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ACID PHOSPHOMONOESTERASE OF HUMAN PROSTATE

PHOSPHATE TRANSFER REACTIONS AND COMPARISON WITH ALKALINE PHOSPHATASE

WLODZIMIERZ OSTROWSKI* AND ERIC A. BARNARD

Departments of Biochemistry and Biochemical Pharmacology, State University of New York at Buffalo, Buffalo, N.Y. 14214 (U.S.A.)

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SUMMARY

The acid phosphomonoesterase I (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) from the human prostate gland was isolated in pure form by an improved procedure. Reaction with [^{32}P]orthophosphate at pH values from 3 to 9, followed by protein precipitation with $HClO_4$, led to no incorporation of phosphate into this enzyme, nor did a substrate affect this. No inhibition by beryllium ion of the enzymic activity occurred, at pH 5 or 7. These two effects are in clear contrast to the behavior of alkaline phosphatase.

The competitive transfer of phosphate from *p*-nitrophenyl phosphate to water or to ethanolamine, catalyzed by phosphatase, was measured. With *Escherichia coli* alkaline phosphatase, the phosphate transferred exchanged to a significant extent with external [^{32}P]orthophosphate, at pH 9. With the acid phosphatase no such exchange occurred, nor did that enzyme catalyze an exchange of orthophosphate with *O*-phosporylethanolamine when hydrolyzing the latter.

The evidence indicates that if a phosphoryl-enzyme intermediate is involved at all in prostatic acid phosphatase catalysis, it is of quite a different type to that in the known alkaline phosphatases.

INTRODUCTION

The acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) from the human prostate gland is an enzyme of interest from a number of viewpoints. The prostatic enzyme shows distinct differences from other known acid phosphatases[1,2], and is of significance in the biochemistry and pathology of the gland[3]. This enzyme, also, is of value as a tool in determinations of terminal phosphate groups in oligonucleotides and phosphoproteins, for which purpose it is required to be rigorously free of other phosphohydrolases. Further, it is, as we demonstrate in the

* Present address: Interfaculty Department of Physiological Chemistry, Medical Academy, Krakow, Poland.

present paper, suitable for study of some enzymic mechanisms of phosphoryl group transfer.

Those studies that have been previously reported on the catalytic properties of this enzyme were not able to utilize a homogeneous preparation. Such a preparation has become available by use of the method of OSTROWSKI *et al.*[4,5], involving several chromatographic steps. Some additional improvements have recently been made in this method, as noted elsewhere by OSTROWSKI[6] and further described in the present report, such that a highly homogeneous final preparation of prostatic acid phosphatase I is obtained. This enzyme has been used for the present studies, and is characterized physically in the succeeding paper[7].

We have made some studies of the phosphoryl group transfer catalyzed by this enzyme, related to the possibility of participation of a phosphoryl-enzyme as an intermediate. Some contrasts in behavior with the well-known alkaline phosphatases are established in this work.

MATERIALS AND METHODS

Preparation of the enzyme

All the operations were carried out at 4°. Acid phosphatase was isolated from hypertrophic human prostate glands (obtained from a Buffalo hospital, and frozen immediately after surgical removal). The initial stages of isolation were by procedures previously described[3] involving extraction in Tween 80 solution, fractionation using $(NH_4)_2SO_4$ and extraction at pH 4. At this stage (fraction F-II of OSTROWSKI AND TSUGITA[4]), the enzyme solution was dialyzed against water for 30 h. A sediment formed and was removed, and the solution was further dialyzed overnight against 0.05 M Tris–HCl–0.1 M KCl, pH 6.5. The centrifuged dialyzate was fractionated on a Sephadex G-100 column (Fig. 1A). The fractions in the central one-third of the single phosphomonoesterase peak were pooled, and the solution was dialyzed against 0.0175 M sodium phosphate buffer, pH 7.0 (24 h) and then adsorbed on a column (1.0 cm × 21 cm) of DEAE-cellulose pre-equilibrated in the latter medium, followed by washing with a further 150 ml and then elution by a 0.0175 M–0.070 M phosphate gradient, pH 7.0–6.0, as described previously[3,5], but using a constant volume mixing chamber containing initially 250 ml 0.0175 M sodium phosphate buffer, pH 7.0, and a microgranular DE-52 DEAE-cellulose (Whatman) column to give improved resolution. A single, essentially symmetric protein peak (centered at 350 ml after the gradient commenced) was obtained, containing all the eluted activity; most of the protein remained bound to the column, and could be subsequently eluted (with only slight activity) when 0.5 M NaCl was applied. The enzyme peak (about 80% in yield) was dialyzed as before and concentrated by adsorption on a small (1 ml per 30 mg protein) DE-52 column in 0.0175 M phosphate, pH 7, and elution with 0.2 M phosphate, pH 6. This solution was re-filtered on Sephadex G-100 (Fig. 2), giving a single sharp peak of the enzyme. This material was used for all the experiments.

Other materials

Alkaline phosphatase of *Escherichia coli* was from Worthington and bovine serum albumin from Armour Co. $NaH_2{}^{32}PO_4$ (used at 220–450 mC/mmole) was from New England Nuclear Corp.; β-glycerophosphate, *O*-phosphorylethanolamine·HCl

and *p*-nitrophenyl phosphate were from Sigma. Ethanolamine was a re-distilled, colorless sample of the free base (Suchard). $BeSO_4 \cdot 4\ H_2O$ was Fisher Reagent grade.

Reaction with [^{32}P]orthophosphate

Essentially the method used by BARMAN AND GUTFREUND[8] was applied. The enzyme (300 μl in water) and the buffer (0.2 M, 100 μl) were mixed, and 3 μl of sodium [^{32}P]phosphate solution added in an ice-bath. After the stated time at 0°, serum albumin (10 mg in 100 μl water) was added as an inert carrier, and 0.1 ml 20% (v/v) $HClO_4$. Using centrifugation at 20 000 rev./min for 15 min, the precipitate was collected, washed twice with 6 ml 5% $HClO_4$, dissolved in 90% (v/v) formic acid (0.5 ml), reprecipitated (in another tube, with 1 ml water added) by addition of 1 ml 20% $HClO_4$, and washed with 4.5 ml 5% $HClO_4$ 5 times. The final pellet was dissolved in 90% formic acid (0.5 ml) and this was added to a vial for liquid scintillation counting[9]. Calibration vials contained the same medium and a [^{32}P]phosphate standard.

Separation of products of the transphosphorylation reactions

O-Phosphorylethanolamine was separated and measured by ion-exchange chromatography on the Beckman-Spinco amino acid analyzer, Model 120B, using the standard 60 cm resin column. This was operated at 35°, equilibrated with 0.10 M sodium citrate–HCl buffer, pH 2.20. The amount of isotope in the effluent was continuously recorded in a flow-cell scintillation counting system as described elsewhere[9]. The amount of *O*-phosphorylethanolamine was read concurrently by integration of the ninhydrin color peak, using a calibration made by chromatography of a standard solution of this compound. The latter coincided in the chromatography, when tested in a mixture, precisely with the product formed in the experiments. The samples withdrawn from the experimental reaction mixtures were each diluted with four times their volume of the pH 2.2 buffer, arresting the enzymic reaction, and were immediately analyzed.

Other methods

Enzymic activity was measured[4] on *p*-nitrophenyl phosphate as substrate, but using the rate of absorbance increase at 405 nm ($\Delta A_{405\ nm}$) in cells thermostatted at 25.0°, recording in a Cary Model 15 spectrophotometer directly (at pH 5), or reading after dilution in alkali[4], to determine the (linear) initial velocity. Concentrations of the enzyme solutions were determined by absorbance measurement at 280 nm, calibrated on a stock solution by refractometry by standard methods[7].

RESULTS

The acid phosphatase preparation used

The acid phosphatase I isolated from human prostate gland was purified by an improved version of the method of OSTROWSKI AND TSUGITA[4], incorporating gel filtration[6] stages (Fig. 1). By several refinements, the preparation has been advanced to the purest yet reported; in the final stage of purification on a Sephadex G-100 column (Fig. 2) it was of constant specific enzyme activity except for the most trailing region of the peak, which was discarded. When the gel filtration as in Fig. 2 was then repeated, the final peak was homogeneous, and this material was entirely homo-

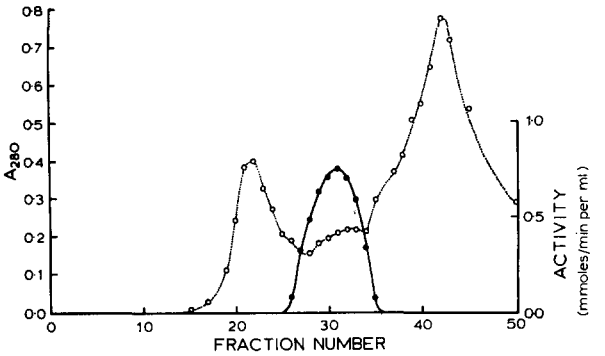

Fig. 1. Gel filtration on a Sephadex G-100 column (3.5 cm × 100 cm). A 10-ml sample of the dialyzate (after fractionation with $(NH_4)_2 SO_4$ and extractions at pH 4), containing about 200 mg protein, was used in each filtration. The medium was 0.05 M Tris–HCl–0.1 M KCl, pH 6.5, at 4°; 5-ml fractions were collected at 20 ml/h. ○----○, protein, by $A_{280\ nm}$; ●—●, activity on *p*-nitrophenyl phosphate, as mmoles of *p*-nitrophenol released per min per ml of enzyme solution. Fractions up to 35 were shown to be devoid of phosphodiesterase[3] activity.

geneous by the criteria of rechromatography on DEAE-cellulose (by the same method as used in the preparation), and of polyacrylamide gel electrophoresis[7]. Phosphodiesterase activity[4] (on bis-*p*-nitrophenyl phosphate) was undetectable, and the specific enzymic activity on *p*-nitrophenyl phosphate (0.01 M) at pH 5.0 was 1680 units (μmoles/min at 25°) per mg protein. This can be compared with a maximum for a mammalian alkaline phosphatase (at its optimum pH, 10, on the same substrate), estimated by "all-or-none" active site titration, of 854 units per mg (ref. 8).

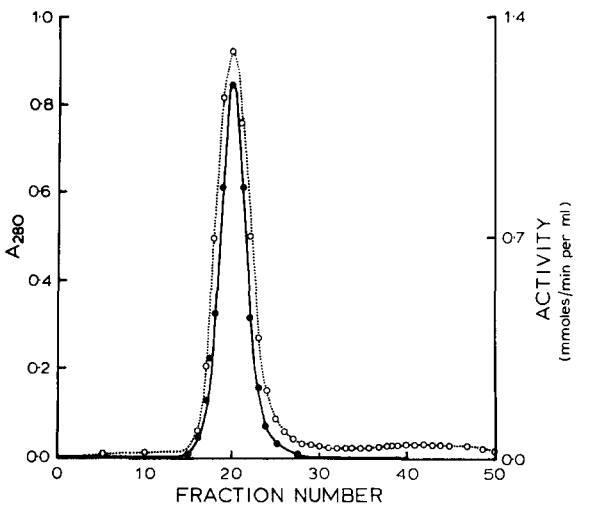

Fig. 2. Refiltration on Sephadex G-100. The enzyme solution after purification as in Fig. 1 (taking the central one-third of the active peak) followed by DE-52 chromatography, was concentrated and 1.5 ml was applied to a 0.9 cm × 150 cm column. Medium and notation as in Fig. 1. 1.5-ml fractions were collected at 10 ml/h.

TABLE I

REACTION OF ACID PHOSPHATASE WITH [^{32}P]PHOSPHATE

The radioactivity finally measured in the precipitated enzyme (see text), after incubation in the conditions shown, is expressed in the last column as moles of phosphate per mole of enzyme, taking the molecular weight as 100 000 (ref. 6). In the control incubations the enzyme was omitted; only amounts above this level are used in making the calculations. In Expt. 1, only two final washes in 5% $HClO_4$ were given (instead of the 5 used later) accounting for the higher residual activity in both test and control precipitates. In Expt. 3, the volumes used were double those stated in the text. In Expts. 1 and 2, the incubation period was 10 min. In a duplicate set in Expt. 1 (other than at pH 9) incubation was for 2 min, when a set of values within the range shown (for pH 3–8) was again obtained. The buffers used (0.05 M final concentration) were citrate (pH 3), acetate (pH 4–6), or Tris (pH 7–9), adjusted with NaOH or HCl.

Expt. No.	*Enzyme (μg)*	*^{32}P added (counts/min)*	*Incubation conditions*	*Counts/min in precipitate*	*^{32}P bound (moles/mole protein)*
1	40	$1.2 \cdot 10^7$	pH 3	766	$0.2 \cdot 10^{-4}$
			pH 4	472	0
			pH 5	497	0
			pH 5	611	0
			pH 6	279	0
			pH 7	215	0
			pH 8	599	0
			pH 9	2642	$1.7 \cdot 10^{-3}$
			Control	744	
2	80	$9.4 \cdot 10^6$	pH 9	499	$3.5 \cdot 10^{-4}$
			pH 4.5, with $4 \cdot 10^{-5}$ M glycerophosphate	353	$1.6 \cdot 10^{-4}$
	0		Control	216	
3	100	$6.0 \cdot 10^6$	pH 9 (3 h)	143	$2.5 \cdot 10^{-4}$
			pH 5 (30 min) → pH 9 (2.5 h)	136	$2.4 \cdot 10^{-4}$
			Control	24	

Reaction of the enzyme with [^{32}P]orthophosphate

The enzyme was incubated with [^{32}P]phosphate at a series of pH values, and then precipitated under acidic conditions[8,10] in which alkaline phosphatase is obtained as the stable phosphorylated derivative. No significant incorporation occurred with acid phosphatase at any pH value (Table I). The minute levels of radioactivity above that of the corresponding control are of the order to be expected for residual adhering phosphate, in view of the enormous numbers of counts per min in the initial incubation mixtures. The phosphorylation of alkaline phosphatase[8,10] shows an inverse pH dependence to that of the enzymic activity, the former occurring maximally at pH near 5. If it behaves equivalently, the acid phosphatase might be expected to show a maximum in its phosphorylation at pH 8–9, where this enzyme[4] has negligible hydrolase activity. However, after incubation periods varying from 2 min to 3 h at pH 9, no significant incorporation occurred, although the enzymic activity is recovered fully after return from those conditions. Prior incubation with [^{32}P]phosphate at pH 5 followed by adjustment of the pH to 9 also yielded no incorporation (Table I). In one experiment, the substrate β-glycerophosphate was also present, again with no in-

TABLE II

EFFECT OF Be^{2+} ON ACID PHOSPHATASE

The enzyme was pre-incubated at 25° with $BeSO_4$ for the period shown (in 0.05 M sodium acetate, pH 5, or 0.05 M Tris–HCl, pH 7) and then assayed at pH 5.0 with substrate containing Be^{2+} at the same concentration. The activity is recorded as the mean of 3 determinations of the initial velocity ($\Delta A_{405\ nm}$ in 60 sec), on *p*-nitrophenyl phosphate as substrate. The quantity of enzyme used in the experiments at pH 7 was about 15% greater than at pH 5.0.

pH	*Incubation (min)*	*Activity*	
		Be^{2+} (10^{-4} M)	*No Be^{2+}*
5.0	10	0.90	0.98
	30	0.94	
	60	0.97	0.91
7.0	10	1.05	1.04
	30	1.11	
	60	1.14	1.11

corporation. Its concentration was such that in the final equilibrium mixture the isotopic dilution was 5-fold, so that any incorporation could still have been easily detected.

Reactions with Be^{2+}

THOMAS AND ALDRIDGE[11] have reported that enzymes which can be phosphorylated by orthophosphate, including two other phosphatases, are also very susceptible to reversible inhibition by Be^{2+}. In the pH range of high activity, treatment with a 100-fold larger concentration of $BeSO_4$ than previously[11] used produced no inhibition of prostatic acid phosphatase (Table II).

Transphosphorylation reactions

Acid phosphatase was permitted to act for various periods of time on *p*-nitrophenyl phosphate in the presence of a high concentration (1.5–1.6 M) of ethanolamine as an acceptor competitive with water. Parallel experiments were performed with *E. coli* alkaline phosphatase. After every incubation, the reaction mixture was fractionated by ion-exchange chromatography, under conditions in which *O*-phosphorylethanolamine is completely separated even from an enormous excess of orthophosphate (Fig. 3).

Phosphorylation of ethanolamine was demonstrated thus in the case of each enzyme. For alkaline phosphatase, this reaction (Fig. 4A) was as expected from the results of WILSON *et al.*[13] on its transphosphorylation reaction. With the acid phosphatase, the acceptor capacity of ethanolamine was very much less, but still appreciable (Fig. 4B).

The reversible hydrolytic reaction of acid phosphatase with *O*-phosphorylethanolamine, which may complicate these measurements, was followed separately, by a similar chromatographic technique (Fig. 5). This hydrolysis was found to be very slow compared to that of nitrophenyl phosphate: an enzyme preparation having an activity of 1850 units/mg in hydrolyzing the latter (0.01 M) has an activity of 12 units/mg

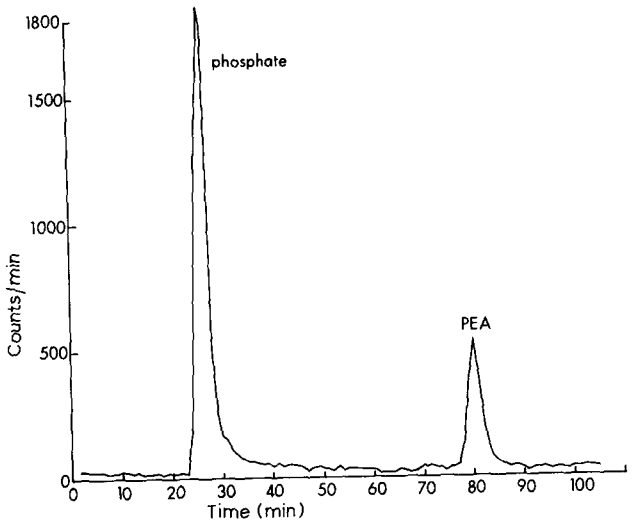

Fig. 3. Separation of *O*-[^{32}P]phosphorylethanolamine (PEA) from excess [^{32}P]phosphate on the amino acid analyzer column, at pH 2.20, 35°. The radioactivity in the effluent, as read continuously in the flow-cell system, is plotted. When a very much larger excess of orthophosphate was present, some tailing of the [^{32}P]phosphate peak was seen due to adsorption on the anthracene in the flow cell[9]; the flow was then temporarily diverted so that almost all of the orthophosphate peak did not pass through the flow cell, which was reconnected 15 min before the [^{32}P]phosphorylethanolamine peak. In those conditions, even 10^5 times more radioactivity in the orthophosphate than in the phosphorylethanolamine peak was completely separated from the latter.

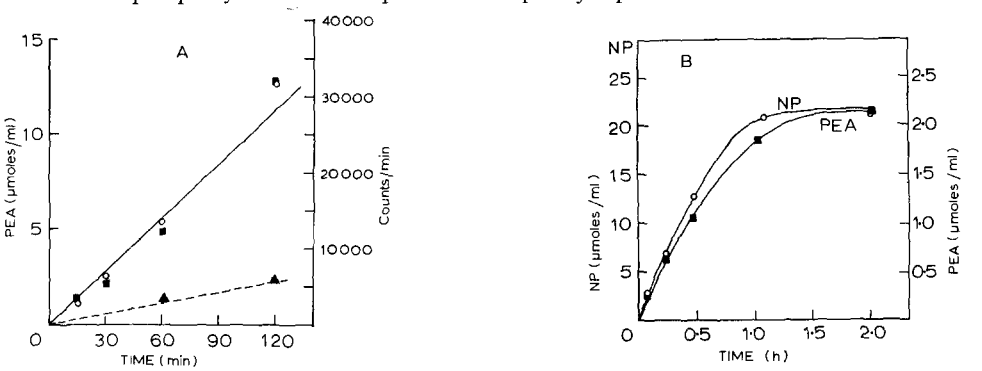

Fig. 4. A. Reaction of alkaline phosphatase (25 μg) with *p*-nitrophenyl phosphate (0.05 M) to form orthophosphate or, with the 1.5 M ethanolamine initially present, *O*-phosphorylethanolamine (PEA; ○—○, μmoles/ml). Radioactivity (■—■, counts/min per ml) is incorporated into the *O*-phosphorylethanolamine from the [^{32}P]orthophosphate present ($3 \cdot 10^6$ counts/min). At the last point shown, the nitrophenyl phosphate decomposition was about 60% complete. The full line corresponds to a constant rate of incorporation of the [^{32}P]phosphate at a level of 0.7% of the *O*-phosphorylethanolamine formed. In the back reaction (▲----▲, counts/min, measured up to 8 h further than shown, to give the linear rate indicated), 0.05 M sodium phosphate replaced the *p*-nitrophenyl phosphate. The pH was 9.0 (ethanolamine–HCl), at 25°. The forward reaction incorporation values (■) are shown corrected for the estimated low level of *O*-phosphorylethanolamine labeling by the back reaction. B. Similar reaction of acid phosphatase at pH 5.0 (0.1 M sodium acetate buffer), using 1.6 M ethanolamine as competitive acceptor. The concentration of the released *p*-nitrophenol (NP, ○—○) was followed spectrophotometrically, and of the product *O*-phosphorylethanolamine (PEA, ■—■) by the analyzer method (each expressed as μmoles/ml). The incorporation of the [^{32}P]phosphate into *O*-phosphorylethanolamine throughout this reaction was below the limit of accurate measurement. Note that at the plateau reached, which corresponds to complete hydrolysis of the substrate, the amount of *O*-phosphorylethanolamine formed is only about one-tenth that of the *p*-nitrophenol liberated.

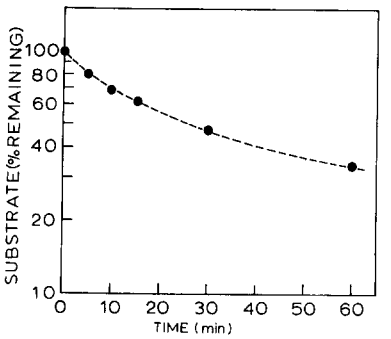

Fig. 5. Hydrolysis of *O*-phosphorylethanolamine (0.02 M) by acid phosphatase, in 0.1M sodium citrate buffer, pH 5.0. The decline from the linear pseudo-first-order plot is attributable to the product inhibition[15] by orthophosphate.

on *O*-phosphorylethanolamine (0.02 M), at pH 5.0, 25°. At these concentrations, the overall maximum velocity is being measured in each case, as shown by similar measurements of the rates in the substrate concentration range 0.001–0.03 M. The reverse reaction, of ethanolamine with phosphate ion, is, as expected, still much slower (Table III).

The transfer reactions were also conducted in the presence of [^{32}P]orthophosphate. With alkaline phosphatase, incorporation of this into *O*-phosphorylethanolamine occurred as the transfer from nitrophenyl phosphate proceeded (Fig. 4A). This incorporation is at a relatively low level, but is highly significant. The direct esterification of ethanolamine by [^{32}P]orthophosphate (termed the back reaction here) was measured separately, and occurred at much too slow a rate to account for the observed exchange (Fig. 4A). Since orthophosphate is a competitive inhibitor[14,15], it was added

TABLE III

FORMATION OF *O*-PHOSPHORYLETHANOLAMINE BY ACID PHOSPHATASE IN THE PRESENCE OF [^{32}P]PHOSPHATE

The amount of *O*-phosphorylethanolamine formed by transfer from *p*-nitrophenyl phosphate was measured, as well as the radioactivity in that amount (third column). Conditions were identical to those used in Fig. 4B, except that sufficient acid phosphatase was added initially to get the faster reaction rate indicated here. The same concentrations of ethanolamine, [^{32}P]phosphate and enzyme were also incubated with 0.05 M orthophosphate similarly, to measure the reverse reaction. The amount of *O*-phosphorylethanolamine formed thus was below the limit of detection in the analyzer ninhydrin system, but was measured by its radioactivity (4th column).

Reaction period (min)	*O-Phosphorylethanolamine formed (μmoles)*	*^{32}P incorporated counts/min*	*^{32}P incorporated in reverse reaction (counts/min)*
5	1.32	200	200
15	1.40		
30	1.46	1020	
120	1.32	1700	1300
120			2080*

* In this incubation, 0.05 M *p*-nitrophenol was added initially.

in the back reaction experiment at the concentration present finally in the transfer reaction; this does not reproduce precisely the conditions in the course of the latter, but since its precursor, *p*-nitrophenyl phosphate, has a K_m value[14–16] similar to or smaller than that of orthophosphate or *O*-phosphorylethanolamine, the back reaction measurement made here will over-estimate, if anything, the contribution of that reaction to the experimental phosphate exchange rate. In the absence of enzyme, no exchange of [^{32}P]phosphate with *p*-nitrophenyl phosphate occurs in the same conditions.

With acid phosphatase, there was no significant orthophosphate exchange during the transfer reaction (Fig. 4B and Table III); the very slight labeling found then was at the rate of the reverse reaction of ethanolamine with orthophosphate. This was confirmed by permitting labeling to continue beyond the completion of the transfer from nitrophenyl phosphate. The labelling of *O*-phosphorylethanolamine then proceeded steadily, to a level sufficiently accounted for by the slow reverse reaction from ethanolamine (Table III). This correspondence became still closer when *p*-nitrophenol was present in the latter case as in the former. The concentration of *O*-phosphorylethanolamine remains nearly constant for a long period (Table III), since its hydrolysis is relatively so slow.

When [^{32}P]phosphate ($4.5 \cdot 10^4$ counts/min) was present with 0.02 M *O*-phosphorylethanolamine in the same conditions as in the transfer experiments of Fig. 4, acid phosphatase catalyzed, as expected, no exchange of ^{32}P into the ester. The latter was separated at intervals from 5 to 60 min, and contained no radioactivity above background; labeling would have been measurable down to an exchange of 0.2% of the phosphate in the ethanolamine phosphate.

DISCUSSION

Prostatic acid phosphatase, in pure form, shows a number of differences from alkaline phosphatase that point to dissimilar catalytic mechanisms. Thus, the acid phosphatase (a) is not phosphorylated by inorganic phosphate at any pH to form a product that is stable to the conditions of isolation of the phosphorylated alkaline phosphatases; (b) is not inhibited by Be^{2+} (inhibition by which has been correlated[11] with enzyme phosphorylation); (c) greatly prefers water to certain acceptors which are efficient with alkaline phosphatases[13], as in the case of ethanolamine (Fig. 4B); and (d) does not equilibrate with added orthophosphate in the course of the normal transferase reaction. While none of these phenomena excludes a phosphoryl-enzyme intermediate, if such exists it is not of the type found with all of the alkaline phosphatases that have been reported upon[8,10,18–21].

With regard to observation (a), it should be noted that the alkaline phosphatases that have been studied in detail from this standpoint, *e.g.* from *E. coli*[10,14] or *Serratia marcescens*[22] or from bovine milk[8] or intestine[18], yield phosphorylated forms that are stable to strong acids. We have used the $HClO_4$ method found by BARMAN AND GUTFREUND[8] to give excellent results with alkaline phosphatase. However, it has been reported[12] that trichloracetic acid precipitation of a less pure preparation of prostatic acid phosphatase after incubation with [^{32}P]phosphate resulted in a product having 0.3 mole ^{32}P per mole of enzyme. It is, of course, possible that a phospho-enzyme form of the prostatic phosphatase is attainable, but is partly or totally decomposed by the

acids to which the corresponding alkaline phosphatase phospho-enzyme is resistant.

The observation (d) above requires more elaboration. Consider the alternative pathways I and II for the action of the enzyme (E) on a phosphate ester (ROP) in the presence of an acceptor molecule (AOH), either *via* a phosphorylenzyme, EP:

$$E\mathrm{H} + \mathrm{ROP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\mathrm{H}\cdot\mathrm{ROP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\mathrm{P} + \mathrm{ROH} \tag{Ia}$$

$$E\mathrm{P} + \mathrm{H_2O} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\mathrm{H} + \mathrm{POH} \tag{Ib}$$

$$E\mathrm{P} + \mathrm{AOH} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E\mathrm{H} + \mathrm{AOP} \tag{Ic}$$

or *via* a single displacement:

$$E\mathrm{H} + \mathrm{ROP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\mathrm{H}\cdot\mathrm{ROP} \underset{k_{-2}}{\overset{k_2 + \mathrm{H_2O}}{\rightleftharpoons}} \mathrm{POH} + \mathrm{ROH} + E\mathrm{H} \tag{IIa}$$

$$E\mathrm{H}\cdot\mathrm{ROP} + \mathrm{AOH} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\mathrm{H} + \mathrm{AOP} + \mathrm{ROH} \tag{IIb}$$

In Mechanism I, external [^{32}P]orthophosphate can equilibrate with the enzyme if the rate k_{-3} is at all appreciable, and in that case the steady-state pool of EP will be labeled, hence labeling the phosphorylated alcohol, AOP, *via* reaction Ic. This seems to be realized in practice in the case of *E. coli* alkaline phosphatase (Fig. 4A). BARRETT *et al.*[21] have provided strong kinetic evidence that phosphate ester hydrolysis by this enzyme involves a phosphoryl-enzyme catalytic intermediate. The present results indicate that that intermediate is indeed in equilibrium with orthophosphate in the course of the catalysis, despite the inability[8,10,18,20] to isolate that intermediate in conditions of high enzymic activity. That inability is to be expected from the known[19,20] high value of k_3 then.

The classical criteria of isotope exchange[22,23] for enzymic double displacement reactions are not usually applied to transferases that are also good hydrolases, since exchange may then occur by complete reversal of the reaction pathway, whatever the nature of the catalytic mechanism. The relative reaction rates for the various steps may, however, be such as still to permit conclusions to be drawn, as was found in the case of 3'-nucleotidase by KOSHLAND[22]. With alkaline phosphatase this is in fact the case, since the introduction of isotope by reaction of orthophosphate with alcohol was much slower than the observed exchange reaction. The fast incorporation of isotope into the *O*-phosphorylethanolamine product requires the presence of (unlabeled) nitrophenyl phosphate, needed to produce a rapid turnover of phosphoryl-enzyme. The equilibration seen in the forward reaction does not, of course, give more detailed information on the mechanism, but a study of the rates of the various exchange reactions (initially and at equilibrium) would help define intermediate steps and relative velocities in Scheme I. This was not pursued here, but it can be noted that the rate of incorporation was such that approx. 0.7% of the molecules of the product were labeled throughout the phase of its initial formation (Fig. 4A), setting a lower limit for k_{-3}. The data can be simply explained by assuming that the finding[24] that phos-

phorylation of *E. coli* alkaline phosphatase at pH 4.6 is much slower with orthophosphate than with nitrophenyl phosphate, extends through the alkaline pH range. There is direct evidence[19] that during ester hydrolysis at alkaline pH the equilibria are such that only a very small fraction of this enzyme is present in the phosphorylated form.

For the acid phosphatase, no exchange reaction of this type could be detected when tests were made during a transfer reaction proceeding at about the same rate. The reaction was, therefore, conducted in conditions such that the product was almost all formed in 5 min (Table III); it was then negligibly labeled, the fractional exchange being 500 times smaller than that found in the alkaline phosphatase case, and even this very low level was all attributable to the reverse reaction. After transfer from nitrophenyl phosphate was completed, the *O*-phosphorylethanolamine continued for much longer times to exchange at this same, slow, reverse reaction rate.

A second type of reverse reaction would be that of the k_{-2} step of Reaction IIa, with the phenol, followed by the transferase Reaction IIb. This would be stimulated by the addition of nitrophenol to the system. Such an effect, slight but probably significant, was detectable in the acid phosphatase reverse reaction measurement (Table III). These two very minor synthesis reactions fully account for the (very slight) labeling occurring in the transferase reaction mixture.

The absence of exchange in the transfer reaction does not in itself totally exclude a phosphoryl-enzyme. The evidence as a whole does show that if a phosphorylated acid phosphatase exists, its status in the reaction scheme is quite different from that for alkaline phosphatase. A large difference between the acid and alkaline phosphatases in the position of the equilibrium (which has been studied in the latter case[25]) between the phosphorylated enzyme (EP) and the Michaelis complex ($E \cdot P_i$) could permit the present findings to be reconciled with a phospho-enzyme pathway.

A recent report[26] on yeast acid phosphatase prefers a single displacement mechanism for that enzyme, based on the lack of enzyme labeling by [^{32}P]phosphate at pH 5, and on the very low degree of transphosphorylation obtainable with acceptors such as methanol. Since prostatic acid phosphatase does give considerable transphosphorylation with alcohols[17], there is some further mechanistic difference between it and the yeast enzyme[26]. Hence, generalizations on the mechanism of action of the phosphomonoesterases should be made only with great caution.

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