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The Reversible Inactivation of Pig Kidney Alkaline Phosphatase at Low pH

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1. Pig kidney alkaline phosphatase is inactivated by treatment with acid at 0°. 2. Inactivated enzyme can be partially reactivated by incubation at 30° in neutral or alkaline buffer. The amount of reactivation that occurs depends on the degree of acid treatment; enzyme that has been inactivated below pH 3·3 shows very little reactivation. 3. Studies of the kinetics of reactivation indicate that the process is greatly accelerated by increasing temperature and proceeds by a unimolecular mechanism. The reactivated enzyme has electrophoretic and gel-filtration properties identical with those of non-treated enzyme. 4. The results can be best explained by assuming that a lowering of the pH causes a reversible conformational change of the alkaline phosphatase molecule to a form that is no longer enzymically active but is very susceptible to permanent denaturation by prolonged acid treatment. A reactivation mechanism involving sub-unit recombination seems unlikely.

It has been suggested that the alkaline phosphatase (EC 3.1.3.1) found in human urine originates from the kidney by a process involving dissociation into sub-units (Butterworth, 1968). The alkaline phosphatase present in the micro-organism *Escherichia coli* has been shown to possess a sub-unit structure (Levinthal, Signer & Fetherolf, 1962; Schlesinger & Barrett, 1965), but experiments on human kidney alkaline phosphatase failed to provide conclusive evidence that inactivation in the presence of such agents as urea and inactivation at low pH was reversible and associated with sub-unit formation (Butterworth, 1968). The present paper describes further studies of the effect of acid on an alkaline phosphatase preparation.

MATERIALS AND METHODS

Enzyme preparation. A 2·6kg. portion of fresh pig kidney cortex was homogenized with 2·6l. of water and then extracted with 1·3l. of butan-1-ol (Morton, 1950). The aqueous extract was separated by centrifugation at 18000*g*, then filtered through Hyflo Super-Cel. Further purification consisted of successive fractionation with acetone at −15° and $(NH_4)_2SO_4$ followed by gel filtration on Sephadex G-200 and chromatography on DEAE-Sephadex at room temperature. Gel filtration was carried out on a column (43cm. × 4cm.) in 0·1M-tris–HCl buffer, pH 7·6. The buffer for DEAE-Sephadex chromatography was 0·05M-tris–HCl, pH 7·6, and the enzyme was eluted from the DEAE-Sephadex column, which measured 30cm. × 2·5cm., by an increasing concentration of NaCl. Because of the limited capacity of the Sephadex and DEAE-Sephadex columns, chromatography was carried out on portions of the active fractions from the acetone and $(NH_4)_2SO_4$ precipitation steps; the remainder was stored at −15° until required. A summary of the purification procedure is given in Table 1.

Assay of alkaline phosphatase. The substrate was 3mM-*p*-nitrophenyl phosphate (British Drug Houses Ltd., Poole, Dorset) in 0·1M-Na_2CO_3–$NaHCO_3$ buffer, pH 10 and I 0·22, and phosphatase activity was determined at 30° by following the increase in E_{400}. Alkaline phosphatase activity was

Table 1. *Purification of pig kidney alkaline phosphatase*

Phosphatase activity is expressed in μmoles of *p*-nitrophenol released/min.; measurements of E_{280} were used as an estimate of protein concentration. The specific activity was increased about 1200-fold by the purification procedure.

	Total enzyme activity	Total protein	Specific activity	Percentage recovery for each stage
Aqueous phase from butan-1-ol–water extraction	17650	152000	0·12	—
45–65% (v/v) acetone ppt.	11520	23200	0·5	65
Supernatant after pptn. of some inert protein by 50–60%-satd. $(NH_4)_2SO_4$	8110	3480	2·33	70
After gel filtration on Sephadex G-200	336	34	9·9	74
Main peak from DEAE-Sephadex chromatography	77	0·54	142·5	65

expressed as μmoles of *p*-nitrophenol released/min./ml. of enzyme solution.

Reversible inactivation at low pH. A stock acidic solution was prepared by adjusting 0·1 M-tris to pH 2·65 with N-HCl. The stock solution was then diluted with water to give the required pH and ionic strength. Measurements of pH at 25° were made on a Dynacap pH-meter (W. G. Pye and Co., Cambridge) and chloride concentrations determined on an EEL chloride meter (Evans Electroselenium Ltd., Halstead, Essex).

Alkaline phosphatase was mixed with the acid solutions at 0°. The mixtures were usually of 2 ml. volume and contained 2–3 units of alkaline phosphatase activity. At timed intervals, portions were withdrawn and residual enzyme activities determined at pH 10 in reaction mixtures containing 5 mM-Mg^{2+}. For reactivation studies, acid-treated enzyme was usually introduced into 0·05 M-tris–HCl buffer, pH 7·6, at 30°, but details of experiments in which the reactivation was studied under other conditions are given in the Results section. When phosphatase activities of inactivated or partially reactivated enzyme were determined, assay times were kept short to minimize the effects of reactivation of enzyme during the assay period.

Determination of K_m. Measurement were made at 30° in Na_2CO_3–$NaHCO_3$ buffer, pH 10, containing 3·3 mM-$MgSO_4$. Acid-inactivated enzyme was reactivated for 45 min. at pH 7·6 and 30° before measurement.

RESULTS

Pig kidney alkaline phosphatase was eluted from Sephadex G-200 as a single peak. Separation of this peak on DEAE-Sephadex indicated that the enzyme preparation was heterogeneous (Fig. 1). Over 90% of the enzyme activity placed on the DEAE-Sephadex column was recovered. The sharp peak eluted from DEAE-Sephadex by the starting buffer constituted approx. 65% of the total enzyme activity recovered and possessed the highest specific activity. Chromatographic fractions constituting the sharp peak were pooled to provide the source of enzyme used in the inactivation studies. Horizontal starch-gel electrophoresis of this enzyme by the method of Smithies (1955) showed that it was homogeneous.

Exposure of alkaline phosphatase to low pH led to a progressive loss of enzymic activity (Fig. 2). Above pH 4 there was negligible loss of activity, but as the pH was lowered the rate of inactivation was markedly increased. The inclusion in the inactivation mixtures of Mg^{2+} (5 mM), Zn^{2+} (10 μM) and $H_2PO_4^-$ (1 mM), either singly or in combination, did not protect the enzyme against acid inactivation.

Enzymic activity lost as a result of acid treatment was partially recoverable by incubation at 30° in 0·05 M-tris–hydrochloric acid buffer, pH 7·6. The amount of activity that could be recovered was dependent on the conditions under which inactivation had occurred: the lower the pH of inactivation and the more prolonged the process, the less was the amount of activity regained at pH 7·6. Fig. 3 shows enzyme reactivation after exposure to acid for 30 min. at 0°. The presence of 5 mM-Mg^{2+} or 10 μM-Zn^{2+} did not increase the rate of reactivation of alkaline phosphatase. Zn^{2+} inhibited alkaline phosphatase activity to some extent, but did not seem to inhibit the reactivation process. EDTA at a concentration of 1 mM, however, did inhibit

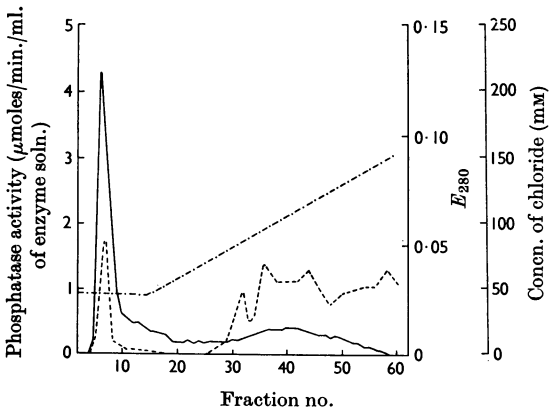

Fig. 1. DEAE-Sephadex chromatogram of pig kidney alkaline phosphatase. ——, Enzyme activity; ------, E_{280}; –·–·–, chloride concentration. Fractions were of 4·8 ml. volume and nos. 5–20 were pooled to provide the enzyme under study.

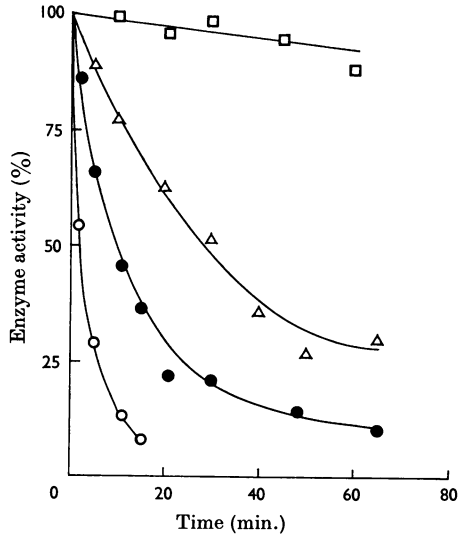

Fig. 2. Inactivation of alkaline phosphatase by acid at 0°. □, pH 3·86 and *I* 0·02; △, pH 3·5 and *I* 0·04; ●, pH 3·23 and *I* 0·08; ○, pH 3·16 and *I* 0·1.

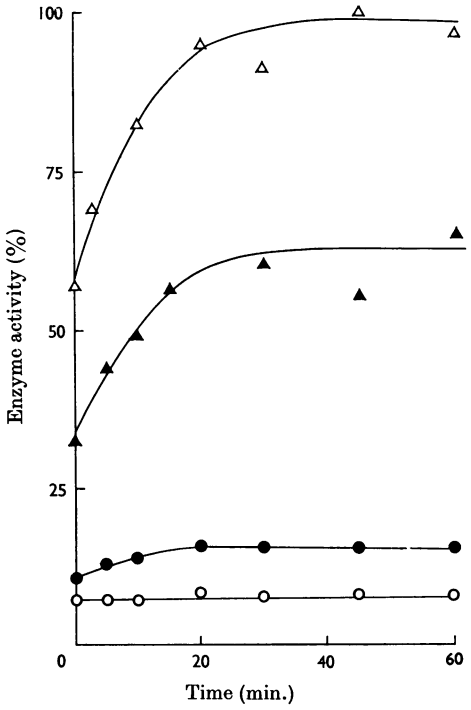

Fig. 3. Reactivation of alkaline phosphatase at 30° and pH 7·6. The enzyme had been treated with acid for 30 min. at 0°. △, Enzyme inactivated at pH 3·5; ▲, enzyme inactivated at pH 3·35; ●, enzyme inactivated at pH 3·23; ○, enzyme inactivated at pH 3·16.

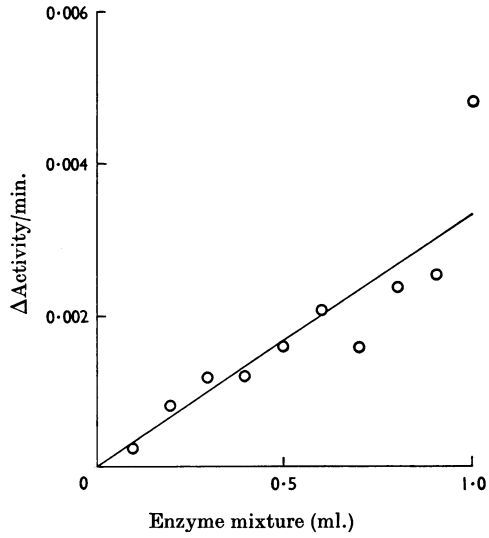

Fig. 4. Effect of enzyme concentration on the rate of reactivation. Enzyme (12 units) was inactivated at pH 3·5 for 10 min. in a reaction mixture of 1 ml., then transferred to 9 ml. of 0·05 M-tris–HCl buffer, pH 7·6, and kept on ice to prevent reactivation. Portions of this mixture were then reactivated at 30°.

reactivation. Reactivation did not seem to be pH-dependent, for enzyme inactivated at pH 3·5 regained lost activity as readily at pH 10 (carbonate–bicarbonate buffer) as at pH 7·6.

The reactivation of enzyme treated at pH 3·5 for 10 min. was temperature-dependent, the initial rate of reactivation being about 2·5-fold faster for a 10° rise in temperature. No reactivation was detectable at 0°. When reactivation was studied as a function of enzyme concentration it was found that the data were in accordance with unimolecular kinetics (Fig. 4).

The rate of migration on starch-gel electrophoresis and separation by gel filtration were unaffected by taking the enzyme through the inactivation–reactivation cycle, but the K_m value for the hydrolysis of *p*-nitrophenyl phosphate was decreased from 0·32 mM to 0·2 mM.

DISCUSSION

Acidification seems to have at least two effects on pig kidney alkaline phosphatase: a reversible effect that occurs under relatively mild conditions, and a permanent inactivation that results from prolonged exposure to pH values below 3·5. Kunitz (1960) found that the acid inactivation of chicken intestinal alkaline phosphatase was associated with a loss of Zn^{2+}, and readdition of Zn^{2+} brought about a rapid return of activity. Though EDTA inhibits reactivation of pig kidney phosphatase, removal of Zn^{2+} does not seem to be the cause of the reversible inactivation of this enzyme since the rate of reactivation is unaffected by the presence of Zn^{2+}. EDTA inhibition of alkaline phosphatase is complex (Conyers, Birkett, Neale, Posen & Brudenell-Woods, 1967), and the prevention of reactivation in the presence of the chelating agent could be the result of an action that is unrelated to ion binding. The inhibition of pig kidney alkaline phosphatase by EDTA is time-dependent and not fully reversible by the addition of Mg^{2+} and Zn^{2+} (P. J. Butterworth, unpublished work).

Electrophoretic and gel-filtration experiments indicate that there is no change in molecular weight of pig kidney alkaline phosphatase during the inactivation–reactivation cycle. Thus sub-unit formation and dimerization with regain of activity after the pattern of *E. coli* alkaline phosphatase seems unlikely. Also, the finding of unimolecular kinetics for the reactivation process seems to

eliminate the possibility of dimerization in the reactivation mechanism. Thus lowering the pH probably causes a conformational change of the pig alkaline phosphatase molecule to a structure that is no longer fully active catalytically. On readjustment to neutral pH there is a return to the active configuration of the enzyme in a reaction the rate of which is greatly enhanced by increasing temperature. Below 10° the rate of reactivation is very slow. Scutt & Moss (1967) obtained evidence that the reactivation of human intestinal alkaline phosphatase is not a single process but involves at least two reactions: one occurring at neutral pH and another at pH 9·9. Reactivation of intestinal enzyme does not occur when acid-treated enzyme is taken straight to pH 9·9. Pig kidney alkaline phosphatase does not show this property and appears to be reactivated as readily at pH 10 as at pH 7·6.

If the first reaction of acid on alkaline phosphatase is to induce a conformational change, then it is possible that the new configuration is more susceptible to denaturation. Such a mechanism would account for the impossibility of obtaining a total regain of enzymic activity after treatment with acid. Alternatively, it could be postulated that more severe acid conditions lead to dissociation into sub-units that are sensitive to rapid denaturation and do not recombine under the conditions of the experiments. Sub-unit formation could result from a loss of Zn^{2+}, as has been demonstrated for *E. coli* alkaline phosphatase (Schlesinger & Barrett, 1965). However, even if sub-units are formed, from the evidence presented in the present paper it seems that dissociation is not the primary cause of the reversible loss of enzymic activity on acidification.

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