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Twentyfold Increase in Alkaline Phosphatase Activity by Sequential Reversible Activation of the Enzyme Followed by Coupling with a Copolymer of Ethylene and Maleic Anhydride

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ABSTRACT

Alkaline phosphatase, APase, (EC 3.1.31) from calf intestine, after shifting the equilibrium by effector molecules towards the dimeric form of the enzyme, was coupled (ratio 1:2, protein: copolymer) to a copolymer of ethylene and maleic anhydride, EMA. The water-soluble APase–EMA was separated from APase and the unbound EMA by DEAE-cellulose ion exchange chromatography. The specific activity of the APase–EMA, compared to APase, increased 26-fold at pH 7.1 and 10-fold at pH 8.6. The pH optimum of APase–EMA was shifted down from pH 9.5 (native APase) to 8.6. This change could be interpreted in terms of polyelectrolyte theory. APase–EMA retained 50–70% of its optimum activity in the pH range 7–8, while APase retained only 5–15% of its optimum activity within the same pH range. Its isoelectric point, *pI*, was 4.2 (APase 6.0) and it migrated on polyacrylamide gel electrophoresis in a single band, anodic movement twice as fast as APase. Parallel with the kinetic measurements, the reactive-enzyme sedimentation method was used to measure $S_{20,w}$ values. $S_{20,w}$ values obtained for APase–EMA, activated APase, and APase dialyzed against water were 6.56S, 6.46S, and 5.17S, respectively. Molecular weights, M_r, were determined by equilibrium sedimentation: the values obtained were 180,000,

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160,000, and 84,500. M_r values of APase–EMA and APase (native) estimated by Sepharose-4B gel filtrations were essentially the same. The above-mentioned values remained unchanged for APase–EMA after intensive dialysis against water, whereas for the activated APase, separation from the effector molecules caused the equilibrium to shift back to the monomeric, very slightly active enzyme with concomitant changes of $S_{20,w}$ to 5.15 and M_r to 82,000.

Index Entries: Immobilized alkaline phosphatase, water-soluble; ligand–copolymer of ethylene and maleic anhydride; dimeric enzyme, with increased enzymatic activity; alkaline phosphatase, increased activity of; activation, of immobilized alkaline phosphatase; coupling, of copolymer with immobilized alkaline phosphatase; ethylene–maleic anhydride copolymer, coupling to alkaline phosphatase; maleic anhydride–ethylene copolymer, coupling to alkaline phosphatase.

INTRODUCTION

Enzyme systems in vivo rarely exist in the free form. Either they are bound to other components of the cells or immobilized in a certain microenvironment, mainly in membranes. Consequently they may possess additional, systemic, properties that are not exhibited by isolated enzymes. Intensive studies were made to couple enzymes to negatively, zero, and positively charged water-soluble polymeric substances and study the properties of the bound enzymes thus obtained (*1–4*).

It has been reported that APase in certain malignant cells bound to membranes in a negatively charged microenvironment. Therefore it was of interest to couple APase to a negatively charged polyelectrolytic substance. The copolymer of ethylene and maleic anhydride, EMA, was chosen for this purpose. In the present study we describe the preparation of the water soluble APase–EMA, its purification, its catalytic and physicochemical properties, and their comparison with those of pure APase before binding to EMA.

MATERIALS AND METHODS

Alkaline phosphatase, APase, Type VII-S lot 121F-8045; *p*-nitrophenyl phosphate (*p*-NPP); Tris (hydroxymethyl) aminoethan e (Tris); β-naphthyl acid phosphate and fast dyes were purchased from Sigma Chemicals. DEAE-cellulose (DE-52) was purchased from Whatman (Maidstone, Kent, UK). Sephadex G-100 and Sepharose-4B from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used were of analytical grade. Aminoethanol was distilled freshly before preparing the buffers.

DEAE-Cellulose Chromatography

DEAE-cellulose was treated as described by Peterson and Chiazze (*5*) and adjusted to pH 7.2 with dilute HCl. The absorbent was then equilibrated with 5 m*M* aminoethanol/HCl, pH 8.0. The sample, dialyzed against the starting elution buffer, was applied to the column (1.5 × 30 cm). Elution, with a stepwise concentration gradient, was then performed with equal volumes of starting buffer and the next chosen concentration (50 mL of each concentration; the concentration steps were 5–50, 50–100, 100–180, 180–250, and 250–500 m*M*). The APase and APase–EMA was eluted from the column collecting 2.0 mL eluate/20 min. The tubes were assayed for APase activity in 0.5*M* Tris-HCl, pH 9.0, using *p*-NPP as substrate and for protein content (*6,7*).

Molecular-Weight Determinations

Sephadex G-100 and Sepharose-4B Gel Filtration

Two sets of marker proteins were used: (1) [^{14}C]methylated protein mixture; (2) a calibration mixture composed of Blue Dextran (M_r 2,000,000), fructose bisphosphate aldolase (M_r 125,000), alkaline phosphatase (*Escherichia coli*) (M_r 80,000), bovine serum albumin (M_r 67,000), egg albumin (M_r 46,000), pepsinogen (M_r 40,800), pepsin (M_r 35,000), soybean trypsin inhibitor (M_r 21,500) and cytochrome C (M_r 11,500). The eluates were monitored at 650 nm for cytochrome C and at 278 nm for the other proteins. Enzymatic activities of fructose bisphosphate aldolase, alkaline phosphatase, and activated pepsinogen were also measured.

Isoelectric Focusing

Isoelectric focusing of APase–EMA and APase was performed in an LKB 8101 isoelectric-focusing column (28 mL) with 2% Ampholines of pH 3.5–10.0 at 800 V for 16 h. A volume of 1.0 mL/tube was collected (*8*). APase activity and pH were measured in each tube.

Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out in 10 and 15% crosslinked polyacrylamide slab gels at pH 8.6 at constant amperage 100 mA/slab gel, for 3 h at room temperature. APase was made visible after incubating the gel with β-naphthyl acid phosphate for 2 h at 35°C. The excess of substrate was removed by washing with water and APase was made visible with Fast Blue diazonium salt as described by Smith et al. (*9*).

Protein Concentration

Protein concentrations were measured by the method of Lowry et al. (*7*) after calibration using bovine serum albumin as standard.

Enzymatic Assay

APase activity measurements were carried out routinely in 0.8 or 0.08*M* Tris-HCl buffer in the pH range of 7.1–9.8. The assay mixture contained 0.2*M* NaCl and 0.02*M* $MgCl_2$ and the enzyme (APase or APase–EMA, 1.0 or 0.1 ng/mL). The reaction was initiated by adding the substrate to the assay mixture to avoid lag time (*10*). The hydrolysis of *p*-NPP was followed spectrophotometrically by measuring the absorption of *p*-nitrophenolate at 400 nm. Correction was made for the change of molar absorption of *p*-nitrophenolate with pH and ionic strength. The initial rates were calculated as described in (*6*). In all experiments the substrate concentration was at least five times higher than the K_m ($\sim 2 \times 10^{-4}M$) (*6*). K_m values were determined for APase–EMA, activated APase, and APase, from Lineweaver reciprocal plot and found to be the same, within experimental error, for the three species $K_m = 10^{-4}M^{-1}$.

Analytical Ultracentrifugation

Molecular weight determinations and reactive enzyme centrifugation were carried out in a Beckman Model E ultracentrifuge as described (*11–15*).

L-Phenylalanine Inhibition

The dependence of inhibition of the enzymatic activity of native APase, activated APase, and APase–EMA on concentrations of L-phenylalanine were carried out according to Gosh and Fishman. (*16*).

Thermal Stability

Thermal stability of the unbound native APase and of APase–EMA were investigated (*17*) at 55 and 65°C. The enzyme preparations were incubated for 1, 5, 10, 15, and 30 min at the above temperature and aliquots were taken to measure the residual activities and for polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Preparation of APase–EMA

APase (4 mg, 4 mL) was dialyzed against 4 L of 0.015*M* $NaHCO_3$, pH 8.6 (or 0.500*M* Tris-HCl, pH 8.6) at cold room temperature for 48 h. The buffers contained also 0.200*M* NaCl, 0.010*M* $MgCl_2$, and 0.005*M* inorganic orthophosphate. These conditions were chosen to shift the equilibrium of monomer $\rightleftharpoons$ dimer enzyme towards the dimeric form (*15*). The dialyzed enzyme (4 mg in 8 mL) was coupled with EMA (4 mg, average M_r 20,000). EMA was added in small portions to the enzyme solution,

keeping the pH constant (8.6) by adding solid $NaHCO_3$. The EMA, which in the beginning was in suspension, dissolved because of the coupling with the protein and hydrolysis of the unreacted anhydride groups of the copolymer. The solution was stirred overnight at 4°C. This preparation was named APase–EMA. It was dialyzed against 0.005*M* aminoethanol/HCl buffer, pH 8.0 at 4°C and subjected to column chromatographic separation. It is pertinent to note that the monomeric form of APase did not react with EMA under the identical reaction conditions described above.

Separation of APase–EMA

The water-soluble APase–EMA preparation was separated from the native enzyme, APase, and from the unbound copolymer EMA by DEAE-cellulose ion exchange chromatography. The chromatographic separation was carried out as described in Materials and Methods. The results of that chromatogram are given in Figs. 1a and 1b. It is apparent that APase–EMA bound to DEAE-cellulose more strongly; it was eluted from the column only at 450 m*M* concentration of aminoethanol/HCl buffer, pH 8.0, while the native APase eluted at a 35 m*M* concentration of the same buffer. APase–EMA migrated on polyacrylamide gel electrophoresis at a faster anodic movement, giving a *distinct single band* as revealed by alkaline phosphatase activity staining on the gel (Fig. 2), with a faster anodic band compared to native APase. Separation of APase–EMA from APase by DEAE-cellulose ion exchange chromatography also resulted in the same electrophoretic pattern (Fig. 3). The single activity band of APase–EMA indicated that the coupling of APase with EMA was not of a random type, i.e., each protein enzyme probably bound with one or two molecules of EMA on the same amino acid residue.

Enzymatic Measurements

The rate of enzymatic hydrolysis of *p*-nitrophenyl phosphate in saturating concentrations of substrate was measured in 0.800*M* Tris-HCl at various pH values (7.1–9.8) in the presence of 0.200*M* NaCl and 0.02*M* $MgCl_2$. The influence of the binding of APase to a negative carrier, EMA, on the rate of hydrolysis of *p*-NPP catalysed by APase–EMA at various pH values is given in Table 1. For comparison the enzymatic hydrolysis of *p*-NPP was also measured using the untreated APase, and the result given in the same Table 1. APase–EMA has an elevated specific activity towards the substrate *p*-NPP. Its specific activity was 20–26 times greater at pH 7.1, and 8–10 times greater at pH 9.0 compared with the native enzyme using identical assay conditions (Figs. 4a, 4b). The optimum activity of APase–EMA was at pH 8.5 and it retained 50–60% of its optimum activity at pH 7.1. The untreated enzyme has an optimum activity at pH 9.5, retaining 10–20% of its optimum activity at pH 7.1 (Fig. 5).

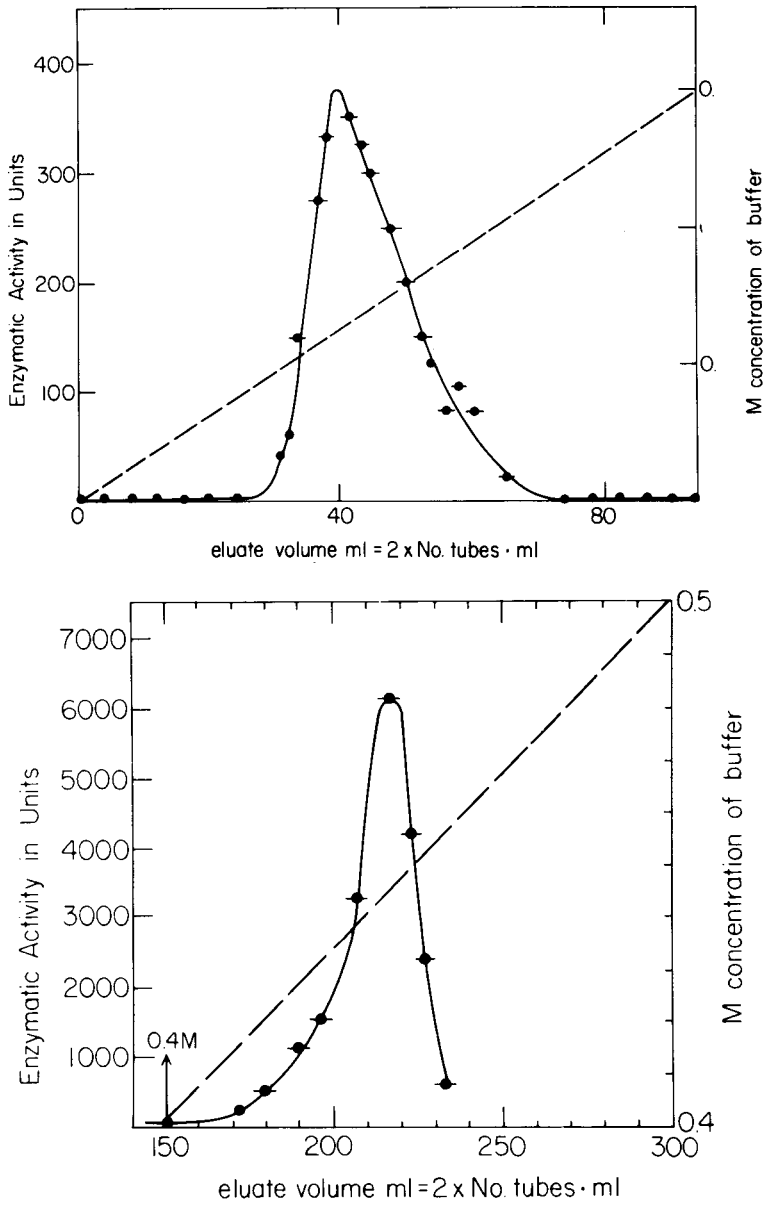

Fig. 1. DEAE-cellulose chromatography of the reaction mixture containing APase and APase–EMA: 4 mL (4 mg) of the above reaction mixture, after dialysis against the starting buffer; 0.01*M* AE/HCl, pH 8.0, buffer was applied to a DEAE-cellulose column (1.5 × 30 cm) preequilibrated with the same buffer. The material was eluted by stepwise gradient concentrations of AE/HCl, pH 8.0 buffer, as described in the Expt. section, collecting 2.0 mL fractions/20 min. APase activity, protein, and buffer content were monitored as described. The specific activity was calculated (Units activity/mg protein) and is presented in Fig. 1a (top) and 1b (bottom). It is apparent that APase and APase–EMA were well-separated by this chromatograph, since APase was eluted at 0.035*M* whereas APase–EMA was eluted at 0.45*M* concentration of the same buffer.

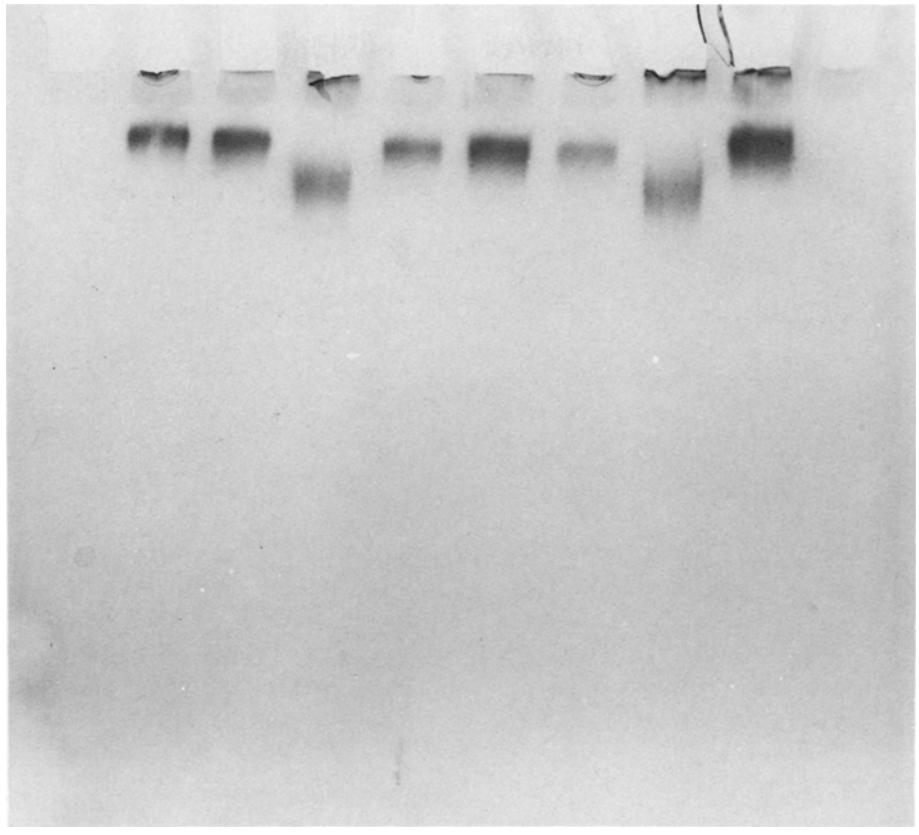

Fig. 2. Polyacrylamide gel electrophoresis of the solutions (containing 0.1 mg/mL total protein). From left to right: APase 10 μL; APase 20 μL; APase–EMA 2 μL; APase 2 μL; APase 20 μL; APase–EMA 10 μL; after DEAE cellulose column separation; APase 20 μL, from the same column. After completion of the electrophoresis the gels were incubated with β-naphthyl acid phosphate as substrate in 0.1*M* Tris-HCl, pH 9.0 buffer at 35°C for 20 min. The substrate was washed off from the gels with water and APase activity band made visible with diazo reagent (*see* Expt. section).

Isoelectric Point PI

The p*I* of APase–EMA preparation was found to be pH 4.2, which is 1.8 pH units more acidic compared to the native enzyme. The p*I* was determined as described under Methods. Concomitantly the p*I* of APase was also measured and found to be 6.0.

APase isoenzyme with p*I* in the range of pH 3.6–4.2 was reported to be present (*18*) in the serum of 68 of 70 patients with histologically proven malignant neoplasm, and in 26 of 70 patients with benign disorders. Even though the p*I* values of this isoenzyme were similar, they

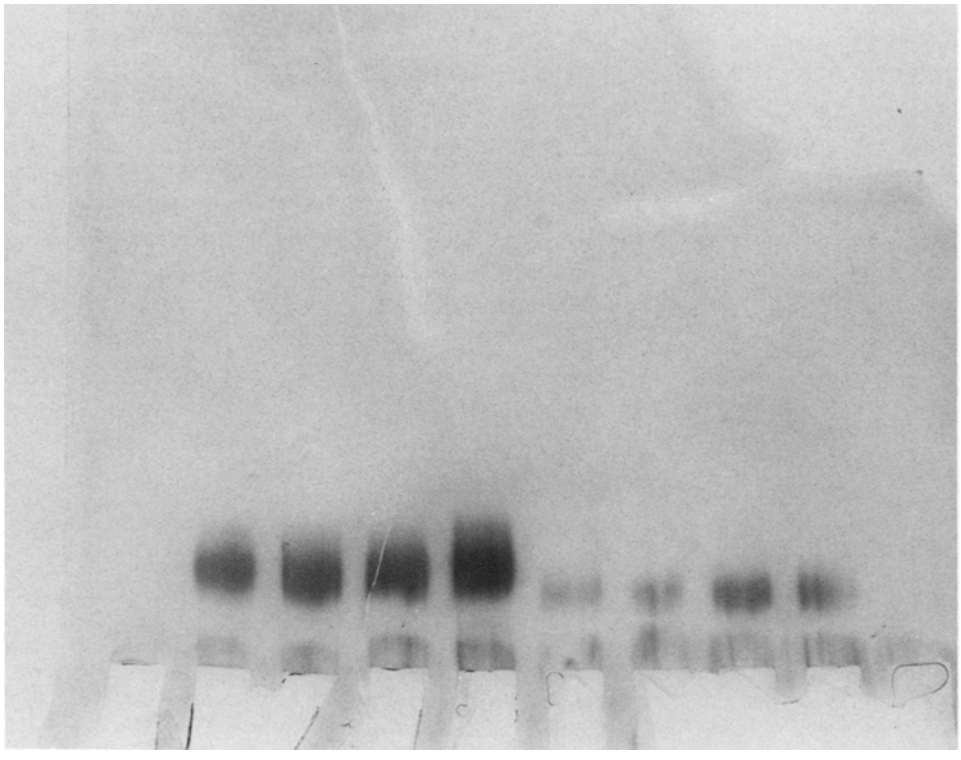

Fig. 3. PAGE of tubes eluted from DEAE-cellulose. From left to right: 10 μL of tubes, Nos. 37, 39, 41, and 43, and 205, 206, 207, and 210. For details, *see* the legend for Fig. 1.

were heterogeneous in respect of stability to heat, and L-phenylalanine and homoserine inhibition. It was established that the introduction of a negatively charged copolymeric substance caused alterations of the kinetic and physicochemical properties of the protein-enzyme to which the ligand is bound. The site, the amino acid residue where the actual coupling takes place, has not yet been identified. In general, the nature of the ligand bound to the isoenzymes is important and has implications for biological aspects and for clinical practice.

L-Phenylalanine Inhibition

Native APase activity was found to be inhibited by L-phenylalanine. The extent of inhibition of APase activity was found to be dependent on the enzyme and L-phenylalanine concentrations, with 0.01*M* L-phenylalanine inhibiting the enzymatic activity of 0.1 ng/mL enzyme by 80%, as revealed by activity measurements. By contrast, APase–EMA activity under similar assay conditions underwent only insignificant inhibition, 10–15%.

TABLE 1
Dependence of Activity on pH[a]

pH	APase-untreated		APase–EMA		$R = \frac{A \text{ of APase–EMA}}{A \text{ of APase}}$
	A, units	*A*/opt. *A*,%	*A*, units	*A*/opt. *A*,%	
7.10	77	18	1900	50	24.7
7.30	89	21	1645	43	18.5
7.50	101	24	1750	45	17.5
7.80	111	26	2430	64	22.0
7.95	128	30	2600	68	20.3
8.20	213	51	3240	85	15.2
8.40	234	56	3355	93	14.3
8.65	304	72	3820	100	12.6
9.00	375	89	3632	95	9.7
9.20	400	95	3032	79	7.6
9.40	410	98	2395	62	5.8
9.60	420	100	2045	53	4.9

[a]The activity of APase and APase–EMA at different pH values was measured under the assay conditions described (*see* Expt. section). The specific activity, *A*, was calculated and expressed in Units, where one Unit is equal to 1 μmol *p*-NPP hydrolyzed/mg enzyme/min at 25°C. The Table includes the percentage of the activity vs the optimum activity retained at various pH values. APase before coupling to the copolymer, and APase–EMA measurements are given. The ratios of the increased activities of APase–EMA vs APase at various pH values are given also. The pH dependence of activity of five different batches of APase before and after coupling with EMA was tested. The results obtained were similar with variations in the experimental error of ±10%. The activities were also measured at pH 6.90, but the experimental errors, arising from the dissociation constant pK_a value of *p*-nitrophenol, gave unreliable results.

Heat Stability

Activity measurements, both in solution and on polyacrylamide gel electrophoresis, revealed that APase–EMA retains its activity for a longer time at 60°C, whereas native APase under similar conditions completely loses its activity (*see* Fig. 6).

Molecular Weight and $S_{20,w}$ Values

The molecular weights of APase–EMA and of APase were established by gel filtration and equilibrium sedimentation, and were found to be 180,000 ± 10,000 and 85,000 ± 10,000, respectively. It is important to note that APase was coupled to EMA after shifting the equilibrium to the dimeric form of the enzyme by effector molecules as described (*15*). The dimeric form of the enzyme has a molecular weight of 160,000 ± 10,000.

Reactive enzyme sedimentation was used to measure $S_{20,w}$ values. The values obtained were 6.72 *S* for APase–EMA and 5.21 *S* for APase.

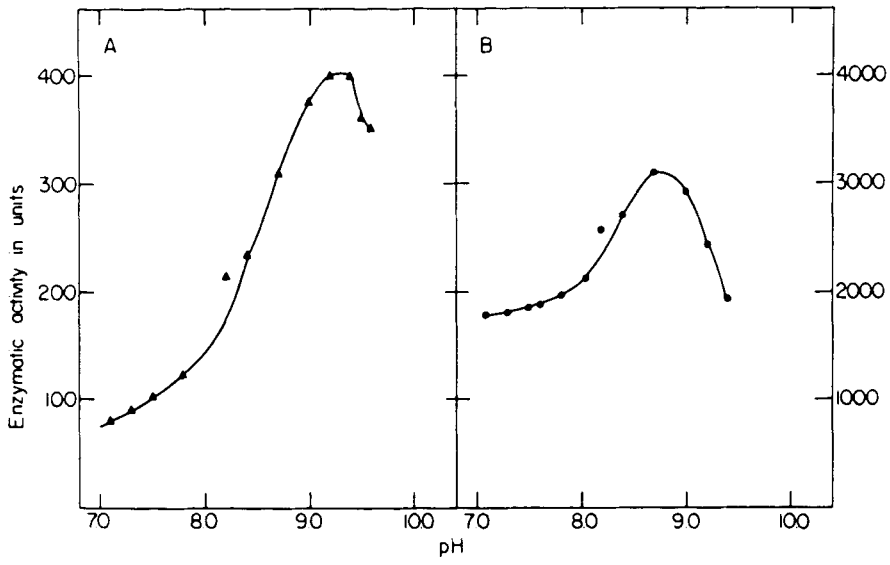

Fig. 4. pH activity curve of APase and APase–EMA. Enzymatic assays were performed as indicated under the Expt. section. The specific activities were calculated and expressed in units per mg, where one unit is 1 μmol *p*-NPP hydrolyzed per min at 25°C under assay conditions specified (*see* Expt. section).

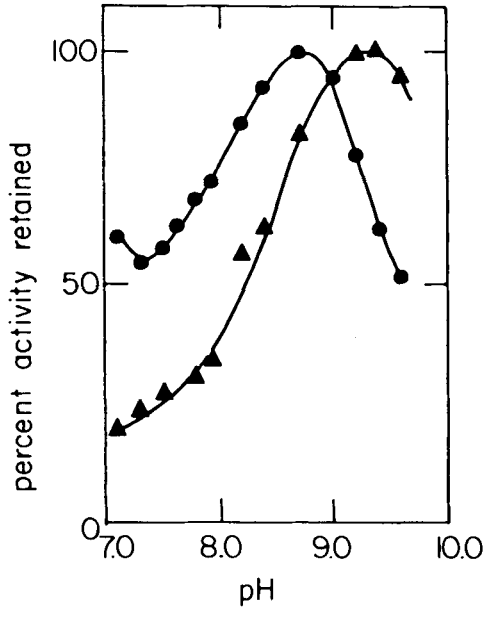

Fig. 5. Percentage of the optimum activity of APase (▲) and APase–EMA (●) retained at various pH values.

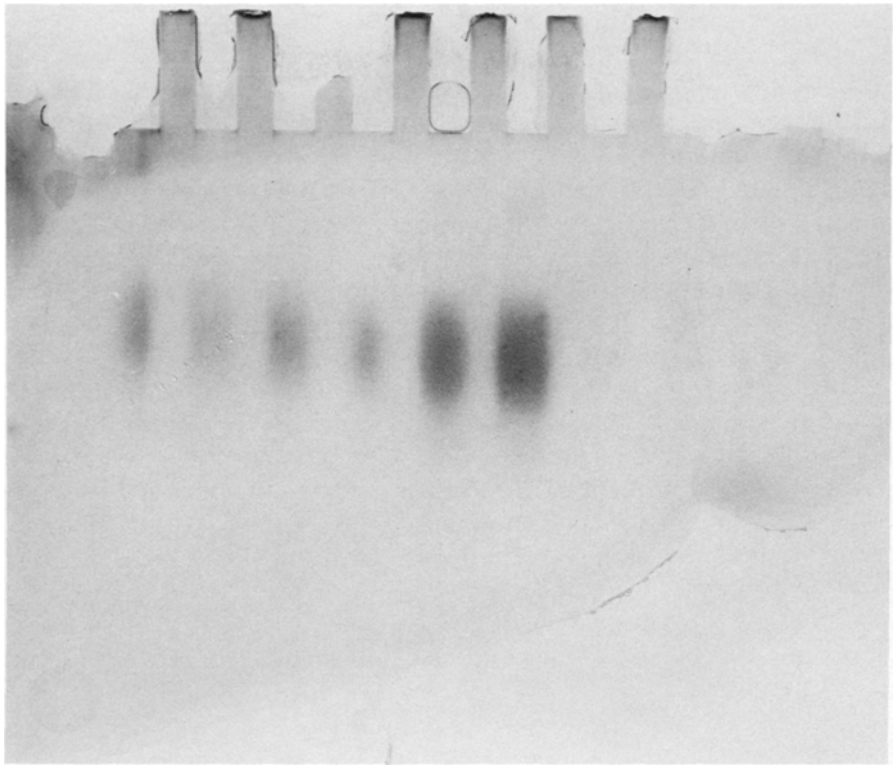

Fig. 6. Heat stability of APase and APase–EMA incubated for 0, 5, 10, 15, and 30 min at 65°C. Aliquots: 5 μL aliquots were removed and subjected to electrophoretic separation. For details, *see* legend Fig. 1. After completion of electrophoresis, the gel was incubated with β-naphthyl acid phosphate in 0.1*M* Tris-HCl, pH 9.0 buffer for 30 min at 35°C. The substrate was washed off and APase activity made visible with diazo reagent. From right to left: APase after 5, 10, 15, 20, and 25 min incubation; APase–EMA after 0, 5, 10, 15, 20, and 25 min. It is apparent that APase–EMA is more stable to exposure to heat compared to native APase.

Both the M_r and $S_{20,w}$ values of APase–EMA remained unchanged after dialysis against 15 m*M* aminoethanol/HCl buffer, pH 8.0. The dimeric form of APase was reported to have $S_{20,w}$ values of 6.3 and 6.5 calculated, respectively, from boundary sedimentation velocity and reactive-enzyme sedimentation measurements. Removal of the effector molecules caused a shift towards the slightly active monomeric enzyme with an $S_{20,w}$ value of 5.3 *S*. L-Phenylalanine, a noncompetitive inhibitor of APase activity that also prevents dimeric enzyme formation (*15*), did not inhibit the activity of APase–EMA. This finding can be explained in that the stable dimeric form of APase has no binding site available for L-phenylalanine.

Isoenzymes are heterogeneous proteins. Yet in spite of this diversity they catalyze the same chemical reactions. The isoenzymes have great potential for the investigation of heterogeneous mixtures of biological

fluids. They have important applications in many areas of biology, e.g., typing tissue and determining cell origins. They also have great prospects in medicine, both for use in diagnosis and in prognosis. Enzymes can be determined in minute quantities and measured with great accuracy.

The clinical importance of differentiating alkaline phosphatase isoenzymes has been recognized for a long time, and has been reviewed elsewhere (*19*). Thus, isoenzymes for clinical purposes are different iated and characterized by their electrophoretic mobility, isoelectric point, molecular size, the pH profile of their activities and, in the case of APase, their L-phenylalanine inhibition.

In our present work we have shown that the above parameters can be changed by binding a polyelectrolytic substance covalently. Additionally, a group of isoenzymes may be homogeneous with respect to certain properties and heterogeneous in other properties, as described recently (*18*). The addition of other parameters to the existing measurements may therefore be desirable. We suggest the study of the specific interaction of enzymes with low (high) molecular weight effector (or inhibitor) substance. Ultracentrifugation methods, especially *reactive enzyme sedimentation*, offer possibilities for studying such interactions in both crude and purified enzymes.

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