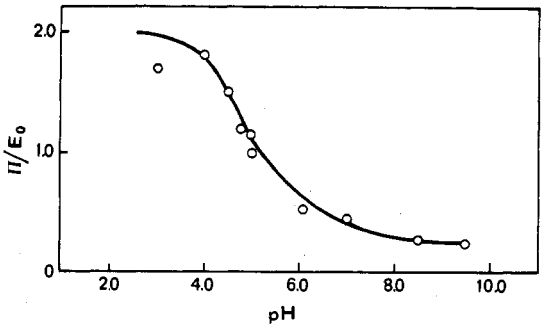

FIGURE 6: pH dependence of phosphorylation of the active centers of the calf intestine alkaline phosphatase with [^{32}P]orthophosphate at 2°. π/E_0 = moles of [^{32}P]phosphate covalently bound (π) per mole of enzyme (E_0). Enzyme concentration, 10 μM; orthophosphate concentration, 40 mM. The time dependence of the phosphorylation was followed at all pH values. Phosphorylation is complete after 0.5 min. No inactivation of the enzyme occurs during the incubation time between pH 4.0 and 10.0.

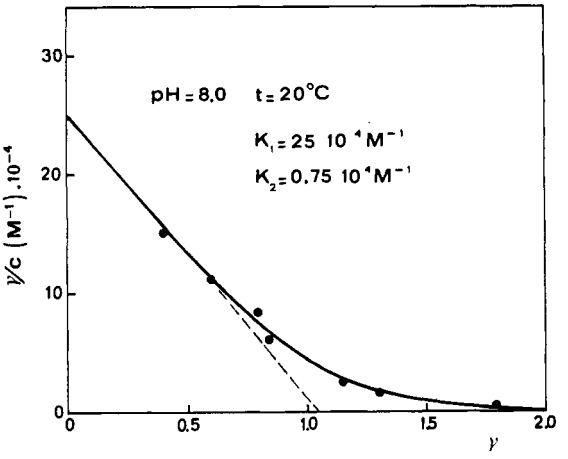

FIGURE 7: Scatchard plot for the binding of inorganic phosphate to the calf intestine alkaline phosphatase; ν, mole binding ratio orthophosphate/dimer; *c*, concentration of free phosphate; 25°, pH 8.0 (0.01 M Tris-Cl buffer), 0.4 M NaCl. [^{32}P]Orthophosphate concentrations were varied from 3 to 200 μM. The curve was calculated as indicated in the Results section.

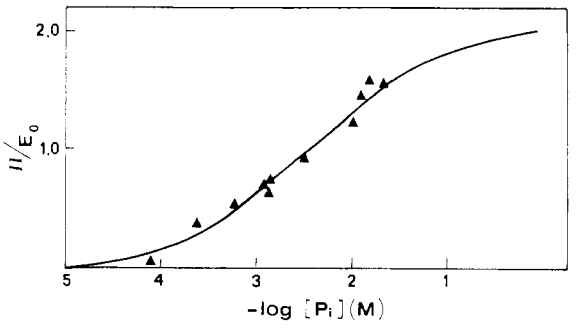

FIGURE 8: [^{32}P]Orthophosphate concentration dependence of the covalent phosphorylation of the calf intestine alkaline phosphatase at 2°, pH 4.0 (acetate buffer). Ionic strength, 0.05; enzyme concentration, 2.5–6.5 μM. π/E_0 = moles of [^{32}P]phosphate covalently bound (π) per mole of enzyme (E_0).

ence of high concentrations of inorganic phosphate. A Scatchard plot of results obtained by Hummel and Dryer's technique is shown in Figure 7. It indicates nonequivalence of the sites. The solid line which describes the experimental points was calculated assuming two sites with different apparent association constants K_1 and K_2 for inorganic phosphate

$$\nu = \frac{K_1c + 2K_1K_2c^2}{1 + K_1c + K_1K_2c^2}$$

where c is the concentration of free phosphate. A good fit between experimental and calculated data is obtained with the following values of K_1 and K_2: $K_1 = 25 \times 10^4$ M^{-1} and $K_2 = 0.75 \times 10^4$ M^{-1}.

Kinetic measurements (Figure 1) indicate a single association constant, $1/K_1 = 25 \times 10^4$ M^{-1}, for the formation of the phosphatase–inorganic phosphate complex at pH 8.0. This value is identical with K_1. Such a result shows that saturation of only one of the two sites, the "tight site," by inorganic phosphate suffices to inhibit competitively the catalysis of organic phosphates by the intestinal enzyme.

Nonequivalence of the active sites is also observed for the covalent phosphorylation of the intestinal phosphatase at acidic pH. The inorganic phosphate concentration dependence of the incorporation of [^{32}P]phosphate at pH 4.2 is presented in Figure 8. The calculated curve indicates two different association constants $K_1 = 0.5 \times 10^2$ M^{-1}, $K_2 = 15 \times 10^2$ M^{-1}. Using inorganic phosphate, we have dealt up to now

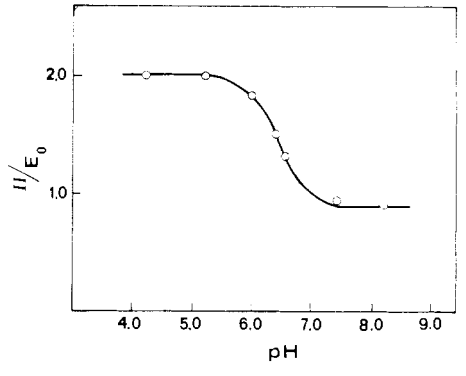

FIGURE 9: pH dependence of AMP-mediated phosphorylation of the active sites in calf intestine alkaline phosphatase under steady-state conditions. Maximal phosphorylation of the enzyme with [^{32}P]-AMP at 2° was obtained with a substrate concentration of 10 mM; enzyme concentration, 2 μM. π/E_0 = moles of [^{32}P]phosphate covalently bound per mole of enzyme. Neither noncovalent binding nor covalent phosphorylation could be observed with the intestinal phosphatase previously inactivated (apophosphatase) by EDTA.

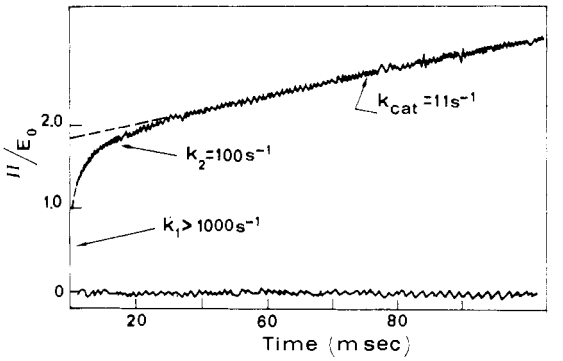

FIGURE 10: Stopped-flow oscilloscope tracing measuring absorbance changes related to 2,4-dinitrophenol appearance during enzymatic hydrolysis of 2,4-dinitrophenyl phosphate. [E_0] = 2.5 μM, [S_0] = 0.5 mM, pH 6.0, 10°. Catalyzed substrate hydrolysis was followed at 360 nm. Spontaneous hydrolysis of the substrate was negligible during measurements.

with equilibrium situations. Figure 9 presents the labeling results obtained after quenching the phosphorylated derivative of intestinal phosphatase formed under steady-state conditions with saturating concentrations of [^{32}P]AMP. Both sites of the enzyme were phosphorylated with high concentrations of substrate below pH 6.0 whereas only one of them could be covalently labeled in the alkaline pH range where the enzyme displays a higher activity. Incorporation of 2.0 covalent phosphates/mol of enzyme was also observed at pH 5.0 using [^{32}P]pyrophosphate (5 mM) instead of [^{32}P]AMP. The fact that only one of the two active sites can be phosphorylated at any instant at alkaline pH indicates that the intestinal alkaline phosphatase can be classified as a half-site enzyme (Lazdunski *et al.*, 1971; Levitzki *et al.*, 1971; Lazdunski, 1972, 1973).

Typical analysis of transient kinetics of the intestinal alkaline phosphatase at pH 6.0 and 8.0 are presented in Figures 10 and 11.

At pH 6.0, a burst of about 2 mol (π/E_0 = 1.85) of 2,4-dinitrophenol/mol of phosphatase is liberated during the pre-steady-state period of the catalyzed hydrolysis of 2,4-dinitrophenyl phosphate. This value is in excellent agreement with the results of Figure 10 which showed that both sites of the enzyme could be phosphorylated at high concentration of

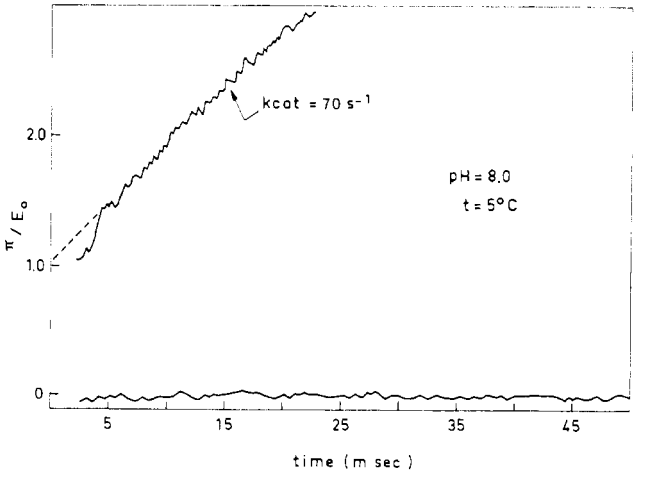

FIGURE 11: Stopped-flow oscilloscope tracing for phosphatase-catalyzed hydrolysis of 2,4-dinitrophenyl phosphate at pH 8.0, 5°. [E_0] = 1 μM, [S_0] = 1 mM. The reaction was followed at 410 nm. Spontaneous hydrolysis of the substrate was negligible during measurements.

AMP ($\pi/E_0 = 1.80$) at pH 6.0. Stopped-flow data also indicate that the sites are not equivalent at pH 6.0 where a biphasic pre-steady-state phase was observed (Figure 10). The "instant" burst of about 1 mol of dinitrophenol/mol of dimeric enzyme shows that the first site is phosphorylated very rapidly ($k_1 > 1000$ sec^{-1}). The second burst of dinitrophenol, which is of about 0.8 mol of dinitrophenol/mol of phosphatase, appears more slowly; it indicates that the second site is phosphorylated at a much slower rate ($k_2 = 100$ sec^{-1}). The value of k_{cat}, the first-order rate constant of the steady state, is one order of magnitude lower than the value of k_2, the first-order rate constant of the slower phosphorylation step in the pre-steady state.

At pH 8.0 a burst of only 1.0 mol of 2,4-dinitrophenol was observed in the transient phase; it was too fast to permit measurement of the rate constant for the phosphorylation step. The fact that only 1 mol of 2,4-dinitrophenol is liberated per mol of dimeric phosphatase, in the transient phase of 2,4-dinitrophenyl phosphate hydrolysis at pH 8.0, corroborates the phosphorylation results presented in Figure 9. It is a further indication of the half of the sites reactivity of the intestinal alkaline phosphatase at alkaline pH.

Table II gives burst values, at several pH, which are in good agreement with phosphorylation results obtained with AMP (Figure 9).

TABLE II:[a] Burst Values at Several pH's.

pH	Burst Size (π/E_0)	k_{cat} (sec^{-1})
4.2	2.0	0.13
5.1	2.0	0.7
6.0	1.7	11
7.1	1.0	48
8.1	0.9	80

[a] 1 mM 2,4-dinitrophenyl phosphate as substrate, 10°.

Discussion

The catalytic mechanism of *E. coli* alkaline phosphatase has been extensively studied in recent years (Coleman, 1971; Lazdunski *et al.*, 1971; Reid and Wilson, 1971; Halford *et al.*, 1972; Lazdunski, 1972, 1973). Therefore, it is of interest to start the discussion by a rapid comparison of the catalytic properties of the intestinal phosphatase with those of the bacterial enzyme. As shown in the previous paper, both phosphatases are dimers and contain the same amount of Zn^{2+}/mol of protein. However, molecular weights are very different and the intestinal enzyme, in contrast with the *E. coli* phosphatase, is a glycoprotein.

The most important catalytic difference between *E. coli* and intestinal phosphatases is the difference in maximal specific activity. At pH 10.5, 25°, the maximal specific activity of the intestinal alkaline phosphatase is 500 μmol min^{-1} mg^{-1} as compared to only 35 μmol min^{-1} mg^{-1} for the *E. coli* phosphatase. V_m *vs.* pH profiles are also different; the maximal activity (V_m) for the *E. coli* phosphatase reaches its highest value (a plateau) at pH 8.5 at 25° whereas maximal V_m is obtained at pH's higher than 10.5 with the eucaryotic phosphatase under the same conditions (Figure 2).

Other catalytic properties of the two enzymes are similar, including the lack of specificity at pH 8.0, the variation of K_m with pH, the activation of the enzymes at high ionic strength at pH 8.0, and the activation of the release of the alcohol product by nucleophiles at the same pH.

The *E. coli* phosphatase is located in the periplasm of the bacteria and is apparently not bound to the cell wall or to the cell membrane (Malamy and Horecker, 1964; Heppel, 1967; Brockman and Heppel, 1968). The *in vivo* catalytic properties of the enzyme have been studied with a constitutive mutant (*E. coli* CW 3747) and found to be nearly identical with the *in vitro* catalytic properties of the pure phosphatase (Lazdunski *et al.*, 1971). In contrast with the *E. coli* phosphatase, the intestinal phosphatase is a particulate enzyme. It is normally bound to the brush border membrane of the intestine. A number of reports in recent years (see, for example, Katchalski *et al.*, 1971) have claimed that catalytic properties of enzymes in the free state should be expected to be markedly different from those of enzyme associated with its membrane matrix. In fact, in the particular case of the intestinal phosphatase, no drastic difference has been observed between free and membrane-bound enzyme. Values of K_m at different pH's are nearly identical for duodenum, jejunum, and ileum membranes and for the free enzyme. Moreover, efficiency of catalysis is increased for both species of phosphatase (free and membrane bound) in the presence of 1 M Tris. The main differences concern the ionic strength effect and the V_m *vs.* pH profiles. Free intestinal phosphatase is markedly activated by high ionic strength at pH 8.0 whereas the membrane-bound phosphatase is slightly inhibited under the same conditions. The optimum V_m values of the free intestinal phosphatase are reached only at very alkaline pH (pH 10.5) as compared to the membrane-bound phosphatase (pH 9.5) (Figure 3). The pH range for maximal activity of the free phosphatase does not correspond at all to physiological pH values in the intestine. The pH of duodenal contents is below 6.5; it increases in the jejunum and reaches maximal values between 7.2 and 7.8 in the ileum (Fordtran and Ingelfinger, 1968). The activity of the membrane-bound phosphatase, in pH conditions corresponding to that of the ileum, is 40–50% the optimal activity which could be attained at pH higher than 10 (Figure 3). It should be remarked in passing that K_m values are minimal and V_m/K_m values maximal in the physiological pH zone of the ileum (Figure 3). Because of the low values of K_m below pH 8.0 ($K_m < 6 \times 10^{-6}$ *M*) and the usually high values of phosphate concentration in intestinal contents (Moog and Glazier, 1972), intestinal alkaline phosphatase most probably works in V_m conditions *in vivo*.

Identical phosphatase activities have been found at pH 8.0, 25°, using membrane vesicles and *p*-nitrophenyl phosphate or 2,4-dinitrophenyl phosphate as substrate. These substrates have very different leaving groups since the p*K*'s of *p*-nitrophenol and 2,4-dinitrophenol are 7.15 and 4.1, respectively. These results demonstrate that the membrane-bound alkaline phosphatase is also characterized by its lack of specificity.

The nonequivalence of the active sites of intestinal alkaline phosphatase has been demonstrated in this work in a number of ways. (a) One high and one low affinity site have been demonstrated for the noncovalent binding of inorganic phosphate at alkaline pH. (b) One high and one low affinity site have been also demonstrated for the covalent phosphorylation of the intestinal phosphatase by inorganic phosphate at acidic pH. (c) Only one of the two sites is phosphorylated at a given time under steady-state conditions at alkaline pH with AMP. (d) Half of the sites reactivity has also been demonstrated with 2,4-dinitrophenyl phosphate and *p*-nitrophenyl phosphate by stopped-flow techniques at alkaline pH. Moreover, analysis of

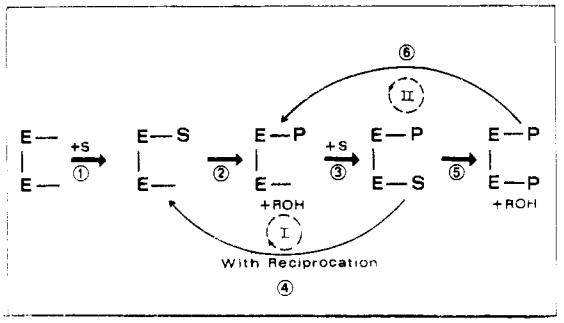

FIGURE 12: A plausible mechanism for the intestinal alkaline phosphatase. E represents a free active site; E–S indicates the noncovalent binding of the substrate; and E–P the covalent phosphorylation of the essential serine residue.

the presteady state at acidic pH has also clearly shown the nonequivalence of the active sites.

The nonequivalence of the sites for the intestinal alkaline phosphatase can be interpreted in two different ways. (a) In spite of the observed molecular weight identity of the subunits (Fosset *et al.*, 1974) they could be different in sequence and they could comprise active sites with different binding and turnover properties. (b) Subunits and active sites are identical but the mechanism of the enzyme is based on the characteristic property of negative cooperativity.

The analogy between the properties of intestinal alkaline phosphatase and *E. coli* alkaline phosphatase (Lazdunski *et al.*, 1971; Lazdunski, 1973) for which there is genetic evidence that the subunits are identical (Rothman and Byrne, 1963) is so strong that it practically rules out the possibility of nonidentical sites in the free enzyme. The second interpretation seems much more likely and will be adopted in the discussion which follows.

The mechanism to be proposed must reconcile two apparently antinomic properties, *i.e.*, negative cooperativity for both substrate binding and transformation which indicates subunit interaction in catalysis, and Michaelis–Menten kinetics. Because Michaelian kinetics are characteristic of monomeric enzyme it is often believed that observations of that type of behavior with oligomeric enzymes indicate functional independence of the subunits. This problem has already been discussed elsewhere (Lazdunski *et al.*, 1971, Lazdunski, 1972, 1973).

In the mechanism of the intestinal alkaline phosphatase coupling between subunits not only occurs at the level of the substrate-binding step but also during catalytic transformation of the substrate (phosphorylation and dephosphorylation of the active site). A mechanism which could explain "kinetic cooperativity" between subunits of the intestinal phosphatase as well as Michaelian kinetics is proposed in Figure 12.

In step 1, one substrate molecule binds to one of the subunits; this binding desensitizes (intersubunit negative cooperativity) the second subunit which is then unable to bind the second substrate molecule. The saturated subunit is phosphorylated in step 2. This phosphorylation induced an intersubunit conformational change which allows the binding of S to the second subunit in step 3 thus forming a hybrid complex with one subunit phosphorylated and the other noncovalently bound to S. Two choices are then offered to the enzyme. The first possibility is to couple substrate binding to the second site (step 3) and dephosphorylation of the first site (step 4). This is a typical flip–flop mechanism (Lazdunski *et al.*, 1971). The second possibility is to couple the phosphorylation of the second site (step 5) with the dephosphorylation of either one of the two sites (step 6) (a typical flip–flop in that case would couple the phosphorylation of the second site with the dephosphorylation of the first one but this is not a necessary property of the mechanism). Such a mechanism would explain both the negative cooperativity for noncovalent binding and for the covalent phosphorylation. It requires the intermediate formation of P–E–E–S. That such a hybrid complex is formed has not been demonstrated in the case of the intestinal alkaline phosphatase but has been clearly established for the *E. coli* phosphatase (Petitclerc *et al.*, 1970; Lazdunski *et al.*, 1971). The characteristic of such mechanisms is that the enzyme never comes back to the free form –E–E–. Some steps which appear in the transient phase will never appear in the steady state. In mechanism I, step 1 will appear only in the transient state and not in the steady state. The steady-state cycle includes only steps 2, 3, and 4. The substrate is introduced in step 3 in the steady-state cycle. Despite the requirement for tight coupling between subunits, the kinetics will be of the Michaelian type (Lazdunski *et al.*, 1971; Lazdunski, 1972).

In mechanism II, steps 1 and 2 will appear only in the transient phase, whereas steps 3, 5, and 6 will appear in the steady state. Again, "kinetic cooperativity" between the 2 active sites is necessary but since the substrate is introduced in only one step in the steady-state cycle (in step 3) again kinetics will be of the Michaelian type.

We have shown previously that at alkaline pH only one of the two sites is phosphorylated under steady-state conditions; that means that the other site is either completely free or noncovalently saturated by S. Therefore two different complexes could accumulate under these conditions, P–E–E– or P–E–E–S. Accumulation of P–E–E– would mean that step 3 is the rate-liniting step both for mechanism I or for mechanism II. Accumulation of P–E–E–S would mean that, in mechanisms I and II, steps 4 and 5, respectively, are the rate-limiting steps. It should be realized that the mechanisms proposed in Figure 12 are oversimplified mechanisms. All steps which appear in these mechanisms include conformational changes. A conformational change is necessary in step 1 to permit negative cooperativity.[1] A conformational change of the whole enzyme molecule is necessary in step 2 to recover the capacity of substrate binding to the second active site in step 3. A conformational change also occurs in step 3 which permits "activation" of the phosphorylated active site so that it can dephosphorylate in step 4. Since coupling of the subunits in catalysis is a necessary property of the proposed mechanism, conformational changes are likely to occur also in steps 4, 5, and 6. In step 5 the asymmetric species P–E–E–S will give an unstable symmetric complex P–E–E–P; the conformational change is necessary in that case to "activate" one of the phosphorylated sites so that it can dephosphorylate and exclude inorganic phosphate in step 6. The conformational change in step 6 corresponds to the transformation of a symmetrical complex P–E–E–P into an asymmetrical complex P–E–E–. A conformational change will also occur in step 4 if the geometry of the phosphorylated active site is different from the geometry of the free active site in S–E–E–.

If step 3 is limiting in mechanism I or II that means that the first-order conformational change triggered by substrate binding to the second subunit is the slowest step in the steady-state cycle. This interpretation would give an easy explanation for the lack of specificity of the enzyme if the conformational

[1] This conformational change might occur by displacement of a preequilibrium between a symmetrical and an asymmetrical form of the free enzyme or by isomerization of the 1:1 enzyme–substrate complex.

changes induced by substrate binding were not dependent upon the nature of the leaving group in the organic phosphate. If step 5 is limiting in mechanism II, this step, which includes a phosphorylation and a conformational change, would have to be controlled in rate by the conformational change. The rate of the conformational change, but not the phosphorylation of the serine residue in the active site, could be quite insensitive to the nature of the leaving group (Trentham and Gutfreund, 1968). No problem of interpretation arises if step 4 is limiting in mechanism I. Step 4 probably comprises a dephosphorylation and a conformational change. Dephosphorylation of the active site will obviously be insensitive to the chemical nature of the leaving group, and the conformational change could again occur at the same rate with very different substrates. Conformational changes which occur in the mechanism of *E. coli* alkaline phosphatase action have been demonstrated in several laboratories (Simpson and Vallee, 1968; Halford *et al.*, 1969; Coleman, 1971; Halford, 1971; Lazdunski *et al.*, 1971). They appear to involve a reorganization of the chelate structure of the active site (Lazdunski, 1973).

The enzyme derivative which accumulates at alkaline pH with inorganic phosphate is practically unphosphorylated. The major complex formed under these conditions is then S–E–E–. Therefore the rate-limiting step for the "hydrolysis" of inorganic phosphate at alkaline pH is step 2.

At acidic pH, the complex which accumulates is P–E–E–P. Step 6 is rate limiting both for inorganic and organic phosphates.

A number of other enzymes with very different catalytic functions are now known to display half of the sites reactivity.

Those that have been studied most carefully, aside from *E. coli* alkaline phosphatase, include alcohol dehydrogenase, malate dehydrogenase muscle glyceraldehyde 3-phosphate dehydrogenase, CTP synthetase, and glutamine synthetase.

An analysis of the literature concerning half-site enzymes and a discussion of flip–flop mechanisms and subunit interactions in catalysis can be found in recent reviews (Lazdunski, 1972, 1973).

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