Biochimica et Biophysica Acta, 523 (1978) 407—421

BBA 68395

AN ESSENTIAL ACTIVE-SITE HISTIDINE RESIDUE IN HUMAN PROSTATIC ACID PHOSPHATASE

ETHOXYFORMYLATION BY DIETHYL PYROCARBONATE AND PHOSPHORYLATION BY A SUBSTRATE

JOHN J. McTIGUE and ROBERT L. VAN ETTEN *

Chemistry Department, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

(Received August 1st, 1977)

Summary

Human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) is a dimeric (α_2) protein that catalyses the hydrolysis of phosphomonoesters. Several reports suggest that a phosphoenzyme intermediate is involved in the mechanism of acid phosphatase. Chemical modification studies and trapping experiments were therefore undertaken in order to ascertain the identity of the amino acid residue(s) involved in the formation of this intermediate. Human prostatic acid phosphatase is inactivated by diethyl pyrocarbonate (second-order rate constant of 7 $M^{-1} \cdot min^{-1}$ at pH 6.2) with an accompanying increase in absorbance at 242 nm due to formation of ethoxyformylhistidyl derivatives. In the presence of competive inhibitors the the rate of inactivation is decreased. Inactivation can be partially reversed by hydroxylamine. The pH curve of inactivation indicates the involvement of a residue having a pK_a of 6.5. Direct evidence for the involvement of a histidine residue in the mechanism was obtained by trapping a covalent phosphohistidyl-enzyme intermediate. Incubation of the enzyme with *p*-nitrophenyl[^{32}P]phosphate leads to incorporation of 0.44 mol ^{32}P/mol enzyme. The denatured phosphoenzyme, which was acid labile but base stable, was hydrolyzed in 3 M KOH and the radioactivity was found to cochromatograph with synthetic τ-phosphohistidine on Dowex-1 ion-exchange resin. These results are consistent with a catalytic mechanism involving histidine as a nucleophile in the formation of a covalent phosphoenzyme intermediate.

* To whom correspondence should be addressed.

Introduction

Human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) catalyzes the hydrolysis of the monoanionic form of a wide range [1] of phosphomonoesters. Evidence indicates that the acid phosphatase-catalyzed hydrolysis of phosphomonoesters involves a common phosphoenzyme intermediate and follows the minimal scheme shown in Eq. 1.

$$E + ROPO_3H^- \overset{K_s}{\rightleftharpoons} E \cdot ROPO_3H^- \underset{ROH}{\overset{k_2}{\rightarrow}} E\text{-}PO_3H^- \xrightarrow[H_2O]{k_3} E + H_2PO_4^- \qquad (1)$$

The evidence for this mechanism includes the expected ordered uni-bi kinetic scheme [2,3], virtually identical V values in the hydrolysis of several different alkyl and acyl phosphate esters as catalyzed by wheat germ [4] and human prostatic acid phosphatases [1], and the observation of transferase activity [5—7]. Other evidence in support of Eq. 1 includes the observation of a burst of *p*-nitrophenoxide ion when wheat germ acid phosphatase is mixed with *p*-nitrophenyl phosphate at pH > 7 under conditions where [S] > K_m [8]. The pH dependence of both K_m and V with several substrates suggests the involvement of a common ionizable group in the ES complex of prostatic acid phosphatase and this has been ascribed to a phosphohistidine-enzyme intermediate [9]. In addition, the potent inhibition of acid phosphatases by early-transition-metal oxyanions is consistent with the action of such ions as transition state analogs, there being a structural resemblance between these ions and the probable trigonal-bipyramidal transition state for formation and hydrolysis of a phosphoramidate intermediate [10]. Convincing evidence for the presence of a phosphohistidine is shown by the isolation of phosphohistidine from the alkaline hydrolyzate of acid phosphatases which had been labeled by incubation with phosphate [11] or with substrates [8,12—15].

Previous reports of chemical modification of the prostatic enzyme indicated that serine [16], tyrosine [17] or tryptophan but not histidine [18] amino acid residues were involved in the catalytic mechanism. More recent investigations have eliminated these residues from consideration [19,20] and photooxidation studies in the presence of Rose Bengal which led to inactivation of the enzyme were held to be consistent with the participation of histidyl residues [20]. However, photooxidation is a rather unselective procedure and the large number of residues modified leave this interpretation open to question. From experiments in which impure enzyme was incubated with inorganic[^{32}P]-phosphate Greenberg and Nachmansohn [19] reported the incorporation of radioactive label into the enzyme and the phosphorylation of an amino acid other than serine [19]. Further attempts to identify the phosphorylated residue were not reported. While some other labeling experiments with homogenous enzyme and inorganic[^{32}P]phosphate led to no incorporation of phosphate into the enzyme [21] the procedure for isolation of the labeled protein involved precipitation with $HClO_4$ which would remove any acid-labile phosphoenzyme component.

The inconsistency of previously reported labeling experiments and the difficulty in interpretation of previous studies of the chemical modification of

prostatic acid phosphatase prompted the present investigation. We describe here the inactivation of the enzyme by diethyl pyrocarbonate and also demonstrate that protein-bound phosphohistidine is formed when the enzyme is incubated with the substrate *p*-nitrophenyl[^{32}P]phosphate. Portions of this study were described in preliminary communications [13,14,22] and subsequently by other investigators [15]. The results provide evidence for the importance of a histidine residue for the activity of the enzyme.

Experimental procedures

Materials. Homogeneous enzyme was obtained essentially by the method described by Ostrowski [23]. *p*-Nitrophenyl[^{32}P]phosphate (spec. act. 1—10 mCi/mol) was obtained from Amersham-Searle Corp. and was used without further purification. Diethyl pyrocarbonate, *p*-nitrophenyl phosphate, L-(+)- and D-(—)-tartrate, trinitrobenzene sulfonate, and pyridoxal phosphate were from Sigma Chemical Co. Dowex AG-1X8 was obtained from Bio-Rad Laboratories and was prepared by thorough recycling and washing [24]. Thiomalic acid was obtained from Evans Chemetics, Inc. τ-Phosphohistidine * was synthesized from phosphoramidate [25] and purified as described by Hultquist et al. [24]. All other materials were analytical reagents or the highest grade available, and were used without further purification.

Enzyme assay. The assay substrate solution was 5 mM *p*-nitrophenyl phosphate in assay buffer (0.10 M citrate/sodium citrate, pH 5.0). A 10 μl sample of enzyme solution diluted in assay buffer was added to 1.0 ml of substrate solution at 25° C. After 1 min the reaction was stopped by the addition of 0.4 M NaOH (2.0 ml). The liberated *p*-nitrophenoxide ion was determined spectrophotometrically at 400 nm. A molar extinction coefficient $\epsilon = 18.3 \cdot 10^3$ l/mol cm was used in the calculations [26]. 6—8 determinations were performed on each enzyme stock solution. One unit of acid phosphatase activity is defined as the amount required to produce 1 μmol of *p*-nitrophenol per min.

Homogeneous enzyme has a specific activity of 280 units/mg. Protein concentrations were determined [27] using crystalline bovine serum albumin as a standard. Absorbance measurements were made on a Gilford 2000 or a Cary 118 spectrophotometer.

Electrophoresis. Electrophoresis in polyacrylamide gels at 7% monomer concentration and pH 9.5 and at 15% monomer concentration and pH 4.3 was performed as per Canal Industrial Corp., Bethesda, Md. The gels were stained for protein with Coomassie Brilliant Blue (1.26 g in 454 ml 50% methanol and 46 ml glacial acetic acid) and destained in 7.5% acetic acid. The gels were stained for enzyme activity by incubation in a solution of 10 mg α-naphthyl acid phosphatase and 10 mg Fast Garnet GBC salt in 10 ml 0.1 M sodium citrate (pH 5.0). Activity staining was stopped by the addition of 7.5% acetic acid.

Modification by pyridoxal phosphate. All procedures involving pyridoxal

* The nomenclature is that recommended by the IUPAC-IUB. The nomencalture π- and τ-phosphohistidine has been recommended to relieve the ambiguity associated with the differing usage of names such as 1- and 3-phosphohistidine in the chemical and biochemical literature. These derivatives are also collectively called N'-phosphonohistidine in the chemical literature.

phosphate were performed at very low levels of room light or in the dark. Enzyme was incubated with 10 mM pyridoxal phosphate in 0.05 M sodium borate (pH 8.0) at 30°C in the presence or absence of 0.1 M L-(+)-tartrate. Aliquots (25 μl) were removed from the inactivation mixture at various times and activity determined by the standard assay procedure except with the addition of 1.5 mM pyridoxal phosphate to the assay substrate solution. Controls were run under the same conditions but without additions.

Modification by trinitrobenzene sulfonate. The reaction mixture contained 2 mM trinitrobenzene sulfonate in 0.05 M sodium borate at pH 8.0, 8.5 and 9.2. The reaction was initiated by the addition of enzyme at a final concentration of 0.1 μM followed by incubation at 25°C in the presence or absence of 0.1 M inorganic phosphate or 0.1 M L-(+)-tartrate. Aliquots (25 μl) were removed from the inactivation mixture at various times, and activity was determined by the standard assay procedure.

Diethyl pyrocarbonate concentration and ethoxyformylation of enzyme. The concentration of commercial reagent was determined by NMR [28] and found to be 7.0 M which agreed with the concentration of neat reagent calculated from the specific gravity (d_4^{20} 1.12). This concentration was higher than the 2.8 M obtained using the spectrophotometric method of Holbrook and Ingram [29]. In the present report 7 M is used for the concentration of the neat reagent. Stock solutions were prepared by dilution with absolute ethanol. The extinction coefficient was determined by reaction of 4 mM reagent with 0.1 mM *N*-acetylhistidine in 0.05 M dimethylglutarate (pH 6.8, I = 0.15 M with NaCl). After 15 min of incubation at room temperature the increase in absorbance at 242 nm was measured and an $\epsilon = 3.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ was determined. The effect of diethyl pyrocarbonate on the activity of the enzyme was investigated by incubation of reagent with enzyme at 25°C. Aliquots were taken periodically and assayed for activity using the standard enzyme assay described above. Pseudo first order rate constants for the initial rate of loss of enzymic activity were obtained from a computer fit of the data to a non-linear least-squares equation. As a control experiment, enzyme was incubated under the same conditions with absolute ethanol added in place of stock diethyl pyrocarbonate solution.

Amino acid analyses. Enzyme samples following modification with diethyl pyrocarbonate were diluted with 1 volume ice-cold 50% acetic acid, dialyzed against 10% acetic acid for 8 h, 5% acetic acid for 12 h and then lyophilized. The lyophilized samples were hydrolyzed for 25 h at 110°C in evacuated glass tubes containing 0.5 ml 6 M HCl. Analyses were performed with a Durrum D-500 amino acid analyzer.

Preparation of ^{32}P-labeled enzyme. When purified or partially purified enzyme (0.2—0.4 mg) is incubated with 2—20 mM *p*-nitrophenyl[^{32}P]phosphate in 0.05 M sodium acetate (pH 5.0) or 0.025 M sodium barbital (pH 7.0) a phosphorylated protein results. The mixing-quenching technique involved the placement of 1 ml of the enzyme and substrate solutions into separate syringes connected by 0.8 mm (internal diameter) Teflon tubing to a Plexiglass tee. The solutions were mixed through the tee and the resulting reaction mixture was squirted through a syringe needle into 3 ml of rapidly stirred 1.0 M NaOH which acted to quench the enzyme reaction. The total mixing time was 1—4 s.

A slower quenching technique involved simply adding 3 ml of 1.0 M NaOH to the enzyme/substrate solution. The quenched mixutre was then applied to a Sephadex G-100 column (2.5 × 50 cm) equilibrated with 0.05 M borate (pH 9.2)/0.25% sodium dodecyl sulfate (SDS)/0.01 M thiomalic acid/1 mM sodium pyrophosphate in order to separate the protein from P_i and unreacted substrate and the eluent was collected directly into scintillation vials and quantitated by counting in a Packard Tricarb liquid scintillation spectrometer model 3320 using Cerenkov radiation. The quantity of incorporated label was determined by integration of the resulting radioactivity curves.

Identification of the labeled amino acid. The labeled protein was precipitated by addition of acetone, allowed to stand at −20° C overnight and centrifuged. The precipitate was hydrolyzed in 4 ml 3 M KOH for 5 h at 110° C, diluted with 50 ml water, applied to Dowex-1 column (1.5 × 26 cm) and eluted with a linear gradient of 0.2—0.8 M $KHCO_3$ and collected directly into scintillation vials and counted. Two radioactive peaks were found. The major peak was rechromatographed on a Dowex-1 column (0.9 × 15 cm) with 2 mg of synthetic τ-phosphohistidine and eluted with a linear gradient of 0.2—0.8 M $KHCO_3$. The amino acid content was quantitated by reacting 0.5 ml from each vial with 0.5 ml ninhydrin solution (2.0 g ninhydrin/62.5 mg hydrindantin/25 ml 4.0 M lithium acetate/75 ml dimethylsulfoxide). After 15 min at 90° C the absorption at 570 nm was determined. The radioactivity was quantitated as described above. The minor peak was identified as inorganic phosphate from its elution position.

Results

Modification studies

After 1 h of incubation pyridoxal phosphate reduced the enzyme activity by 10% and no protection was afforded by the presence of L-(+)-tartrate. At the three pH values studied, trinitrobenzene sulfonate completely inactivated the enzyme but the rate of inactivation was the same in the presence or absence of a competitive inhibitor. These results provide no evidence for an active-site lysyl residue.

The reaction of diethyl pyrocarbonate with prostatic acid phosphatase results in the rapid loss of enzymic activity (Fig. 1). The initial pseudo first-order rate constant is independent of enzyme concentration (0.2—20 μM), but is linearly dependent on the modification reagent concentration giving a second-order rate constant of $7\ M^{-1} \cdot min^{-1}$ (correlation coefficient 0.98, $n = 6$).

To determine if the modified residue(s) is present at or near the active site, competitive inhibitors were added to the modification reaction mixture. Both L-(+)-tartrate and inorganic phosphate protected the enzyme against inactivation. L-(+)-Tartrate in relatively low concentrations ($3 \times K_i$) decreased the rate of inactivation 3-fold whereas inorganic phosphate at a higher concentration ($25 \times K_i$) decreased the rate of inactivation 8-fold (Fig. 2). The non-inhibitory D-(−)-tartrate did not affect the rate of inactivation. These results suggest that the amino acid residues whose modification results in inactivation of the enzyme are present at or near the active site.

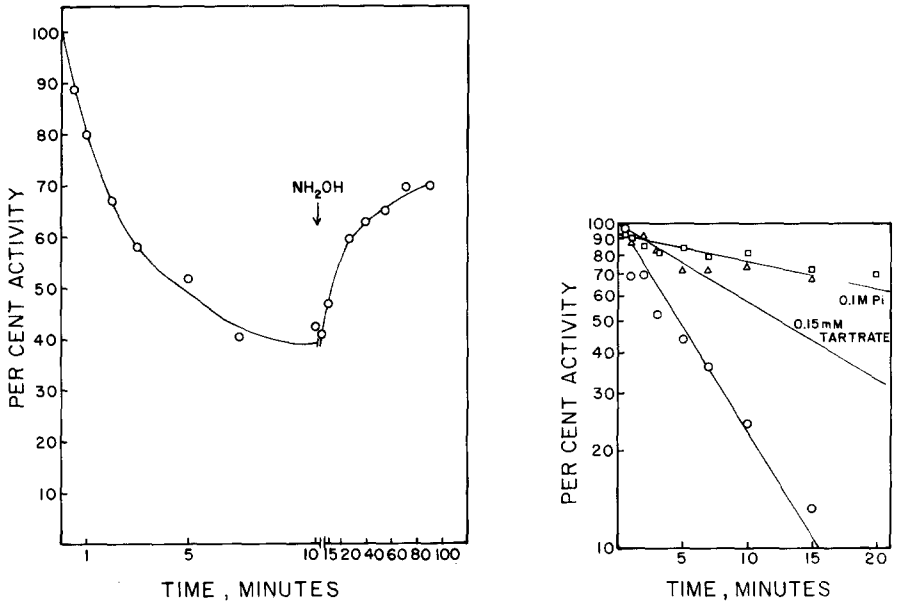

Fig. 1. The inactivation of acid phosphatase with diethyl pyrocarbonate and reactivation with hydroxylamine. The enzyme (0.2 μM) was mixed with 10 mM reagent in 0.025 M sodium barbital pH 6.9 (I = 0.15 M with NaCl) at 25°C. After 10 min NH_2OH was added to a final concentration of 0.2 M. Aliquots were withdrawn at various times and assayed directly by the standard assay. Activity was the percentage of activity in a control of native enzyme treated in an identical manner using absolute ethanol in place of diethyl pyrocarbonate solution.

Fig. 2. Competitive inhibitor protection against inactivation of acid phosphatase with diethyl pyrocarbonate. Inorganic phosphate (□, 0.1 M), L-(+)-tartrate (△, 0.15 mM), and D-(—)-tartrate (○, 0.15 mM) were mixed with phosphatase in 0.025 M sodium barbital pH 6.5 (I = 0.15 M with NaCl). Diethyl pyrocarbonate was added to a final concentration of 8 mM and aliquots were taken at various times and assayed by the standard assay. A control experiment in which no phosphate or tartrate was added gave the same rate of inactivation as with D-(—)-tartrate.

Diethyl pyrocarbonate is a relatively specific modification reagent at pH 6 for histidyl residues [30,31]. However, in neutral or weakly alkaline media other amino acid residues can react [30—33]. To determine if histidine modification was responsible for the inactivation, control experiments were performed. The measured rate of inactivation of the enzyme should depend on the degree of protonation of the presumed histidine [34]. The rate constant for inactivation k_{app} should therefore be correlated with the second-order rate constant k_2 for the modification of the unprotonated histidine, the apparent dissociation constant K_a of the acidic form of histidine and the H^+ concentration by the equation $1/k_{app} = (1/k_2 + [H^+]/k_2K_a)$. The intercept and slope obtained from a plot of $1/k_{app}$ vs. $[H^+]$ (Fig. 3) were used to calculate an apparent pK_a of 6.5. This value is similar to the pK_a reported for histidyl residues in other enzymes [29,35] which could be modified by diethyl pyrocarbonate. The biphasic nature of the pH dependence is probably caused by the modification of amino acid residues other than histidine at pH values greater than 7.3 [34] and therefore the correlation shown in Fig. 3 employed the points obtained at pH values less than 7.2. Subsequent investigations of the modification of

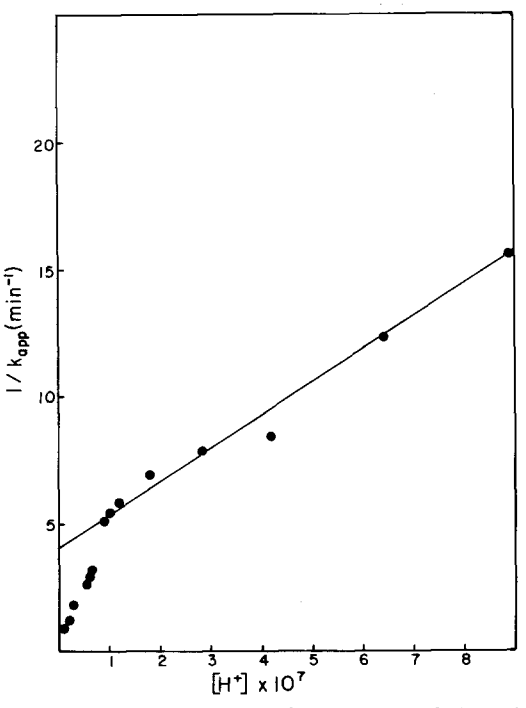

Fig. 3. The pH dependence of inactivation of prostatic acid phosphatase with diethyl pyrocarbonate. All reactions were conducted at 25°C in 0.05 M dimethyl glutarate (I = 0.15 M with NaCl). The second-order rate constant (k_2) was obtained from the pseudo first-order rate constant for inactivation (k_{app}) and the concentration of diethyl pyrocarbonate and plotted as $1/k_{app}$ vs. $[H^+]$.

human prostatic acid phosphatase were carried out at pH values below 7.

Reactivation of diethyl pyrocarbonate-inactivated enzymes by treatment with hydroxylamine is generally taken to indicate that an ethoxyformyl-histidyl bond is being cleaved, since the more stable ethoxyformyl-lysyl derivative does not undergo significant reaction during the usual time of such reactivation experiments [32]. When enzyme was inactivated and the reaction stopped with approx. 40% activity remaining it was found that the addition of 0.2 M NH_2OH only partially (about 25%) reactivated the enzyme (Fig. 1). The inability to fully reactivate the enzyme with NH_2OH may be due to imidazole ring-opening via a Bamberger reaction [36]. To test this possibility the amino acid content of the modified and reactivated enzyme was determined. The modified protein was hydrolyzed under acid conditions which should regenerate histidine from ethoxyformyl histidine. However, the number of histidyl residues in the modified enzyme had decreased by 2—3. The content of other residues remained the same.

The ethoxyformylation of a histidyl residue causes an increase in absorbance at 242 nm [37] while the modification of a tyrosyl residue results in a decrease in absorbance at 280 nm [30]. The 80% inactivation of enzyme leads to no decrease in 280 nm absorbance but results in an increase in the 242 nm absorbance which is quantitatively consistent with the formation of ethoxy-

formyl-histidyl derivatives of 12 of 27 histidine residues present in the enzyme [38] and no modification of tyrosyl residues (Fig. 4). To ascertain if the absorbance change could be correlated with the modification of a histidine residue located specifically at the active site the absorbance change at 242 nm was followed in the presence of the inhibitory L-(+) and non-inhibitory D-(−) forms of tartrate (Fig. 5). The reference cuvette contained 1.5 mM L-(+)-tartrate (12 × K_i) while the sample cuvette had 1.5 mM D-(−)-tartrate. It may be noted that when the difference in activity between the two solutions is 50% (approx. 12 min) the absorbance difference (lower curve, Fig. 5) corresponds to the modification of one histidine at the active site. Further inactivation of the enzyme in the D-(−)-tartrate-containing solution leads to a still greater difference in absorbance which corresponds to the modification of another histidine in this dimeric enzyme [6,14], presumably in the other subunit. Because the enzyme in the L-(+)-tartrate-containing solution is slowly being inactivated, a difference of 100% in activity and a corresponding difference in modification of two histidine residues could not be attained. The addition of hydroxylamine decreases the 242 nm absorbance and partially reactivates the enzyme but this decrease in absorbance and the concomitant regeneration of histidine cannot be exactly correlated with the reactivation of the enzyme indicating that some other process may be affecting enzyme activity. Nevertheless, these results indicate that the modification of an active site histidine residue results in inactivation of human prostatic acid phosphatase.

Labeling of enzyme with p-nitrophenyl[^{32}P]phosphate.

The purity of the protein used in the labeling experiments was determined from the specific activity of the enzyme. A single band of activity and of protein in disc gel electrophoresis experiments at two pH values corresponded to

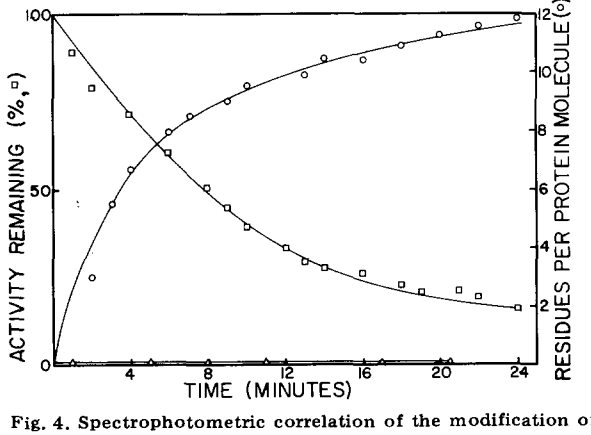

Fig. 4. Spectrophotometric correlation of the modification of histidine residues with the inactivation of acid phosphatase (18 μM) by diethyl pyrocarbonate (5 mM) in 0.05 M dimethyl glutarate (pH 6.8, I = 0.15 with NaCl) at room temperature. The reaction was followed with time in a Gilford 2000 spectrophotometer with the reference cuvette containing absolute ethanol and the sample cuvette containing diethyl pyrocarbonate. Modification of histidine was followed by the increase in absorption at 242 nm (○) using $\Delta\epsilon_{242}$ = 3500 and modification of tyrosine residues was followed by the decrease in absorption at 280 nm (△). At various times, aliquots were withdrawn, diluted in ice-cold dimethyl glutarate buffer and assayed for activity (□) by the standard assay.

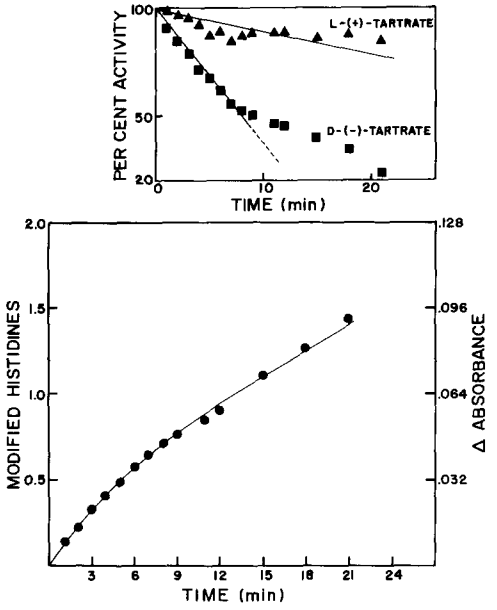

Fig. 5. Correlation of the difference in absorbance at 242 nm (•) with the inactivation of acid phosphatase (18 μM) by diethyl pyrocarbonate (5 mM) in the presence of L-(+)- or D-(—)-tartrate. The reaction was followed with time in a Cary 118 spectrophotometer at room temperature in 0.05 M dimethyl glutarate, pH 6.8, with the sample cuvette containing 1.5 mM D-(—)-tartrate and the reference cuvette containing 1.5 mM L-(+)-tartrate. At various times, aliquots were withdrawn, diluted in ice-cold dimethyl glutarate buffer and assayed for activity by the standard assay (upper curve). The calculation of the number of modified histidines at the active site was based on a molar extinction coefficient of 3500 $M^{-1} \cdot cm^{-1}$ for ethoxyformyl histidine at 242 nm (lower curve).

a specific activity of 280 μmol/min per mg. Therefore, an enzyme of specific activity 280 was taken to correspond to pure protein. Active site burst titrations were not used to determine enzyme purity because of the difficulty in interpreting such bursts at substrate concentrations below K_m [8,9].

Enzyme preparations having specific activities of 175—280 units/mg were labeled with *p*-nitrophenyl[^{32}]phosphate, quenched in 1.0 M NaOH (see Experimental procedures) and placed on a column of SephadexG-100. Chromatography showed that the protein peak was coincident with a peak of radioactivity (Fig. 6). Table I shows the quantitative results from several labeling experiments in which an average of 0.44 mol ^{32}P/mol enzyme was incorporated. It is noteworthy that the amount of incorporation parallels the increase in purity (specific activity) of the enzyme; that a non-catalytic protein, bovine serum albumin, is not labeled when incubated under the same conditions, and that incorporation is significantly reduced by the presence of a competitive inhibitor ($300 \times K_i$) or by very long reaction times corresponding to total substrate hydrolysis. In addition slightly increasing the reaction time and slowing the speed of quenching of the reaction leads to decreased incorporation. Increasing the pH of the reaction mixture to 7 does not significantly affect the percent incorporation.

The stability of the phosphoenzyme is dependent on pH as is seen in Fig. 7. Each point on the curve represents the percentage of hydrolysis of the phos-

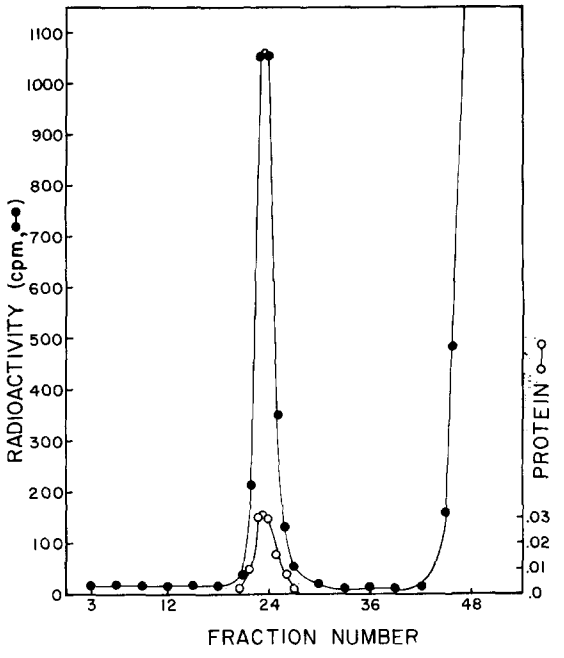

Fig. 6. Separation of ^{32}P-labeled acid phosphatase from substrate and other reaction products on Sephadex G-100. The enzyme was labeled by *p*-nitrophenyl[^{32}P]phosphate and chromatographed as described under Experimental Procedures. The radioactivity was quantitated as counts/min per vial (●, cpm) and the protein was quantitated as the difference in abosrbance at 280 nm. Fractions (4 ml) were collected.

phoenzyme after 20 h of dialysis at room temperature against the stated buffer at the designated pH. It is evident that the denatured phosphoenzyme is an acid-labile, base-stable compound. Such pH vs. stability profiles are charac-

TABLE I

COVALENT LABELLING OF PROSTATIC ACID PHOSPHATASE WITH *p*-NITROPHENYL [^{32}P]-PHOSPHATE

The enzyme was isolated and analyzed as described in Experimental Procedures.

Protein	Purity (%)	Reaction time	Quenching technique	Incorporation (%)
Bovine serum albumin		2 s	rapid *	0.1
Acid phosphatase	100	30 m	rapid	1
Acid phosphatase	100	15 s	slow **	24
Acid phosphatase	100	2 s	rapid	46
Acid phosphatase + 0.1 M L-tartrate	100	2 s	rapid	1
Acid phosphatase + 0.1 M D-tartrate	100	2 s	rapid	36
Acid phosphatase	100	2 s	rapid	39 ***
Acid phosphatase	60	2 s	rapid	24
Acid phosphatase	80	2 s	rapid	36
Acid phosphatase	90	2 s	rapid	43
Acid phosphatase average of 25 expts.		2 s	rapid	44 ± 6 †

* Typically 1—4 s (see Experimental procedures).

** >5 s.

*** 0.025 M sodium barbital buffer, pH 7.0.

† Standard deviation of the mean.

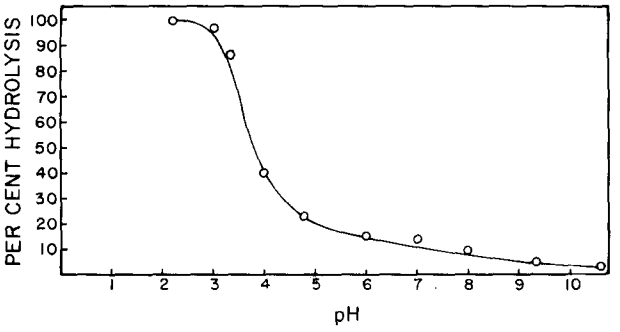

Fig. 7. Effect of pH on the decomposition of denatured phosphoenzyme. Fractions under the radioactivity protein peak of a Sephadex G-100 column (as in Fig. 6) were pooled and 2-ml (4000 cpm) samples were placed in dialysis bags and dialyzed against 4 l of the stated buffer at the designated pH for 20 h at room temperature. The radioactivity in 2 ml of dialysis buffer was subtracted from the radioactivity in the dialysis bag and this quantity was divided by the radioactivity in the control (2 ml of undialyzed pooled fractions) to give the percent hydrolysis of the phosphoprotein. The buffers were 0.1 M and used at the following pH: 2.2, 3.0 and 3.5, glycine/HCl; pH 4.0 and 4.8, sodium acetate; pH 6.0, sodium maleate; pH 7.0, Tris/sodium maleate; pH 8.0, Tris; pH 9.3, Sephadex G-100 column buffer; pH 10.6, glycine/NaOH.

teristic of phosphohistidine [39], but not of the acid-stable, base-labile *O*-phosphoserine [40], or of the U-shaped stability curves for acyl phosphates [41]. The pH vs. stability data suggest that the phosphoryl group in phosphoenzyme is attached to the imidazole moiety of a histidine residue.

In order to confirm that the phosphorylated amino acid is phosphohistidine and to establish the location of the phosphoryl linkage in the histidine moiety, the labeled protein was precipitated with acetone, hydrolyzed in KOH and chromatographed on Dowex-1 (see Experimental procedures). The elution profile of the hydrolysate shows two radioactive peaks (Fig. 8). The first radioactive peak (representing approx.20% of the applied radioactivity) was identified as inorganic phosphate because of its elution position and is probably due to partial hydrolysis of the phosphohistidine [39]. The major radioactive peak representing the remainder of the applied radioactivity had the same elution

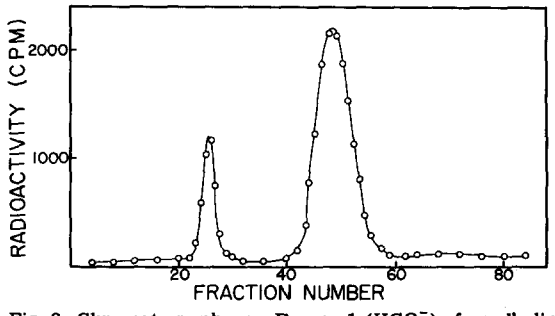

Fig. 8. Chromatography on Dowex-1 (HCO_3^-) of an alkaline hydrolyzate of ^{32}P-labeled enzyme. A sample of ^{32}P-labeled phosphoenzyme prepared, isolated and hydrolyzed as described under Experimental Procedures was absorbed to a column (1.5 × 25 cm) of Dowex 1-X8 (HCO_3^-) at room temperature. Elution was performed with a linear gradient of 0.2—0.8 M $KCHO_3$. Total elution volume was 400 ml collected in fractions of 5 ml. The radioactivity was quantitated by Cerenkov radiation.

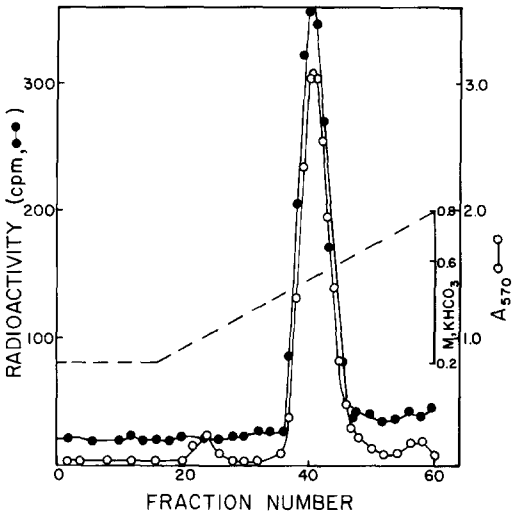

Fig. 9. Rechromatography on Dowex-1 (HCO_3^-) of the major peak of radioactivity from the chromatography in Fig. 8 and synthetic τ-phosphohistidine on a column, 0.9 X 14 cm, of Dowex 1-X8 (HCO_3^-). Elution was performed with a linear gradient of 60 ml of 0.2 M to 60 ml of 0.8 M $KHCO_3$. 2-ml fractions were collected and analyzed for radioactivity (●, cpm) and amino acid content (○) as described under Experimental Procedures.

position as synthetic τ-phosphohitidine and was rechromatographed on Dowex-Dowex-1 with added synthetic τ-phosphohistidine. The elution profile of this mixture clearly shows that the product resulting from alkaline hydrolysis of ^{32}P-labeled phosphoenzyme is eluted at the same position as τ-phosphohistidine (Fig. 9). The small ninhydrin-positive peak has the same elution position as histidine and is probably due to the breakdown of the synthetic τ-phosphohistidine.

Discussion

Diethyl pyrocarbonate reacts with several protein amino acid side chains including imidazole, phenolates, sulfhydryls, and α- and ϵ-amines [30,32] but under neutral or slightly acid conditions the specificity for histidine is greatly increased [30]. Other experiments support only the modification of histidine as being responsible for the loss of activity of the enzyme upon modification with diethyl pyrocarbonate. These control experiments include modification by dithiobis-(2-nitrobenzoic acid) (unpublished results). The rate of inactivation by dithiobis-(2-nitrobenzoic acid) was the same in the presence and absence of competitive inhibitors indicating that an essential cysteinyl residue is not present at or near the active site *. At the experimental pH values used in the inactivation of enzyme by diethyl pyrocarbonate there was no change in absorbance at 280 nm so it is unlikely that tyrosyl residues are being modified. The possible ethoxyformylation of lysyl residues is suggested by the inability

* However, it should be noted that Bittencourt and Chaimovich [42] have reported that a low molecular weight acid phosphatase from bovine brain contains an essential cysteine residue.

to fully reactivate the modified enzyme with hydroxylamine; such a conclusion has been reached for the case of tryptophan synthetase [43]. However, experiments with trinitrobenzene sulfonic acid and with pyridoxal phosphate provide no evidence for active site lysyl residues. Therefore, another interpretation of the lack of full reactivation by NH_2OH is provided by the Bamberger cleavage of the imidazole ring [36,44]. Amino acid analyses of the modified and NH_2OH-reactivated enzymes showed a decrease of 2—3 histidine residues and some protection from loss of histidine by the presence of the inhibitory form of tartrate. The results indicate a loss of histidine content upon modification by diethyl pyrocarbonate similar to the results reported for modification of alcohol dehydrogenase and of ribonuclease [36]. Therefore, the lack of reactivation by NH_2OH cannot be regarded as an absolute criterion for distinguishing between the modification of histidine and of lysine, especially when a relatively high concentration of modification reagent is used or when a large number of histidines are modified. Another criterion for distinguishing between the diethyl pyrocarbonate modification of histidine and of lysine is the second-order rate constant [34]. For the modification of prostatic acid phosphatase the second-order rate constant of $7\ M^{-1} \cdot min^{-1}$, although low, is consistent with histidine modification and is similar to the rate constant ($10\ M^{-1} \cdot min^{-1}$) for inactivation of thermolysin [33]. (If the concentration is determined by the spectrophotometric technique of Holbrook and Ingram [29] rather than the NMR method of Lee and van Etten [28] a lower diethyl pyrocarbonate concentration would be estimated and a second-order rate constant for inactivation of $18\ M^{-1} \cdot min^{-1}$ would be calculated.)

Consequently, the control experiments support the concept that inactivation of the phosphatase is due to the specific modification of an active site histidine residue at $pH < 7$. This specificity for histidine modification at the active site was confirmed by (a) the decrease in rate of inactivation in the presence of competitive inhibitors, (b) the partial reversal of inactivation by NH_2OH, (c) the dependence of the reaction rate on a residue with a pK_a equal to 6.5, (d) a second-order rate constant that is consistent with modification of a histidyl residue with fairly ordinary pK_a and (e) by the comparison of the absorbance of the native and modified enzyme at 242 nm in the presence of two stereoisomeric forms of tartrate which allowed investigation of histidine modification at the active site. The use of a stereoisomer of a competitive inhibitor proved very advantageous in investigating modification at the active site because it provided a control for non-specific interactions (ionic strength effects, etc.) or chemical reactivity between the active site protector and the modifier or the enzyme. This type of control has only rarely been utilized in chemical modification experiments and would seem to be particularly useful when a relatively high concentration of the active site protecting reagent is required.

Direct evidence for the participation of histidine in the catalytic activity is obtained from the incorporation of ^{32}P from a substrate into the enzyme. This incorporation parallels the increase in the specific activity of the enzyme but does not represent the stoichiometric incorporation of 2 mol ^{32}P/mol enzyme, assuming two active sites per enzyme molecule [6,14,45]. The lack of stoichiometric incorporation could be due to negative cooperativity between the subunits similar to that reported for alkaline phosphatase [46] or due to the inabil-

ity to stop the dephosphorylation reaction in the time before the enzyme completely denatured in the quenching medium. A test of this explanation will come with the planned application of rapid mixing-rapid quenching methods and instrumentation to this system.

The site on the enzyme which covalently binds the phosphoryl group is taken to be a histidyl residue because of the shape of the pH vs. stability profile (Fig. 7). Curves which exhibit acid lability but base stability are typical of synthetic [39] and enzyme-linked phosphoramidates [47]. A further, powerful argument for the identification of the phosphoenzyme as a phosphohistidine derivative rests on the isolation of the phosphorylated amino acid residue from an alkaline hydrolyzate of the labeled protein and the identification of that labeled amino acid as τ-phosphohistidine. This, taken together with the transphosphorylation to an alcohol [6] is most readily interpreted as indicating that the phosphohistidine intermediate lies on the direct pathway between substrate and product in the mechanism of action of the enzyme.

In summary, the results of this study in which the chemical modification of one histidine per enzyme active site leads to inactivation and in which a phosphoenzyme intermediate was isolated and chemically characterized as containing τ-phosphohistidine suggests that histidine is acting as a nucleophile in the mechanism of prostatic acid phosphatase-catalysed hydrolysis of phosphomonoesters.

Acknowledgements

This research was supported by grant CA 10585 from the National Cancer Institute of the U.S. Public Health Service. The enzyme was obtained in part from material supplied by Professor Dr. Ulrich Hachmeister of Universitäts Klinikum Giessen, German Federal Republic and in part from material supplied by Dr. T.I. Malinin through the support of U.S. National Prostatic Cancer Project grant CA 15480. The authors thank Mr. William Kohr for the amino acid analyses.

References

1 Kilsheimer, G.S. and Axelrod, B. (1957) J. Biol. Chem. 227, 879—890
2 Hsu, R.Y., Cleland, W.W. and Anderson, L. (1966) Biochemistry 5, 799—807
3 Belfield, A., Ellis, G. and Goldberg, D. (1972) Enzymologia 43, 91—106
4 Hickey, M.E., Waymack, P.P. and van Etten, R.L. (1976) Arch. Biochem. Biophys. 172, 439—448
5 Axelrod, B. (1948) J. Biol. Chem. 172, 1—13
6 Ostrowski, W. and Barnard, E.A. (1973) Biochemistry 12, 3893—3898
7 Morton, R.K. (1955) Disc. Faraday Soc. 20, 149—156
8 Van Etten, R.L. and Hickey, M.E. (1977) Arch. Biochem. Biophys. 183, 250—259
9 Van Etten, R.L. and McTigue, J.J. (1977) Biochim. Biophys. Acta 484, 386—397
10 Van Etten, R.L., Waymack, P.P. and Rehkop, D.M. (1974) J. Am. Chem. Soc. 96, 6782—6785
11 Igarashi, M., Takhashi, H. and Tsuyama, N. (1970) Biochim. Biophys. Acta 220, 85—92
12 Hickey, M.E. and Van Etten, R.L. (1972) Arch. Biochem. Biophys. 152, 423—425
13 Van Etten, R.L. and McTigue, J.J. (1972) Abstracts of the 164th National Meeting of the American Chemical Society, New York, N.Y. 27 Aug—Sept. 1972. Abstr. Biol. 93
14 Van Etten, R.L. and McTigue, J.J. (1973) Abstracts of the 9th International Congress of Biochemistry, Stockholm, July 1973, Abstr. 2P5
15 Ostrowski, W. (1976) Abstracts of the 10th International Congress of Biochemistry, Hamburg, July, 1976, Abstr. 04-3-366

16 Greenberg, H. and Nachmansohn, D. (1964) Biochem. Biophys. Res. Commun. 67, 1353—1359
17 Bobrzecka, D., Ostrowski, W. and Rybarska, J. (1968) Acta Biochim. Pol. 15, 369—379
18 Bobrzecka, K., Rybarska, J. and Ostrowski, W. (1969) Acta Biochim. Pol. 16, 11—15
19 Greenberg, H. and Nachamansohn, D. (1965) J. Biol. Chem. 240, 1639—1646
20 Rybarska, J. and Ostrowski, W. (1974) Acta Biochim. Pol. 21, 377—390
21 Ostrowski, W. and Barnard, E.A. (1971) Biochim. Biophys. Acta 250, 131—142
22 McTigue, J.J. and van Etten, R.L. (1977) Fed. Proc. 36, 635
23 Ostrowski, W. (1968) Acta Biochim. Pol. 15, 213—225
24 Hultquist, D.E., Moyer, R.W. and Boyer, P.D. (1966) Biochemistry 5, 322—332
25 Stokes, N.H. (1893) Am. Chem. J. 15, 198—214
26 Biggs, A.I. (1954) Trans. Faraday Soc. 50, 800—802
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
28 Lee, G.D. and van Etten, R.L. (1975) Arch. Biochem. Biophys. 166, 280—294
29 Holbrook, J.J. and Ingram, V.A. (1973) Biochem. J. 131, 729—738
30 Muhlrad, A., Hegyl, G. and Toth, G. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. 19—29
31 Pradel, L.A. and Kassab, R. (1968) Biochim. Biophys. Acta 167, 317—325
32 Melchior, W.B. and Fahrney, D. (1970) Biochemistry, 9, 251—258
33 Burstein, Y., Walsh, K.A. and Neurath, H. (1974) Biochemistry 13, 205—210
34 Cousineau, J. and Meighten, E. (1976) Biochemistry 15, 4992—5000
35 Thome-beau, F., Le-Thi-Lan, Olomucki, A. and Van Thoai, N. (1971) Eur. J. Biochem. 19, 270—275
36 Loosemore, M.J. and Pratt, R.F. (1976) FEBS Lett. 72, 155—158
37 Ovadi, J., Libor, S. and Elodi, P. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. 2, 455—458
38 Derechin, M., Ostrowski, W., Galka, M. and Barnard, E.A. (1971) Biochim. Biophys. Acta 250, 143—154
39 Hultquist, D.E. (1968) Biochim. Biophys. Acta 153, 329—340
40 Engstrom, L. (1961) Biochim. Biophys. Acta 52, 49—59
41 Walsh, C.T. and Spector, L.B. (1971) J. Biol. Chem. 246, 1255—1261
42 Bittencourt, H.M. and Chaimovich, H. (1976) Biochim. Biophys. Acta 438, 153—158
43 Miles, E.W. and Kumagai, H. (1974) J. Biol. Chem. 249, 2843—2851
44 Rogers, T.B., Gold, R.A. and Feeney, R.E. (1977) Biochemistry 16, 2299—2305
45 Luchter-Wasyl, E. and Ostrowski, W. (1974) Biochim. Biophys. Acta 365, 349—359
46 Petitclerc, C., Ladzunski, C., Chappelet, D., Moulin, A. and Ladzunski, M. (1970) Eur. J. Biochem. 14, 301—308
47 Norman, A.W., Wedding, R.T. and Black, M.K. (1965) Biochem. Biophys. Res. Commun. 20, 703—709