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ISOLATION OF τ-PHOSPHOHISTIDINE FROM A PHOSPHORYL-ENZYME INTERMEDIATE OF HUMAN PROSTATIC ACID PHOSPHATASE *

WŁODZIMIERZ OSTROWSKI

Institute of Medical Biochemistry, N. Copernicus Academy of Medicine, Kopernika Str. 7, 31-034 Kraków (Poland)

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Summary

The carbethoxylation of prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) was accompanied by modification of histidine residues and the inactivation of the enzyme. These findings are consistent with photoinactivation experiments described earlier (Rybarska, J. and Ostrowski, W. (1974) Acta Biochim. Polon. 21, 377—390). Prostatic acid phosphatase was phosphorylated at alkaline pH using *p*-nitrophenyl [^{32}P]phosphate as substrate. Phosphoryl enzyme is stable in alkaline solutions and undergoes dephosphorylation at acidic pH. After hydrolysis of phosphoryl enzyme in strong alkaline solution, a single phosphoryl amino acid was isolated from hydrolyzate and identified as the τ-phosphohistidine.

Introduction

Covalent phosphoryl-enzyme intermediates in reactions catalysed by phosphomonoesterases are now generally accepted. In alkaline phosphatases an *O*-phosphorylserine derivative was found and identified [1,2], whereas in acid phosphatases from rat liver [3], wheat germ [4,5] and microsomes [6] *N'*-phosphorylhistidine was suggested as the phosphoryl intermediate. Also several independent lines of evidence indicated that covalent phosphorylhistidine intermediate is involved in the mechanism of catalytic function of human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) [4,7,8].

In the present paper we describe isolation of τ-phosphohistidine from the trapped covalent phosphoryl-enzyme obtained upon reaction of the pure prostatic acid phosphatase with *p*-nitrophenyl [^{32}P]phosphate as substrate in an appropriate condition.

* Parts of this study were presented as a preliminary meeting communication [9,10].

Materials and Methods

Acid phosphatase with specific activity of 240—250 units/mg protein was purified from human hypertrophic prostate gland [11]. τ-Phosphohistidine was obtained by direct phosphorylation of L-histidine [12] with orthophosphate amide, prepared according to Brauer [13]. A crystalline preparation was characterized by chromatographic methods [14], high voltage electrophoresis [15] and spectrometry in ultraviolet and infrared regions [16]. *p*-Nitrophenyl [^{32}P]-phosphate, 7.1 Ci/mol was purchased from the Radiochemical Centre, Amersham, U.K. Sephadex G-10, G-25 and G-100 were from Pharmacia Uppsala, Sweden. Dowex 1 X 8 (200—400 mesh) was the product of Serva, Heidelberg, G.F.R.

Enzymic activity determination. Acid phosphatase activity was measured with 0.02 M solution of *p*-nitrophenylphosphate in 0.05 M citrate buffer (pH 5.0) under conditions described previously [11].

Carbethoxylation of acid phosphatase. Carbethoxylation of the enzyme was performed by adding a 5—100 molar excess of diethylpyrocarbonate [17] in 99% alcohol to the protein solution in 0.1 M potassium phosphate buffer (pH 6.0 at 30° C). To measure activity of the modified phosphatase, 2-μl aliquots were withdrawn and added to the substrate solution and incubated for 60 s at 37° C.

Formation of the carbethoxylated enzyme was followed by spectrophotometric measurements at 242 nm [18].

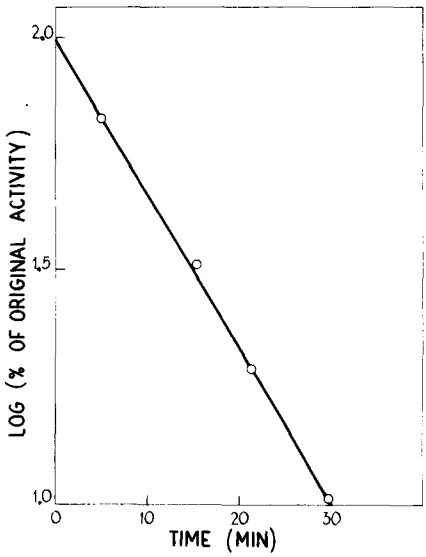
Phosphorylation of acid phosphatase. Phosphorylation of the enzyme with *p*-nitrophenyl [^{32}P]phosphate at pH 7.5—8.5 and isolation of [^{32}P]phosphohistidine from an alkaline hydrolyzate was done according to a modified procedure of Igarashi et al. [3]. Purified acid phosphatase (0.27—0.50 mg) was dialyzed against 0.1 M Tris acetate buffer (pH 7.5 or 8.5). The enzyme was incubated with 2 mM *p*-nitrophenyl [^{32}P]phosphate for 5 min at room temperature. The reaction was stopped by addition of 0.2 ml 1 M NaOH; 50 μl 10% SDS and 0.3 ml 1 M Tris/acetate buffer (pH 7.5) and 2 mg human serum albumin were added. The mixture was separated on a Sephadex G-25 column (115 × 1 cm) run in 0.025 M Tris/acetate (pH 7.5) 0.25% SDS. The fractions were read at 280 nm and the radioactivity was measured in a scintillation counter.

Other methods. Histidine was determined by the method of Macpherson [19]. Phosphohistidine content was calculated from the difference between the histidine value obtained after hydrolysis of the sample in 1 N HCl at 100° C for 5 min. Inorganic phosphate was assayed according to Lowry and Lopez [20]. Protein concentration was determined by the method of Lowry et al. [21] based on calculation from the absorption coefficient for the studied enzyme, $A^{1\%}_{1cm,280} = 14.4$ [22].

Results

Effect of diethylpyrocarbonate on the activity of prostatic phosphatase

Preliminary evidence on the role of histidine residues in the catalytic activity of prostatic acid phosphatase obtained by the experiments with photooxidation was published earlier [23]. In this study more information on the modifi-



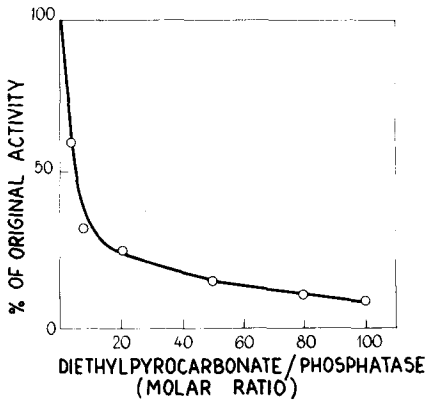


Fig. 1. Kinetic of inactivation of prostatic phosphatase by dethylpyrocarbonate at 100-fold excess of the reagent. 0.1 M phosphate buffer (pH 6.0, at 30° C).

Fig. 2. Inactivation of prostatic phosphatase as a function of diethylpyrocarbonate concentration. Suitable amounts of the reagent were added to 25 μg enzyme in 0.5 ml and after 30 min incubation of 30° C, 2-μl aliquots were taken for the assay of enzymic activity.

cation of histidine residues and the enzyme activity was obtained by applying carbethoxylation. When prostatic acid phosphatase was incubated at pH 6.0 with 100-fold molar excess of diethylpyrocarbonate there was rapid inactivation of the enzyme (Fig. 1). The decrease was complete after 30 min incubation. At the two-fold molar excess of diethylpyrocarbonate to each histidine residue present in the enzyme there was 85% inactivation within 10 min of the treatment (Fig. 2). Characteristic changes in the difference spectrum of carbethoxylated enzyme showed that histidine residues were modified [17]. Amino acid analysis of prostatic phosphatase indicates that the enzyme contains a total 27 histidine residues per 102 000 molecular weight [24]. Treatment of the enzyme with excess of diethylpyrocarbonate reveals that 10-13 histidines are reacting, as determined on the increase of the absorption of the modified enzyme at 242 nm [25].

When the phosphatase was inactivated in 50%, in the presence of 5-fold molar excess of diethylpyrocarbonate : enzyme and then treated with 0.1 and 1 M hydroxylamine at pH 7.0 [26] reactivation of enzymic activity was not observed.

Covalent labeling and characterization of [^{32}P]phosphatase

Gel filtration in alkaline SDS solution of acid phosphatase treated with *p*-nitrophenyl [^{32}P]phosphate, separated a protein peak coincident with a peak of radioactivity (Fig. 3). No radioactivity resulted when bovine serum albumin was substituted for the enzyme. The quantitative results of experiments carried out at pH 7.5 and 8.5 are shown in Table I. The amount of ^{32}P bound indicated

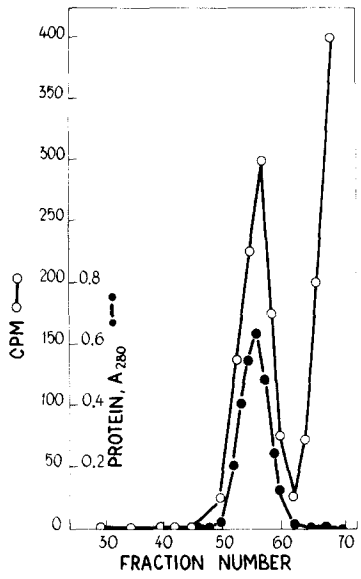


Fig. 3. Gel filtration of ^{32}P-labeled prostatic phosphatase on a column of Sephadex G-25.

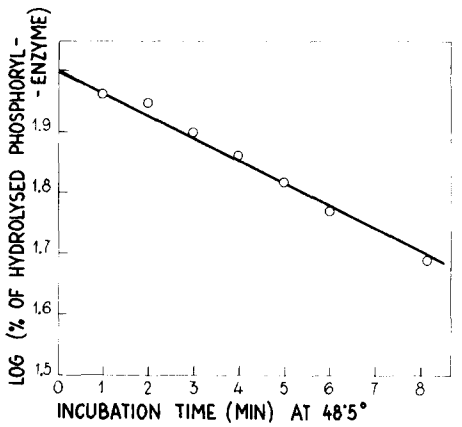


Fig. 4. Rate of hydrolysis of ^{32}P-labeled prostatic phosphatase in 1 N HCl. The phosphoryl-phosphatase (pooled fractions from Fig. 3) was incubated at indicated conditions in ultrathermostate, 1 ml samples were withdrawn, $^{32}P_i$ was precipitated as ammonium molibdate complex and radioactivity determined in scintillation counter.

that about 0.6 phosphoryl group is combined with one molecule of enzyme protein.

The ^{32}P-labeled enzyme was stable to strong alkaline conditions and labile in acids. To determine stability of phosphoryl protein, the sample was incubated in 1 N HCl at 48.5° C for several min. Samples were withdrawn at 30-s intervals and the amount of $^{32}P_i$ liberated was determined. A first-order rate constant, K_d, of the acid hydrolysis was found to be 0.085 min^{-1} (Fig. 4) and this value is in good agreement with that of synthetic τ-phosphohistidine [12].

Isolation and identification of N-phosphohistidine

To isolate the phosphohistidine from ^{32}P-labeled phosphatase, the radioactive protein fractions (Fig. 3) obtained were precipitated with 4 vols. acetone and heated in 3 M KOH at 100° C for 3 h in a sealed glass tube. The hydrolyzate was diluted with water, 10 μmol synthetic τ-phosphohistidine were added and chromatographed on Dowex 1 X 8 column. In all fractions radioactivity, acid labile phosphate and phosphohistidine were estimated. More as 70% of the

TABLE I

INCORPORATION OF ^{32}P INTO HUMAN PROSTATIC ACID PHOSPHATASE

pH	^{32}P incorporated (mol/mol enzyme)
7.5	0.61
8.5	0.63

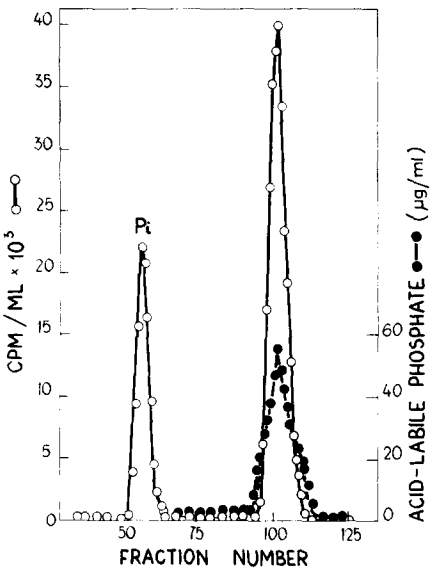


Fig. 5. Chromatography on Dowex 1 X 8 column (1.6 X 26 cm) of alkaline hydrolyzate of 250 μg [^{32}P]phosphatase, with 10 μmol of standard added. Elution was performed with a linear gradient of 0.2—0.8 M $KHCO_3$; 300 ml in each reservoir. Fraction volume, 2 ml.

radioactive material was eluted at the second peak with the reference compound added (Fig. 5). The first peak containing the rest of radioactivity was identified by paper chromatography and electrophoresis as inorganic phosphate. Fractions of the second peak were pooled, diluted with water and rechromatographed on a small Dowex 1 X 8 column in the same conditions. Only one peak of radioactivity was obtained coincident with bound phosphate and bound histidine. This peak was pooled, desalted on a Sephadex G-10 column and lyofilized. A small sample of the isolated radioactive material was hydrolyzed with 3 N HCl at 110° C for 6 h, the hydrolysate was dried and applied to Beckman 120B amino acid analyzer. The analysis showed full yield of free histidine and no other amino acids. Another sample of the lyofilized pool was examined by paper chromatography [14] and high voltage paper electrophoresis [15]. The R_F value (0.62) and electrophoretic mobility of radioactive phosphohistidine were identical as those of standard. When radioactive spot from the chromatogram was eluted, hydrolyzed in HCl and rechromatographed in the same conditions, radioactivity was found in the place of inorganic phosphate.

The above described results confirm the identity of the labeled compound isolated from prostatic acid phosphatase with τ-phosphohistidine.

Discussion

On the basis of the transphosphorylation reaction [7], inhibition with DFP [27] and titration of active sites [4,7], it was found that the mechanism of prostatic acid phosphatase activity consists of the formation of a catalytic covalent P-E intermediate. This intermediate was stable in alkaline pH and labile in acidic solutions, which points to the presence of histidine in the catalytic site of the studied enzyme [12]. Because some evidence exists for histidine residues

in the catalytic mechanism of several acid phosphatases isolated from plant and animal tissues, the described investigations were undertaken to demonstrate that phosphoenzyme formation in the case of prostatic phosphatase is due to protein-bound phosphohistidine.

As was found earlier in our laboratory [23], the pH profile for photoinactivation of prostatic phosphatase is quite compatible with the theoretical ionization curve for imidazole group. The photoinactivation of prostatic phosphatase was markedly reduced in the presence of substrates or L-(+)-tartrate as competitive inhibitor. The experiments on photooxidation did not give completely conclusive evidence that those histidines which are responsible for the loss of activity of the enzyme are part of the catalytic site rather then being of general structural importance.

The importance of histidine residues for the enzymic activity was further supported by studies in this paper on carbethoxylation of prostatic phosphatase. Diethylpyrocarbonate preferentially alters histidine and lysine but is unreactive with methionine, cysteine or tryptophan residues [28,29] under the conditions employed in this work. Treatment of prostatic phosphatase with excess of diethylpyrocarbonate under non-denaturing conditions at pH 6.0 reveals that 10—13 histidine residues are reacting as judged by the increase of the absorption at 242 nm. This value is consistent with the amount of photooxidized histidine in the presence of Rose Bengal [23], and the results obtained by McTigue and Van Etten [30]. It is general opinion, that at any rate, no other ethoxyformylated amino acid side-chain except histidine can be responsible for a maximum in this wavelength range [18]. Evidence in favour of specific modification of histidyl residues by dethylpyrocarbonate is that another reagent with apparent histidine specificity — Rose Bengal photooxidation produces almost the same effects in the same conditions as diethylpyrocarbonate. The discrepancy in this statement is that inactivation due to carbethoxylation is irreversible when hydroxylamine was added to the inactivated enzyme. The same experiment carried out by McTigue and Van Etten [30] gave only 25% reactivation. This may be due to inactivating activity of hydroxylamine itself as it was found by us in another experiment.

The importance of histidine residues for the catalytic activity of prostatic phosphatase was resolved by studies of phosphorylated enzyme obtained in the presence of ^{32}P-labeled substrate. From gel filtration in denaturing alkaline solution or incubation in 1 N HCl, clear evidence was obtained that phosphorylphosphatase is stable in alkali and unstable in acid what is consistent with the acid-lability of *N'*-phosphorylhistidine [15]. Finally, radioactive product obtained from the hydrolyzed [^{32}P]-labeled enzyme, when treated with strong acid and examined in amino acid analyzer, gave full yield of free histidine and no other amino acids.

The experiments on the quantitative incorporation of ^{32}P into the enzyme molecules show that there is no stoichiometric reaction between studied substrate and prostatic phosphatase. Assuming two active sites per enzyme molecule from "burst" experiments [7] and structural data [31], 2 mol of ^{32}P per mol of enzyme should be incorporated. From Table I it is seen that less than 1 mol ^{32}P is bound per mol of enzyme and this result is in agreement with the observation of McTigue and Van Etten [30] and our kinetic data on transphos-

phorylation reaction [7] and recently obtained in the presence of various nucleotides as a substrates [32]. This difference can be explained by negative cooperativity between two subunits of the enzyme [33] or by the course of experimental procedure.

The above described results establish that the reaction catalyzed by prostatic acid phosphatase proceeds through a covalent phosphoryl-histidine intermediate. Since similar products were isolated from acid phosphatase of rat liver [3] and wheat germs [4] it seems that the *N*-phosphorylhistidine intermediate is a common transient compound in the mechanism of catalytic action of these group of enzymes.

Acknowledgements

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