PHOSPHOPROTEIN PHOSPHATASE ACTIVITY OF HUMAN PROSTATE ACID PHOSPHATASE*

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Human prostate acid phosphatase (EC 3.1.3.2) has been shown to dephosphorylate different phosphoproteins with the maximum rate at pH 4.0 - 4.5. The activity with phosvitin is distinctly higher than with β-casein, casein and most of all than with riboflavin-binding protein.

The native phosvitin is homogeneous on isoelectric focusing with pI value of 2.1, whereas phosvitin partially dephosphorylated (in about 15%) by the prostate acid phosphatase shows multiple bands with pI values of 3.5 - 6.8 or higher. The phosphate groups bound to serine residues are removed enzymatically twice as fast as phosphothreonine residues.

The apparent $K_m$ value for phosvitin was $2.4 \times 10^{-7} \text{M}$, and is by three orders of magnitude lower than $K_m$ of p-nitrophenyl phosphate ($2.9 \times 10^{-4} \text{M}$). The competitive inhibitors of prostate acid phosphatase, fluoride and $\text{Na}^\text{(+)tartrate}$, show the same $K_i$ values for phosvitin and $p$-nitrophenyl phosphate.

Human prostate acid phosphatase is a typical acid phosphomonomohydrolase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) which catalyses dephosphorylation of a large variety of phosphoric acid monoesters, but it does not hydrolyse di-, triesters, phosphoamidates and ATP (Ostrowski, 1980). Also it was observed that this enzyme catalyses dephosphorylation of phosphoproteins (Perlmann, 1955; Schmidt, 1961), and oligo- and polynucleotides (Stachelin, 1964; Dziembor & Ostrowski, 1971). This property of prostatic phosphatase may be utilized in human semen for modification of different phosphoproteins involved in fertilization (Mann, 1964).

The main objective of this work was to learn more about the function of prostatic enzyme as phosphoprotein phosphatase. The results obtained may be useful in understanding the physiological role of this enzyme; in the experiments phosvitin from hen egg-yolk was used as a model.

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[175]
Reagents. Casein and protamine were from B.D.H. (England). DL-\(O\)-Phosphoserine, DL-\(O\)-phosphotreonine, bovine serum albumin and \(p\)-nitrophenyl phosphate, sodium salt, were purchased from Sigma (St. Louis, U.S.A.). Ovoalbumin was from Serva (Heidelberg, West Germany), Ampholine from LKB (Stockholm, Sweden), histone proteins from calf thymus were from POCh (Gliwice, Poland) and \([\gamma^{32}\text{P}]\text{ATP}\) was from Amersham (England). All other chemicals used in this work were of analytical grade.

Methods

Enzymes and substrates. Prostate acid phosphatase, of highest purity, was prepared from hypertrophic human prostate as described previously (Ostrowski, 1968; Ostrowski & Barnard, 1971). Riboflavin-binding protein was isolated from egg-white (Rhodes et al., 1959), phosvitin from egg-yolk (Joubert & Cook, 1958), \(\beta\)-casein from fresh skim milk (Hipp et al., 1952) and protein kinase from rat liver (Rikans & Rudden, 1976). Dephosphorylated phosvitin was prepared by alkaline hydrolysis (Thorburg & Lindall, 1977), and the enzymatic phosphorylation of dephospho-phosvitin was performed using rat liver protein kinase and \([\gamma^{32}\text{P}]\text{ATP}\) (Rikans & Rudden, 1976). The content of total phosphorus in the ashed protein samples was determined according to Chen et al. (1956).

Determination of phosphoprotein phosphatase activity. The reaction mixture in the total volume of 0.5 ml contained phosphoprotein substrate (about 100 nmol of the protein-bound phosphate) in 0.05 M-acetate buffer (final concentration), pH 4.0, and 0.2 to 1.0 IU of prostatic phosphatase. After 60 min incubation at 37\(^\circ\)C, the reaction was terminated by adding 0.1 M-silicocytic acid and P\(_{1}\) released was assayed in protein-free supernatant. When \([\text{32P}]\text{phosvitin}\) was used as a substrate, the enzyme activity was calculated from radioactivity of the supernatant determined in the scintillation counter (Packard, Tri-carb., C 2425; scintillation cocktail: 50 mg PPO and 2 mg POPP in 10 ml of toluene). In the case of non-radioactive protein substrates, P\(_{1}\) was determined according to Meisler & Langan (1969). With \(p\)-nitrophenyl phosphate as a substrate, the enzymatic activity was determined as described previously (Ostrowski, 1968).

Isoelectric focusing. Phosphoprotein samples (3 mg protein) were electrophoresed in LKB column (volume 110 ml), in the pH gradient made up with 1% Ampholine, and stabilized with sucrose (Haglund, 1967). The current (300 V, 3 mA) was applied for 48 h, and the fractions of 2.5 ml were collected. Absorbance at 280 nm and pH were measured in each fraction.

High-voltage paper electrophoresis. To determine the content of phosphoamino acids in phosphoproteins, the protein sample was hydrolysed in 6 M-HCl at 105\(^\circ\)C for 4 h, and after evaporation of hydrochloric acid phosphoamino acids were separated on Whatman 3 \(MM\) filter paper in formic acid/acetic acid/water system (25: 78: 897, v/v), pH 1.9. Electrophoresis was carried out at 22 mA, 25 V/cm for
4 h in the cooled chamber (Donella Deana et al., 1979). Dried paper strips were cut into 5 mm pieces and the radioactivity of $[^{32}\text{P}]$phosphoamino acids was determined in scintillation counter. Standard phosphoamino acids were stained with ninhydrin (Allerton & Perlmann, 1965).

Protein was determined by the method of Lowry et al. (1951).

RESULTS

Substrate specificity. Under our experimental conditions (protein-bound P at the concentration of 100 nmol) phosvitin proved to be the best substrate for prostatic phosphatase (Table 1). However, it has been found that, due to a lower content of P in the other phosphoproteins tested, lower enzymatic activity with these substrates might result, at least partially, from a higher concentration of protein at the same substrate level of phosphate.

Table 1

Dephosphorylation of phosphoproteins by human prostate acid phosphatase

Phosphoprotein (100 nmol of protein-bound phosphate, 10 - 600 μg protein) in 0.5 ml of 0.05 M-acetate buffer, pH 4.0, was incubated with 2 IU of prostatic phosphatase at 37°C for 60 min. The reaction was stopped by adding 0.1 ml of 0.1 M-silicotungstic acid and phosphate was determined as described in Methods.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Phosphoprotein</th>
<th>Activity (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosvitin</td>
<td>45.0</td>
</tr>
<tr>
<td>2</td>
<td>Casein</td>
<td>10.2</td>
</tr>
<tr>
<td>3</td>
<td>β-Casein</td>
<td>19.0</td>
</tr>
<tr>
<td>4</td>
<td>Riboflavin binding protein</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Kinetic data. Dephosphorylation of phosvitin, catalysed by prostate acid phosphatase is shown in Fig. 1. As can be seen even after 3 h incubation of this substrate with very active phosphatase, approximately 40% of phosphate remained bound with protein. This suggests that some phosphate groups of phosvitin are buried in the protein structure and are inaccessible to the enzyme.

![Fig. 1. Dephosphorylation of phosvitin by human prostate acid phosphatase. Phosvitin (0.5 mg) was incubated in 2 ml of 0.1 M-acetate buffer, pH 4.1, at 37°C with 25 μg of the enzyme protein (4 IU) for the time indicated. P$_t$ released was determined as described in Methods.](image-url)
Fig. 2. Effect of pH on human prostate acid phosphatase with: p-nitrophenyl phosphate (▲), phosvitin (○), riboflavin binding protein (●) and β-casein (△). The buffer solutions used were: 0.05 M-glycine/HCl (pH 2.0 - 3.5), acetate (pH 3.8 - 5.5), Tris/HCl (pH 6.5 - 9.0). Other conditions as in Methods.

Fig. 3. Lineweaver-Burk double reciprocal plot for prostate acid phosphatase in the presence of phosvitin with (●) and without (○) L(+) -tartrate (10⁻³ M) as the competitive inhibitor. In the inset the double reciprocal plot for p-nitrophenyl phosphate as a substrate. Reactions were performed at pH 4.0 for phosvitin and at pH 5.0 for p-nitrophenyl phosphate, in 0.05 M-acetate buffer.
The effect of pH on prostate acid phosphatase activity was determined with p-nitrophenyl phosphate, phosvitin, β-casein and riboflavin flavoprotein (Fig. 2). The enzyme in 50 mM-acetate buffer showed a broad optimum at pH 5.0 with p-nitrophenyl phosphate, and rather sharp optima at 4.0 to 4.5 with the remaining protein substrates.

The double reciprocal plot of the enzyme activity vs. phosvitin concentration (Fig. 3) shows that the apparent $K_m$ value, at pH 4.0, is $2.4 \times 10^{-7}$ M, i.e. it is three orders of magnitude lower as compared with the $K_m$ value for p-nitrophenyl phosphate ($2.9 \times 10^{-4}$ M). The $V_{\max}$ values are $0.35 \times 10^{-9}$ and $88 \times 10^{-9}$ mol/min for phosvitin and p-nitrophenyl phosphate, respectively.

Human prostate acid phosphatase is competitively inhibited by fluoride and L(+)-tartrate (Reiner et al., 1955; Kilsheimer & Axelrod, 1957). Inhibition of the enzyme by these ions and phosvitin and p-nitrophenyl phosphate as substrates (Fig. 4) was very similar. The $K_i$ values calculated for phosvitin and p-nitrophenyl

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**Fig. 4.** Inhibition of prostate acid phosphatase by fluoride (A) and L(+)-tartrate (B) with phosvitin (○) and p-nitrophenyl phosphate (●) as substrates. Concentration of phosvitin was 0.08 mg/ml in 0.05 M-acetate buffer, pH 4.0, and of p-nitrophenyl phosphate, 20 mM at pH 5.0. Other conditions as in Methods.
phosphate in the presence of fluoride and L(+)tartrate were $1.8 \times 10^{-5}$ M and $2.5 \times 10^{-4}$ M, respectively.

Isoelectric focusing experiments. Phosvitin has 108 phosphate groups (Ahmed et al., 1975), was homogeneous during isoelectric focusing, and showed one symmetrical peak with pI of 2.1 (Fig. 5A). When phosvitin was partially dephosphorylated by prostatic phosphatase, and about 15% of bound phosphate was released, multiple bands appeared with pI values higher than with native protein: pI ranged from pH 3.5 to 6.8 (Fig. 5B). As can be seen from Fig. 5, partially dephosphorylated phosvitin represents a mixture of species of different content of bound phosphate. Thus, isoelectric focusing can be used for monitoring dephosphorylation and for isolation of the protein fractions of various degree of phosphorylation.

Fig. 5. Isoelectric focusing of native phosvitin (A) and phosvitin partially dephosphorylated by prostate acid phosphatase (B). In the latter case about 15% of the protein-bound phosphate was removed. For details see Methods. ---, $A_{280}$; — — —; pH gradient.

Susceptibility of phosphoserine and phosphothreonine residues to enzymatic dephosphorylation. Since phosphate groups in phosvitin are bound in the form of serine and threonine monoesters (Taborsky, 1974), the activity of prostate acid phosphatase towards these two phosphoamino acids was investigated. First phosvitin was dephosphorylated by alkaline hydrolysis (Thornburg & Lindall, 1977),
and then rephosphorylated using rat liver nuclear protein kinase and \( \gamma^{32}\text{P}} \)ATP (Rikans & Ruddon, 1976). After treatment of \([^{32}\text{P}} \)phosvitin with prostate acid phosphatase, partially dephosphorylated protein was hydrolysed in 6 m-HCl, separated by high-voltage electrophoresis and radioactivity of the remaining \([^{32}\text{P}} \)serine and \([^{32}\text{P}} \)threonine was determined (cf Materials and Methods). As can be seen in Fig. 6, under the conditions applied phosphoserine in phosvitin was dephosphorylated in about 75% whereas at the same time phosphothreonine lost only 27% of its phosphate groups. This indicates that of the two phosphoamino acids present in phosvitin, the phosphoric monoester linkage of phosphoserine is hydrolysed at much higher rate by prostate acid phosphatase than the phosphothreonine monoester bonds.

**DISCUSSION**

The results reported in this paper show that human prostate acid phosphatase acts on phosphoprotein substrates and is able to dephosphorylate some of them at high rate. It is noteworthy that pH optimum for dephosphorylation of phosphoproteins was 4.0 - 4.5, whereas the optima with p-nitrophenyl phosphate or other low molecular mass substrates (Hollander, 1971) are above pH 5.0. This property of prostatic phosphatase is contrary to that of potato acid phosphatase which dephosphorylates \( \alpha_2 \)-casein at pH 6.0, and p-nitrophenyl phosphate at pH 5.5 (Bingham et al., 1976).

Substrate specificity of human prostate acid phosphatase resembles that of potato acid phosphatase (Bingham et al., 1976) and alkaline phosphatase from
E. coli (Mellgren et al., 1977); all three enzymes are both active with p-nitrophenyl phosphate and phosphoproteins. Although p-nitrophenyl phosphate is hydrolysed much faster than phosphoproteins, some phosphatases dephosphorylate phosphoproteins at high rate, when assayed at their pH optima. For instance, potato acid phosphatase dephosphorylates at considerable rate αs1-casein and phosvitin (Bingham et al., 1976), whereas alkaline phosphatase from E. coli—bovine heart glycogen synthase D and rabbit skeletal muscle phosphorylase kinase (Mellgren et al., 1977), and prostate acid phosphatase—phosvitin and β-casein (Table 1). Prostatic phosphatase shows very high affinity for phosvitin which in fact is three orders of magnitude higher than for p-nitrophenyl phosphate (2.4x10⁻⁷ M and 2.9x10⁻⁴ M, respectively). This might suggest that dephosphorylation of phosphoproteins is a main biological role of this enzyme in vivo.

The mechanism of dephosphorylation of low and high molecular mass substrates seems to be the same since there are practically no differences in the Kᵢ values for fluoride and 1(+)-tartrate with low molecular mass substrate and phosvitin. The comparative electrofocusing data on native and partially dephosphorylated phosvitins revealed complexity of phosphoprotein structure which on dephosphorylation breaks into a number of protein species of different pH. On the other hand, our results point to the differences in susceptibility of serine and threonine phosphate monoesters to the action of prostatic phosphatase. Owing probably to these differences and different location of phosphate ester bonds in protein molecule the pattern of dephosphorylation products is so complex and not all of the phosphate groups could be split off from the protein moiety of phosvitin.

As it was shown in this paper and by other authors (Clari et al., 1975; Bingham et al., 1976; Mellgren et al., 1977) acid and alkaline phosphomonoesterases might function in vivo as phosphoprotein phosphatases. It is of considerable interest that protein kinase, recently found in human seminal fluid (Wilson et al., 1982), phosphorylates phosvitin and lysine-rich histones. Also bovine spermatozoa contain phosphoprotein phosphatase which may play a significant role in dephosphorylation of bovine sperm proteins (Tang & Hoskins, 1975). Thus the phosphorylation/dephosphorylation system of proteins in seminal fluid in higher mammalian species might be of physiological significance.

REFERENCES


FOSFOPROTEIDOWA AKTYwność KWAŚNEJ FOSFATAZY STERCZU LUDZKIEGO

Streszczenie

Stwierdzono, że kwaśna fosfataza sterczu ludzkiego (EC 3.1.3.2) defosforyluje fosfoproteidy z najwyższą prędkością przy pH 4.0 - 4.5. Spośród badanych substratów, najbardziej efektywnymi okazały się fosfowityna i β-kazeina.

Natywna fosfowityna jest białkiem homogennym podczas izoelektrycznego ogniskowania, a jej punkt izoelektryczny wynosi 2.1. Fosfowityna zdefosforylowana częściowo (w ok. 15%) działaniem kwaśnej fosfatazy ze sterczu ludzkiego wykazuje kilka frakcji o punktach izoelektrycznych 3.5 - 6.8 lub wyższych. Grupy fosforanowe związane z resztami serynowymi są usuwane enzymatycznie dwa razy szybciej w porównaniu do reszt fosfotreoninowych.

Wartość $K_m$ dla fosfowityny wynosi $2.4 \times 10^{-2}$ m i jest trzy rzędy wielkości niższa w porównaniu z $K_m$ dla $p$-nitrofenylo fosforanu ($2.9 \times 10^{-4}$ m).

Kompetytywne inhibitory kwaśnej fosfatazy sterczu, fluorek i L(+-)-winian, posiadają te same stałe inhibicji dla substratów: fosfowityny i $p$-nitrofenylo fosforanu.

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