IZYDOR APOSTOŁ, RADOSŁAWA KUCIEL, EWA WASYLEWSKA
and WŁODZIMIERZ S. OSTROWSKI

PHOSPHOTYROSINE AS A SUBSTRATE OF ACID AND ALKALINE
PHOSPHATASES*

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

A new spectrophotometric method for following dephosphorylation of phosphorysine has been described. The absorption spectra of phosphotyrosine and tyrosine were plotted over the pH range from 3 to 9. The change in absorbance accompanying the conversion of phosphotyrosine to tyrosine was the greatest at 286 nm. The difference absorption coefficients were calculated for several pH values. Dephosphorylation of phosphotyrosine by acid phosphatases from human prostate gland, from wheat germ and potatoes obeys the Michaelis-Menten equation, whereas alkaline phosphatases calf intestine and E. coli are inhibited by excess of substrate.

Acid and alkaline phosphatases from various plant and animal tissues have been extensively studied but their physiological function remains unknown. A wide variety of low- and high-molecular weight esters can serve as substrates for these enzymes (Hollander, 1971; Reid & Wilson, 1971; Fernley, 1971).

Lately, attention has been drawn to the control of some metabolic processes by phosphorylation of tyrosine in cellular proteins (Levinson et al., 1980; Gill & Lazar, 1981; Erneaux et al., 1983; Ek et al., 1982; Roth & Cassel, 1983; Ganzano et al., 1983; Zick et al., 1983). Tyrosine phosphorylation is reversible but phosphotyrosyl-specific phosphatases have not been as extensively studied as tyrosine kinases. A few specific phosphotyrosyl-protein phosphatases were described (Chernoff & Li, 1983; Chernoff

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et al., 1983), but "unspecific" alkaline phosphatases from intestine, liver and *E. coli*, and prostatic acid phosphatase dephosphorylate phosphotyrosyl proteins, too (Swarup et al., 1981; Li et al., 1984). Dephosphorylation of free phosphotyrosine by the above-mentioned enzymes was not investigated.

A structural resemblance of phosphotyrosine to the commonly used substrate of acid and alkaline phosphatases, *p*-nitrophenylphosphate, encouraged us to test this amino acid ester as a substrate of phosphatases.

In this paper we propose a simple assay procedure for phosphatase activity with phosphotyrosine as a substrate. Both types of the investigated phosphatases, i.e. acid and alkaline phosphatases, hydrolyse phosphotyrosine but their kinetic behaviour is different.

**MATERIALS AND METHODS**

**D,L-Tyrosine** was obtained from Reanal (Hungary). Prostatic acid phosphatase was prepared from hypertrophic human prostatic glands according to Ostrowski & Barnard (1971). *E. coli* alkaline phosphatase type III, acid phosphatase from wheat germ type I and acid phosphatase from potatoes were purchased from Sigma (St. Louis, MO, U.S.A.). Calf intestinal phosphatase was obtained from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were of analytical grade.

Phosphotyrosine was prepared from **D,L-tyrosine** and phosphoric acid anhydride according to Rothenberg et al. (1978). The elementary composition and paper chromatography analysis (Fig. 1) indicate high purity of the synthesized product. Treatment of 1.5 mM solution of phosphotyrosine with 1 M-HCl at 100°C resulted in hydrolysis of 40% amino acid phosphate within 1 h. In the solution of 1 M-NaOH, after 1 h of heating at 100°C, 20% of phosphotyrosine phosphate was hydrolysed.

**Spectrophotometric assay for phosphatases using phosphotyrosine as substrate.** The absorption spectra of phosphotyrosine at pH range from 1 to 13 are shown in Fig. 2A. For comparison the absorption spectra of tyrosine in the same range of pH are presented in Fig. 2B. For phosphotyrosine solution in 0.1 M-NaOH the maximum absorption is at 270 nm, with the molar absorption coefficient $8.9 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$. In 0.1 M-HCl solution the molar absorption coefficient at the maximum absorption wavelength 266 nm is $5.6 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$.

The molar absorption coefficients determined for tyrosine are in good agreement with the data already reported (Beaven & Holiday, 1952), namely: $\varepsilon = 2.33 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 293.5 nm in 0.1 M-NaOH; $\varepsilon = 1.34 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 274.5 nm in 0.1 M-HCl. The most significant difference in absorption spectra in the pH range from 3 to 9 was observed at 286 nm (cf.
Figs. 2A and 2B). The values of molar absorption coefficients for tyrosine and phosphotyrosine at 286 nm are listed in Table 1. The differences in absorption spectra of these two compounds were taken advantage of in the direct spectrophotometric assay of phosphotyrosine hydrolysis. The change in absorbance accompanying the conversion of phosphotyrosine to tyrosine is shown in Fig. 3, in the reaction catalysed by prostatic acid phosphatase at pH 5. The direct spectrophotometric assay at 286 nm is particularly convenient for measuring initial velocities for kinetic studies (Fig. 3B).

Fig. 1. Chromatography of phosphotyrosine and tyrosine. Phosphotyrosine and tyrosine were spotted on Whatman 3MM paper and developed in butanol/formic acid/water (70:15:15, by vol.). The amino acids were located by spraying with isatine. A, Tyrosine; B, phosphotyrosine; C, mixture of tyrosine and phosphotyrosine; D, products of phosphotyrosine hydrolysis by the human prostatic acid phosphatase.

In a typical assay of phosphatase activity we used 2 ml of phosphotyrosine at the concentration range from 0.1 mM to 10 mM in the chosen buffer solution. Phosphatase (5-20 µg) was added to this solution to initiate the reaction, and the absorbance at 286 nm was recorded. Initial velocities, in terms of concentrations, can be calculated using the data presented in Table 1.

Alternatively, the enzymatic reaction might be stopped by adding NaOH to 0.1 M concentration, and the concentration of tyrosine calculated. The
Fig. 2. Absorption spectra of phosphotyrosine (A) and tyrosine (B) at the following pH values: 1, 0.1 M-HCl; 3, 0.1 M-citrate buffer, pH 3.0; 4, 0.1 M-citrate buffer, pH 4.0; 5, 0.1 M-citrate buffer, pH 5.0; 6, 0.1 M-citrate buffer, pH 6.0; 7, 0.1 M-Tris/HCl, pH 7.0; 8, 0.1 M-Tris/HCl, pH 8.0; 9, 0.1 M-glycine/NaOH buffer, pH 9.0; 13, 0.1 M-NaOH
Table 1

Molar absorption coefficients of tyrosine and phosphotyrosine at 286 nm at different pH values

The coefficients were determined in the indicated 0.1 M buffers.

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer</th>
<th>$\varepsilon_{286}$ for tyrosine* (M$^{-1}$ cm$^{-1}$)</th>
<th>$\varepsilon_{286}$ for phosphotyrosine (M$^{-1}$ cm$^{-1}$)</th>
<th>$\Delta\varepsilon_{286}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>HCl</td>
<td>820</td>
<td>20</td>
<td>800</td>
</tr>
<tr>
<td>3.0</td>
<td>citrate</td>
<td>820</td>
<td>40</td>
<td>780</td>
</tr>
<tr>
<td>5.0</td>
<td>citrate</td>
<td>820</td>
<td>60</td>
<td>760</td>
</tr>
<tr>
<td>7.0</td>
<td>Tris/HCl</td>
<td>820</td>
<td>90</td>
<td>730</td>
</tr>
<tr>
<td>8.0</td>
<td>Tris/HCl</td>
<td>820</td>
<td>90</td>
<td>730</td>
</tr>
<tr>
<td>9.0</td>
<td>Glycine/NaOH</td>
<td>950</td>
<td>100</td>
<td>850</td>
</tr>
</tbody>
</table>

* Taken from Dawson et al. (1969).

Fig. 3. The absorption spectra of phosphotyrosine in the course of its hydrolysis. The reaction mixture consisted of 0.1 M-citrate buffer, pH 5.0, and 0.7 mM-phosphotyrosine. The reaction was initiated by adding 5 μg (20 μl) of human prostatic acid phosphatase. Absorption spectra: A, at zero time (1); and after 0.7 min (2), 1.7 min (3), 2.7 min (4), and 4 min (5). B, Changes in absorbance at 286 nm.
value of molar absorption coefficient for tyrosine at 293.5 nm is $2.33 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (Dawson et al., 1969).

Kinetic parameters of enzymatic reaction were calculated by the optimization method of Rosenbrock & Storey (1966) on the K 202 microcomputer.

Absorption spectra were measured in UV VIS Specord Spectrophotometer, at 20°C.

RESULTS

Hydrolysis of phosphotyrosine by prostatic acid phosphatase. The initial velocities of the prostatic acid phosphatase catalysed reaction were measured as a function of the concentration of phosphotyrosine (0.1 mm to 10 mm) and as a function of pH (3.0 to 8.0). The values of $K_m$ and $V$ were determined from the relation $1/v_o$ versus $1/S_o$. The variation of these values with changes in pH is shown in Fig. 4. $V$ values change slightly

![Graph](image)

Fig. 4. The variation of log $V$ (A) and log $K_m$ (B) values depending on pH for the reaction of phosphotyrosine hydrolysis by prostatic acid phosphatase. The reaction mixture contained phosphotyrosine in the concentration range from 0.1 mm to 10 mm. The reaction was initiated with 5 µg (20 µl) of prostatic acid phosphatase and the change in absorbance at 286 nm was recorded. $K_m$ and $V$ values were calculated from the relation $1/v_o$ versus $1/S_o$. Buffers: 0.1 M-citrate in the pH range from 3 to 6 (a) and 0.1 M-Tris/HCl at pH from 6 to 8 (b)
between pH 3 and 5, but increase sharply in more alkaline solutions (Fig. 4A). \(K_m\) value at pH 5.0 is \(2.85 \times 10^{-3}\) M (Fig. 4B). It is one order of magnitude higher than \(K_m\) for \(p\)-nitrophenylphosphate at the same pH (Van Etten & McTigue, 1977).

Phosphotyrosine hydrolysis by potato acid phosphatase and wheat germ acid phosphatase was assayed at pH 5.5. The \(K_m\) values 7.1 \times 10^{-4} \) M for potato acid phosphatase and 4 \times 10^{-4} \) M for wheat germ acid phosphatase were obtained.

Hydrolysis of phosphotyrosine by alkaline phosphatases from E. coli and calf intestine. The effect of phosphotyrosine concentration on the initial reaction rates was investigated. Plots of \(v_0\) versus \(\log S_o\) give bell-shaped curves for both enzymes (Figs. 5A and 6A), indicating inhibition by the substrate excess (Bardsley et al., 1980). In this case the course of the enzyme-substrate complex formation can be described as follows:

\[
E + S \xrightleftharpoons[k_{-3}]{k_{+1}} ES \xrightleftharpoons[k_{-3} + nS]{k_{+3} + nS} E + P
\]

where ES is active complex of enzyme and substrate and \(ES_{(n+1)}\), inactive complex of enzyme and substrate. If the \(ES_{(n+1)}\) complex is completely inactive the reaction rate is given by the equation of Brestkin et al. (1961):

\[
v_0 = \frac{k_{+2} \frac{k_{+1}}{k_{-1} + k_{+2}} [E] [S_o]}{1 + \frac{k_{+1}}{k_{-1} + k_{+2}} [S_o] + \frac{k_{+1}}{k_{-1} + k_{+2}} \frac{k_{+3}}{k_{-3}} [S_o]^{n+1}}
\]

In the above equation [\(E\)] is concentration of enzyme; \([S_o]\), initial concentration of substrate; \(k_{-2}[E] = V\), maximal velocity of reaction; \(k_{-1} / k_{-1} + k_{+2} = 1/ K_m\), reciprocal of “apparent” Michaelis constant; \(k_{+3} / k_{-3} = 1/ K_{ss}\), reciprocal of equilibrium constant of the inactive complex dissociation. This equation can be described in terms of Michaelis-Menten kinetic constants as follows:

\[
v_0 = \frac{[S_o] \cdot V}{K_m' + [S_o] + \frac{[S_o]^{n+1}}{K_{ss}}}
\]
Fig. 5. A. The initial rate \( (v_0) \) of the reaction catalysed by intestinal alkaline phosphatase \( (7 \times 10^{-7} \text{ M}) \) as a function of substrate concentration \( (\log S_0) \). The hydrolysis of phosphotyrosine in the concentration range of \( 3.75 \times 10^{-5} \text{ M} \) to \( 7.5 \times 10^{-3} \text{ M} \) was performed in a 0.1 M \( \text{Mg}^2+ \)/NaOH buffer, pH 8.75, containing 0.01 M Mg(CH\(_2\)COO)\(_2\) and 0.001 M ZnCl\(_2\). B. The relationship between the initial reaction rates \( (v_0) \) \text{ versus enzyme concentration} \( (\log [E]) \) and concentration of substrate \( (\log S_0) \). The values of \( K_m \) \( (2.3 \times 10^{-4} \text{ M}) \) and \( K_m \) \( (1.7 \times 10^{-3} \text{ M}) \) were calculated from equation (3). The thick line indicates the experimental curve.

The values of the parameters of eq. (3) for the initial reaction rate \( (v_0) \) \text{ versus} starting concentration of substrate \( [S_0] \) were calculated by the optimalization method of Rosenbrock & Storey (1966). The results are presented in Table 2. The calculated \( K_m \) values are \( 2.3 \times 10^{-4} \text{ M} \) for the alkaline intestinal phosphatase and \( 0.8 \times 10^{-4} \text{ M} \) for \textit{E. coli} phosphatase.

The experimental plots \( v_0 \) \text{ versus} \log \( S_0 \) for both investigated enzymes are presented in Fig. 5A (intestinal phosphatase) and in Fig. 6A (phosphatase from \textit{E. coli}), and compared with the theoretical relationship given by the equation (3), (Figs. 5B and 6B). The approximation of experimental points indicates their consistence with the theoretically calculated curves.
Fig. 6. A. The initial rate \( v_0 \) of the reaction catalysed by phosphatase from *E. coli* \((8 \times 10^{-7} \text{ M})\) as a function of substrate concentration \( \log [S_0] \). For conditions of the reaction see Fig. 5. B. The relationship between the initial reaction rates \( v_0 \) versus enzyme concentration \([E]\) and substrate concentration \( \log [S_0] \). The values of \( K_m \) \((0.8 \times 10^{-4} \text{ M})\) and \( K_{ss} \) \((0.3 \times 10^{-8} \text{ M})\) were calculated from equation (3). The thick line indicates the experimental curve.

### Table 2

*Values of parameters of equation (3) calculated by the optimization method of Rosenbrock & Storey (1966)*

Dephosphorylation of phosphotyrosine was catalysed by intestinal alkaline phosphatase and alkaline phosphatase from *E. coli* in 0.1 M-glycine/NaOH buffer, pH 8.75, containing 0.01 M-Mg\((\text{CH}_3\text{COO})_2\) and 0.001 M-ZnCl₂.

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Enzyme conc. ((\text{M}))</th>
<th>( n+1 )</th>
<th>Composition of inactive complex</th>
<th>( K_m ) ((\text{M}))</th>
<th>( K_{ss} ) ((\text{M}))</th>
<th>( V ) ((\text{M} \times \text{min}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf intestine</td>
<td>( 7 \times 10^{-7} )</td>
<td>2</td>
<td>ES₂</td>
<td>( 2.3 \times 10^{-4} )</td>
<td>( 1.7 \times 10^{-3} )</td>
<td>( 1 \times 10^{-4} )</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>( 8 \times 10^{-7} )</td>
<td>4</td>
<td>ES₄</td>
<td>( 0.8 \times 10^{-4} )</td>
<td>( 0.3 \times 10^{-8} )</td>
<td>( 0.38 \times 10^{-4} )</td>
</tr>
</tbody>
</table>
DISCUSSION

A simple and rapid method for following dephosphorylation of phosphotyrosine by alkaline and acid phosphatases has been described. The activity of the phosphatases can be assayed spectrophotometrically over a wide range of pH values. The results presented indicate that the ability to catalyse hydrolysis of phosphotyrosine is a common feature of acid and alkaline "unspecific" phosphatases. Phosphotyrosine dephosphorylation by potato acid phosphatase, wheat germ acid phosphatase and prostatic acid phosphatase obeys Michaelis-Menten equation over a wide range of substrate concentration. The $K_m$ values for the latter enzyme are considerably higher than the constants reported for $p$-nitrophenylphosphate and other low molecular weight substrates (Van Etten & McTigue, 1977) but dephosphorylation of phosphotyrosine might be directly followed even in the acidic region of pH.

Deviations from Michaelis-Menten kinetics have been observed in the reaction catalysed by alkaline phosphatases (calf intestinal and *E. coli*); this points to an inactive complex formation (Brestkin et al., 1961; Bardsley et al., 1980; Del Arco et al., 1984). From the simplified form of the kinetic equation (Brestkin et al., 1961) it has been calculated that alkaline intestinal phosphatase, composed of two subunits (Malik & Butterworth, 1977), forms an inactive ES$_2$ complex with phosphotyrosine. In our experimental conditions, alkaline phosphatase from *E. coli* occurs as a tetramer (Reynolds & Schlesinger, 1969), and it forms an ES$_4$ inactive complex with phosphotyrosine. This suggests that inactive complexes are formed when all subunits of the enzyme are at the same time saturated with phosphotyrosine.

We conclude that the method described here allows to assay acid and alkaline phosphatases under a wide range of conditions and is a useful tool for kinetic studies.

REFERENCES


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