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Intestinal Alkaline Phosphatase. Physical Properties and Quaternary Structure†

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ABSTRACT: Calf intestine alkaline phosphatase prepared in pure form is homogeneous by the criteria of disc gel electrophoresis and sedimentation velocity ultracentrifugation. It is a glycoprotein of 140,000 molecular weight with an absorbancy index, $A^{1\%}_{278}$, of 7.6, and a specific volume, $\bar{V}$, of 0.756. Analysis of the subunit structure in 5–6 M guanidine hydrochloride by column chromatography and sedimentation equilibrium and in sodium dodecyl sulfate gel electrophoresis indicates that the alkaline phosphatase is a dimer comprising two very similar or identical subunits of 69,000 molecular weight. The enzyme contains 4 g-atoms of zinc/mole of protein. This paper also presents data concerning amino acid composition, sugar content, and enzyme stability.

Although considerable work has been devoted recently to the molecular properties, subunit composition, and to the catalytic mechanism of *Escherichia coli* alkaline phosphatase, much less has been done with alkaline phosphatases from mammalian cells. A number of these enzymes (*e.g.*, from intestinal mucosa, kidney, liver, placenta, milk, bone, etc.) have been extensively studied (Fernley, 1971) but most data were obtained with partially purified enzyme preparations.

Pioneer work concerning the purification and the determination of the molecular properties of the calf intestine phosphatase has been carried out by Portmann and Engström. These studies (Portmann, 1957; Engström, 1961) gave an approximate value of the molecular weight of the enzyme (about 100,000), but information concerning the subunit structure was still lacking. We describe in this paper the main molecular properties of this enzyme. The next paper (Chappelet-Tordo *et al.*, 1974) will be devoted to the analysis of its mechanism of action.

Materials and Methods

Calf intestine alkaline phosphatase, degree of purity I, was purchased from Boehringer Mannheim. Guanidine hydrochloride was purchased in its "ultra pure form" from Heico. All other reagents were of the highest grade commercially available; [^{32}P]orthophosphate was a product of The Commissariat à l'Energie Atomique, Département des Radioéléments.

The purification technique is an adaptation of the method previously described by one of the authors (Lazdunski and Ouellet, 1961) from partially purified commercial preparations. The alkaline phosphatase obtained from Boehringer was dialyzed overnight against Tris-Cl buffer (0.05 M) at pH 8.6 and applied to a column (18 × 1 cm) of DEAE-cellulose (Type 20, Carl Schleicher and Schuell Co.), equilibrated with the same buffer. The molarity of the elution buffer is then increased stepwise and the alkaline phosphatase is eluted from the column with a Tris-Cl solution 0.1 M at pH 8.6. A typical elution diagram is presented in Figure 1.

Protein concentrations were routinely determined from absorbance measurements at 278 nm. $A^{1\%}_{1\,cm}$ = 7.6 was obtained from different estimates: by dry-weight determination and by the refractive index increment method described by Babul and Stellwagen (1968). For this last technique, the ultracentrifuge with interference optics and a double-sector synthetic-boundary cell at a rotor speed of 4000 rpm was used. Calculations were based on a fringe number of 4.05 for a protein solution of 1 mg/ml (Babul and Stellwagen, 1968).

Kinetic Measurements. The hydrolysis of *p*-nitrophenyl phosphate was followed spectrophotometrically at 410 nm with a Gilford 2400 apparatus equipped with a thermostated cell holder as previously described (Lazdunski and Lazdunski, 1966).

Sedimentation Experiments. Sedimentation velocity and equilibrium experiments were performed in a Spinco Model E ultracentrifuge equipped with a titanium AN-H rotor and double sector cells.

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All runs were made at 20° and the observed sedimentation coefficients were corrected to values corresponding to water at 20° and zero concentration ($s_{20,w}$). High-speed equilibrium–sedimentation runs were performed by the procedure of Yphantis (1964) using a 3-mm column.

The partial specific volume for the native enzyme at 20° was taken from measurements with a digital precision densitometer (DMA O2C Anton Paar K.G. Graz Austria) giving reproducible results for different concentrations of protein taken between 0.9 and 2 mg/ml.

For experiments in 5 M guanidine hydrochloride the protein (0.7 mg/ml) was dialyzed 96 hr at 4° against a 50 mM Tris-Cl buffer at pH 7.50 containing 15 mM 2-mercaptoethanol and 5 M guanidine hydrochloride with one change of the equilibration buffer at 48 hr. The density of the buffer, 1.1229, was measured with the precision densitometer.

Gel Filtration Experiments. Gel Filtration of the Native Enzyme. Estimation of molecular weight of the native alkaline phosphatase was carried out according to Andrews (1965).

A column (100 × 1.4 cm) was filled with Sephadex G-150 equilibrated and eluted with 10 mM Tris-Cl (pH 7.50)–0.4 M NaCl. The column was run at 20° and 0.9-ml fractions were collected. Proteins for calibration (about 3 mg) were separately chromatographed with Blue Dextran dye marker. Elution volumes of proteins were determined by absorbance at 280 nm.

Gel Filtration with 6 M Guanidine Hydrochloride. The method of Fish *et al.* (1969) was used for the estimation of the molecular weight of calf intestine alkaline phosphatase by gel filtration in 6 M guanidine hydrochloride. Dextran Blue was used to measure the void volume, V_0, and DNP-alanine to measure the interstitial volume V_i. A Sepharose 4B column (Pharmacia) (100 × 1 cm) was equilibrated and eluted with pH 8.0, 10 mM Tris-Cl containing 6 M guanidine-HCl. The column was operated by ascending filtration, with a flow rate of 6 ml/hr. The standard proteins for calibration and the ^{32}P-labeled phosphatase obtained as described below were first dialyzed for 12 hr against the same buffer containing 2% 2-mercaptoethanol.

In a typical run, 1 ml containing 2 mg of one reference protein or 0.5 mg of ^{32}P-labeled phosphatase with Dextran Blue and DNP-alanine was passed through the column and elution positions were determined by optical density at 280 nm for the standards and by radioactivity count for the ^{32}P-labeled phosphatase. The logarithm of the molecular weights were plotted against $K_d = (V_e - V_0)/(V_i - V_0)$ (V_e is the elution volume of the protein).

Labeling of the enzyme with [^{32}P]orthophosphate was carried out as previously described for the *E. coli* alkaline phosphatase (Lazdunski *et al.*, 1969) by mixing the calf intestine phosphatase (0.5 mg) with 0.1 M [^{32}P]orthophosphate for 20 sec in acetate buffer 0.2 M at pH 4.0. The phosphoprotein was then rapidly denatured and precipitated with concentrated HCl. The precipitate was dissolved by adding 0.8 ml of 10 mM Tris-Cl buffer (pH 8.0) containing 6 M guanidine hydrochloride and 2% 2-mercaptoethanol. After pH adjustment to 8.0 the sample was applied to the Sepharose 4B column.

Disc Gel Electrophoresis in 0.1% Sodium Dodecyl Sulfate. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Weber and Osborn (1969) with minor modifications.

Proteins reduced and carboxymethylated using standard techniques (Hirs, 1957) were prepared for electrophoresis by denaturation in 0.01 M sodium phosphate (pH 7.2) containing 1% sodium dodecyl sulfate with or without 1% 2-mercaptoethanol. Samples were incubated overnight at 25° and then processed as follows: 5–10 μg of each protein was applied to 5% polyacrylamide gels. Electrophoresis runs (8 mA/tube) lasted 4 hr at room temperature in 0.1 M sodium phosphate (pH 7.2)–0.1% sodium dodecyl sulfate.

Amino Acid Analysis. Amino acid compositions were determined by the procedure of Moore and Stein (1963) using

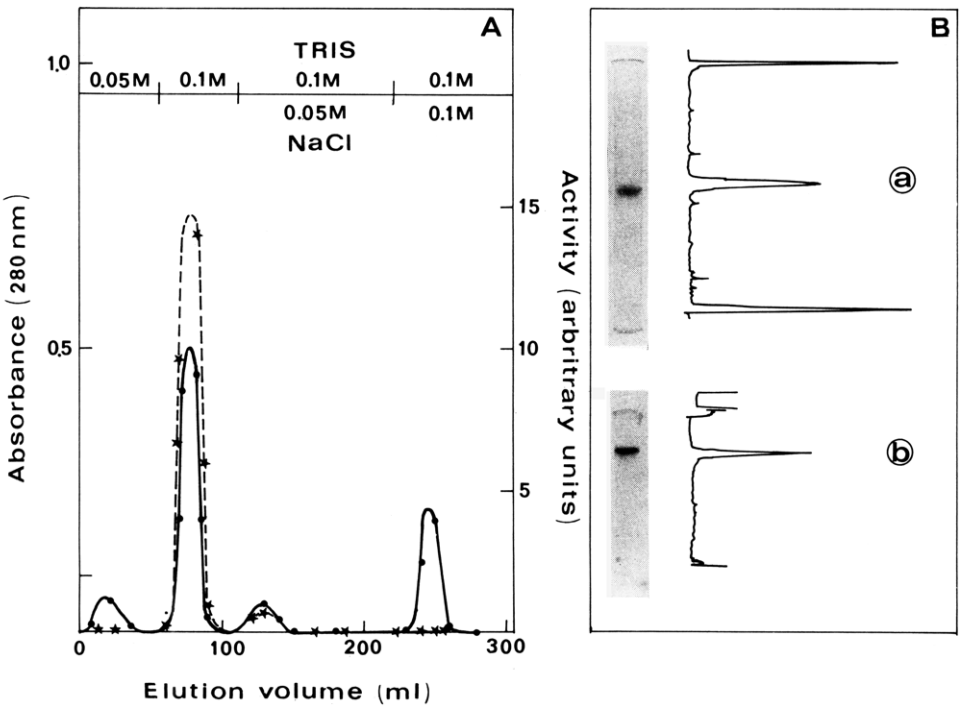

FIGURE 1: (A) Purification of the intestinal alkaline phosphatase on DEAE-cellulose. Enzymatic activity and absorbance profiles are represented by dotted and solid lines, respectively. (B) Gel electrophoresis and densitometric scanning profiles. Migration is from top to bottom. (a) Sodium dodecyl sulfate (0.1%) gel electrophoresis of the enzyme. The proteins are stained with Coomassie Blue. (b) Polyacrylamide gel electrophoresis of the native intestinal alkaline phosphatase. The proteins are stained with Amido Black.

TABLE I: Physical Properties of Calf Intestine Alkaline Phosphatase (t, 20°).

State of Protein	pH	$s_{20,w}$ (S)	$\bar{V}$	Mol Wt	$A^{1\%}_{278\,nm}$	A_{278}/A_{260}
Native	8.0	6.45	0.756	140,000[a]	7.6	1.87
	7.5			138,000[b]		
				138,000[c]		
Subunits in acidic medium	2.0	2.0				
Subunits in 5–6 M guanidine hydrochloride	7.5			60,000[a]		
	8.0			66,000[b]		
Subunits in 0.1% sodium dodecyl sulfate	7.2			69,000[d]		

[a] Sedimentation equilibrium, $\bar{V}$ taken as 0.756 ml/g. [b] Gel chromatography. [c] Amino acid analysis. [d] Disc gel electrophoresis.

norleucine as an internal standard (Walsh and Brown, 1962). Hydrolyses were performed *in vacuo* at 105 ± 1° for periods of 24, 48, and 72 hr. Hydrolyzed samples were analyzed on a Beckman Model 120 C automatic amino acid analyzer.

Half-cystine was determined as cysteic acid following performic acid oxidation as described by Moore (1963). Sulfhydryl groups were determined either by the procedure of Ellman (1959) on 6 M guanidine hydrochloride denatured enzyme, or by carboxymethylation with [^{14}C]iodoacetic acid. Tryptophan was determined by spectrophotometric methods as described by Bencze and Schmid (1957) or Spies and Chambers (1967). Neutral sugars were assayed by the phenol–sulfuric acid procedure of Dubois *et al.* (1965). Amino sugars were determined on the amino acid analyzer.

Optical rotatory dispersion measurements were carried out at 20° with a Fica 1 spectropol spectropolarimeter using cells of 10-mm path length. Optical rotation data were expressed as $[m]_\lambda$, the mean residue rotation in deg cm^2 dmol^{-1}. A mean residue molecular weight of 114 was used in these calculations.

Inactivation by EDTA. The enzyme was incubated (0.06 mg/ml) at pH 8.0 in 0.1 M Tris-Cl buffer at 25° with 2–10 mM EDTA. At various times, aliquots were assayed for activity at pH 8.50 in a Tris-Cl buffer containing 0.4 M NaCl and 1 mM EDTA to prevent reactivation of the enzyme. The presence of the substrate provided the necessary protection against further EDTA inactivation during activity measurement.

Zinc Content. In a first set of determinations, the zinc content was evaluated directly on the purified enzyme. In another set of experiments, the purified enzyme was first incubated in 0.1 M Tris-Cl buffer (pH 8.0) containing 1 mM $ZnCl_2$. Zinc in excess was removed by chromatography on a Sephadex G-25 column equilibrated with zinc-free buffer as described for the *E. coli* alkaline phosphatase (Lazdunski and Lazdunski, 1969). Quantitative determinations of zinc content were made by atomic absorption spectrophotometry with a Eel atomic absorption spectrophotometer at an excitation wavelength of 278 nm.

Results

Figure 1 shows evidence obtained by disc electrophoresis analyses that the intestinal alkaline phosphatase with which the work was carried out is a pure enzyme.

Some of the physicochemical properties of the enzyme are presented in Table I.

The molecular weight of the native enzyme was determined by sedimentation equilibrium studies using the meniscus depletion method of Yphantis (1964). The absence of curvature in the plots of log f *vs.* x^2 indicates that the alkaline phosphatase is homogeneous with respect to molecular weight. The average molecular weight of the native enzyme obtained from four independent determinations at concentrations of enzyme from 0.5 to 0.8 mg/ml was found to be 140,000.

This result is in good agreement with the value of 138,000 determined by Sephadex G-150 chromatography. An identical elution pattern was obtained when the enzyme was passed on the same column equilibrated with 10 mM Tris-Cl–0.4 M NaCl–1 mM orthophosphate at pH 7.5.

Under these conditions the alkaline phosphatase appears in the form of the enzyme–orthophosphate complex (Chappelet-Tordo *et al.*, 1974). This experiment shows that no dissociation of the alkaline phosphatase occurs in the presence of substrates.

Subunit Structure of the Calf Intestine Alkaline Phosphatase. Three different techniques—sedimentation equilibrium, gel filtration in concentrated guanidine hydrochloride solutions, and disc gel electrophoresis in sodium dodecyl sulfate—were used to determine the presence and size of subunits for

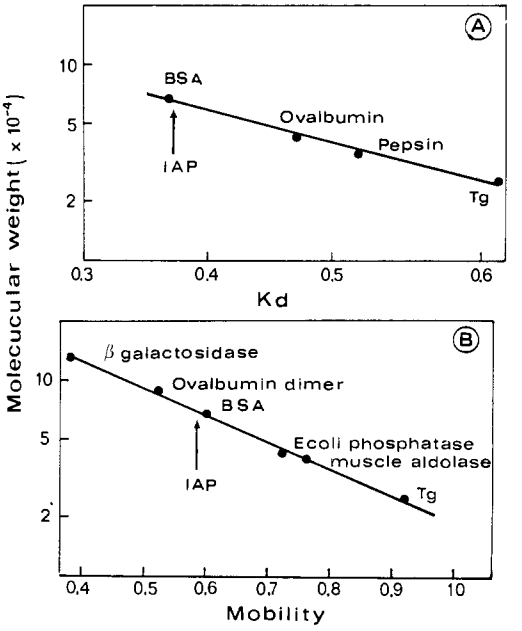

FIGURE 2: Determination of the molecular weight of the subunits of the intestinal alkaline phosphatase: (A) calibration curve for gel filtration in 6 M guanidine hydrochloride; (B) calibration curve for 5% polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The distribution coefficient K_d and the electrophoretic mobilities are plotted against the logarithm of molecular weights.

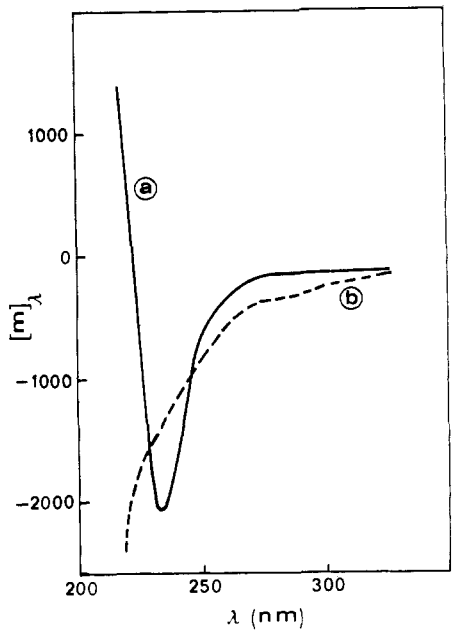

FIGURE 3: Optical rotary dispersion spectra of intestinal alkaline phosphatase: (a) enzyme concentration 0.1 mg/ml, pH 7.6, 20°; (b) 0.1 mg/ml of phosphatase in 10 mM Tris-Cl–5.4 M guanidine-HCl; pH 7.6, 20°. Spectra were measured 60 min after dilution of the enzyme in guanidine-HCl and remained unchanged after 24 hr.

the calf intestine phosphatase. Acrylamide gel electrophoresis of the intestinal alkaline phosphatase in the presence of sodium dodecyl sulfate gave a single band (Figure 1). The migration of this band corresponded to a molecular weight of 69,000 (Figure 2B). This value strongly suggests that the enzyme is a dimer made up of two identical or extremely similar subunits.

TABLE II: Amino Acid Composition of the Calf Intestine Alkaline Phosphatase.

Amino Acid	No. of Residues (moles/125,000 g of amino acids; nearest integer)
Lysine	53
Histidine	32
Arginine	102
Aspartic acid	128
Threonine	75
Serine	47
Glutamic acid	103
Proline	61
Glycine	90
Alanine	120
Half-cystine[a]	10
Valine	88
Methionine	25
Isoleucine	30
Leucine	74
Tyrosine	41
Phenylalanine	31
Tryptophan[b]	13
Total residues	1123
% hexosamines (w/w)	6
% neutral sugars (w/w)	6

[a] Determined as cysteic acid. [b] Determined from ultraviolet spectra and the tyrosine value as described under Methods.

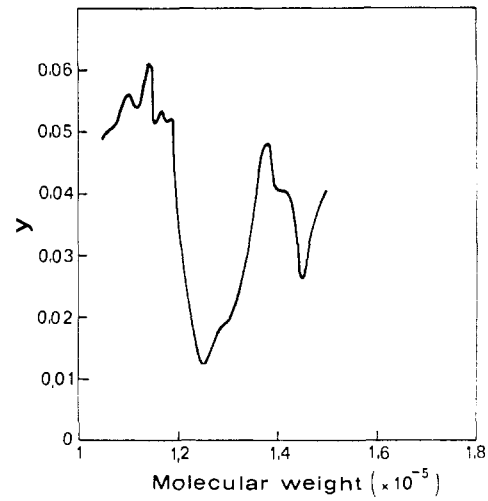

FIGURE 4: Curve of $y = \Sigma[(n_i/N_i) - 1]^2$ (Delaage, 1968). The analysis of x μmoles of a protein sample gave the number of μmoles x_i of amino acid i; $n_i = x_i/x$. N_i represents the number of amino acid residues of type i in 1 mol of protein. The correct value of x, that is of n_i, is that which gives a minimum for y. Therefore, the minimum in the curve represents the most probable value of the molecular weight.

The molecular weight of the subunit obtained from gel filtration in the presence of 6 M guanidine hydrochloride is 66,000 (Figure 2A); it is in good agreement with the gel electrophoresis determination.

Sedimentation equilibrium data for the alkaline phosphatase in guanidine hydrochloride give a linear plot of log f *vs.* x^2 with a slope different from that obtained with the native enzyme. It indicates again that there are subunits and that they have an identical or very similar molecular weight. This molecular weight was determined to be 60,000. This low value as compared to molecular weights of 66,000–69,000 obtained by other techniques is probably due to uncertainties concerning the exact partial specific volume of the protein in guanidine hydrochloride. In fact, calculations were carried out assuming identical partial specific volumes in guanidine hydrochloride solution and in water (Ullmann *et al.*, 1968).

Figure 3 presents the optical rotatory dispersion characteristics of the native enzyme and of subunits obtained by exposure to concentrated solutions of guanidine hydrochloride: the Cotton trough at 233 nm was abolished in the denaturing medium as previously observed for the guanidine hydrochloride mediated dissociation of *E. coli* alkaline phosphatase (Reynolds and Schlesinger, 1967).

Amino Acid, Carbohydrate, and Metal Composition. The amino acid composition of the intestinal alkaline phosphatase is presented in Table II. Treatment of the denatured enzyme in 6 M guanidine hydrochloride with [^{14}C]iodoacetic acid or the Ellman reagent shows the presence of two free sulfhydryl groups per dimer; the content of half-cystines (Table II) indicates therefore that there are four disulfide bridges per mole of protein. In the native enzyme no SH group is available to iodoacetic acid or the Ellman reagent.

The intestinal alkaline phosphatase is known to be a glycoprotein (Engström, 1961). The purified enzyme does indeed contain neutral and amino sugars. Carbohydrates comprise approximately 12% of the total weight of the protein

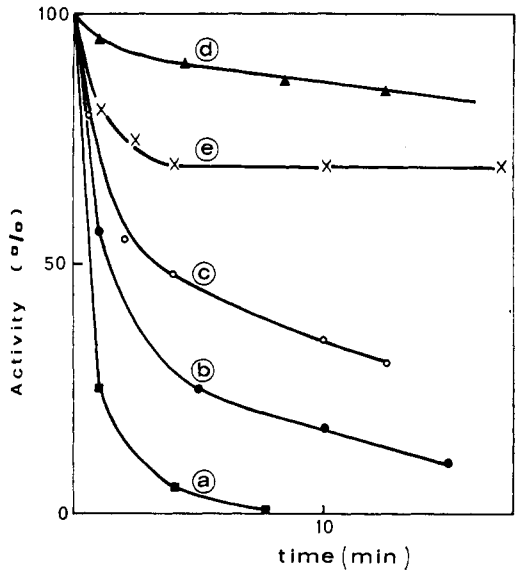

FIGURE 5: pH inactivation of intestinal alkaline phosphatase. The enzyme was incubated (0.02 mg/ml) in 0.1 M solution of the appropriate buffer: (a) pH 3.5, (b) pH 4.0, (c) pH 5.0, (d) pH 5.5, (e) pH 5.0 in the presence of 1 mM inorganic phosphate. At various times aliquots were assayed for activity at pH 9.5 in 0.1 M ethanolamine–Cl buffer containing 0.4 M NaCl. The half-life of the enzyme at pH 5.0 increased from 2.5 to 7.7 min when the temperature was changed from 35 to 15°.

(Table II). No sialic acid could be detected either in previous purified preparations (Engström, 1961) or in phosphatase-containing brush-border membranes of the jejunum (Louvard *et al.*, 1973).

The theoretical analysis of the amino acid composition according to Delaage (1968) is presented in Figure 4; it indicates a molecular weight of 125,000; correction for carbohydrate content gives a value of 138,000, in excellent agreement with gel filtration and sedimentation experiments. Table I summarizes the different estimates of the molecular weight of the intestinal alkaline phosphatase.

The intestinal alkaline phosphatase is known to be a zinc-containing enzyme (Engström, 1961; Fernley, 1971). We have shown in this work that it comprises 4 ± 0.2 g-atoms of zinc/mole of protein of 140,000 molecular weight. It is inactivated by EDTA as was previously observed with partially purified samples (Hofstee, 1955, Morton, 1957). The time course of inactivation follows first-order kinetics (k_{des} = 5 M^{-1} sec^{-1} at pH 8.0, 25°) at high concentrations of EDTA (2–10 mM) and low concentrations of enzyme (0.06 mg/ml), as was previously observed with the alkaline phosphatase of *E. coli* (Lazdunski *et al.*, 1969). Removal of the essential Zn^{2+} ions from the native enzyme does not unmask the two buried –SH groups for reaction with 5,5′-dithiobis(2-nitrobenzoate).

Enzyme Stability. The purified calf intestine alkaline phosphatase was found to retain full activity for at least 2 months during storage at pH 8.0 and 4° at a protein concentration of 1 mg/ml and in a 0.1 M Tris-Cl buffer containing 0.1 M NaCl.

The purified enzyme was found to have greatest stability in the pH range 7.5–9.5. It is inactivated rapidly at acidic pH. Protection against acidic denaturation is observed in the presence of inorganic phosphate. This is shown in Figure 5.

The mechanism of inactivation appears to be similar to that of the alkaline phosphatase of *E. coli* at acidic pH which has been extensively studied (Schlesinger, 1965; Lazdunski and Lazdunski, 1966).

Conclusion

The intestinal alkaline phosphatase is a dimeric glycoprotein of 138,000–140,000 molecular weight. The enzyme is formed of two very similar or identical monomers. It comprises two buried cysteines/mole of dimer. It is a zinc-containing enzyme with 4 g-atoms of the metal/mole of protein. These properties can be compared with those of the *E. coli* enzyme, which was the most thoroughly studied alkaline phosphatase. The latter phosphatase does not contain sugars or free –SH groups and has a molecular weight of only 86,000 (Schlesinger, 1965). However, it is also a dimeric protein (Rothman and Byrne, 1963), with 4 g-atoms of Zn^{2+}/mole of enzyme (Simpson and Vallee, 1968). Stability properties in EDTA solutions and at acidic pH are also extremely similar.

The molecular weight of the intestinal alkaline phosphatase is similar to that of the alkaline phosphatase of human placenta (mol wt, 125,000) which is also a dimeric enzyme (Gottlieb and Sussman, 1968; Harkness, 1968).

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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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