Studies on kinetic properties of acid phosphatase from nuclei-free rat liver homogenate using different substrates

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Kinetic properties of rat liver acid phosphatase were evaluated using the conventional synthetic substrates sodium beta glycerophosphate (βGP) and p-nitrophenyl phosphate (PNPP) and physiologically occurring phosphate esters of carbohydrates, vitamins and nucleotides. The extent of hydrolysis varied depending on the substrates; phosphate esters of vitamins and carbohydrates were in general poor substrates. Kinetic analysis revealed the presence of two components of the enzyme for all the substrates. Component I had low $K_m$ and low $V_{max}$. Opposite was true for component II. The $K_m$ values were generally high for βGP, PNPP and adenosine diphosphate (ADP). Amongst the nucleotides substrates AMP showed high affinity i.e. low $K_m$. The increase in enzyme activity in general at high substrate concentration seems to be due to substrate binding and positive cooperativity. AMP which showed highest affinity was inhibitory at high concentration beyond 1mM. The results suggest that in situ the nucleotides may be the preferred substrates for acid phosphatase.

The pivotal studies of de Duve and colleagues led to the characterization of lysosomes as the membrane-limited subcellular organelles which contain acid hydrolases. It has now been recognized that these organelles in a cell contain about 70 different types of acid hydrolases. In their earlier work de Duve et al. demonstrated that most of the cellular acid phosphatase activity (EC 3.1.3.2) concentrated in this subcellular organelle; sodium beta glycerophosphate (βGP) was used as the substrate for demonstrating the acid phosphatase activity. The enzyme also hydrolyzes the non-specific phosphatase substrate p-nitrophenyl phosphate (PNPP).

It is now recognized that acid phosphatases are a family of enzymes that is widespread in nature and are found in many animal and plant systems. However, despite considerable amount of work, the function of acid phosphatase is not yet fully understood. Nevertheless, acid phosphatases have gained importance as clinical diagnostic tools in the detection of gynaecological conditions, metastasizing prostate cancer, bone conditions including rheumatic osteoarthritis, bone cancer or metastasis, osteogenesis imperfecta, liver diseases such as Goutcher's disease, hyperparathyroidism and chronic renal failure.

The lysosomes function in situ as scavenging organelles and help in degradation of macromolecules of cellular origin and from invading microorganisms. The process involves formation of phagolysosomes or endocytic vesicles in the cell interior. The latter vesicles then fuse with primary lysosomes with given function to form secondary lysosomes, which are considered to be the site of catalytic activity. It may hence be anticipated that under physiologic conditions the enzyme acid phosphatase may be involved specifically in the dephosphorylation of naturally/physiologically occurring phosphate esters. Therefore studies of the kinetic properties of the enzyme employing naturally occurring phosphate esters rather than with conventionally used synthetic substrates such as βGP or PNPP are desirable. The kinetic properties of acid phosphatases from plant and fungal sources have been described. However, these are not relevant in the context of lysosomal function in the animal cell. Thus in depth studies on the kinetic properties of lysosomal acid phosphatase from animal source are desirable.

With a view to illustrating this point in preliminary studies the kinetic properties of lysosomal acid phosphatase from rat liver have been examined. For these studies βGP and PNPP have been employed as the conventional substrates, which are synthetic non-physiological substrates, as reference points. Additionally, studies using phosphate esters of carbohydrates, vitamins and the purine nucleotides have also been undertaken.
Materials and Methods

Enzyme source—Adult male albino rats of Charles-Foster strain weighing 200-250 g were used. The animals were killed by decapitation and the liver was quickly excised and transferred to a beaker containing chilled (0°C-4°C) 0.25 M sucrose. The tissue was minced, washed repeatedly to remove adhering blood and then homogenized in chilled 10 mM NaCl solution using a Potter Elvehjem type glass-Teflon homogenizer to obtain 10% (w/v) homogenate. The homogenate was subjected to centrifugation at 650 g for 10 min. to remove nuclei and cell debris and the post-nuclear fraction was used as the source of the enzyme. It is anticipated that this hypotonic 10 mM NaCl solution would lyse thelysosomal membranes and thus release all the enzyme activity.

Assay of acid phosphatase activity—The assay medium contained in a total volume of 0.4 ml, 0.1 M sodium acetate buffer pH 5 and the substrate concentrations as indicated in the individual experiments. Post-nuclear supernatant (50 μl Ca. 1 mg protein) was used as the source of the enzyme. After preincubation at 37°C for 2-3 min, the reaction was initiated by adding the substrate and after 10 min of incubation the reaction was terminated by adding 0.1 ml of 10% (w/v) SDS solution for all the substrates except for PNP. The released inorganic phosphorus was determined by the method of Fiske and Subba Row19. When PNP was the substrate the reaction was terminated by adding 2 ml of 0.2 N NaOH and the amount of p-nitrophenol released was estimated by measuring the absorbance at 420 nm 19.

For substrate kinetics analysis the concentration of the individual substrate was varied over the range of 0.1-10 mM; for AMP, the range was 0.1-1 mM. K_m and V_max were determined from the Lineweaver-Burk and Eadie-Hofstee plots19. The data were computer analyzed using Sigma Plot version 5.0.

The enzyme activity is expressed as nmoles of Pi or PNP released/hr/mg protein.

Protein estimation was according to the method of Lowry et al19, with bovine serum albumin used as the standard.

Results and Discussion

In the initial experiment the relative efficacy of the acid phosphatase to hydrolyze the various phosphate esters, both synthetic and natural was tested. In these experiments the substrate concentration was kept fixed at 5 mM. The results are given in Table 1. As can be seen, of the two synthetic substrates, the enzyme hydrolyzed PNPP with a two-fold higher efficiency.

Thiamine pyrophosphate (TPP) was a poor substrate whereas the results with riboflavin 5’-monophosphate (FMN) were variable in that detectable activity was noted only in some experiments. This is not surprising because TPP and FMN are known to be catalyzed by cytosolic enzymes which act near neutral pH 20-21.

Amongst the phosphate esters of the carbohydrates tested, fructose 1,6-bisphosphate (FBP) was hydrolyzed with highest efficiency followed by glucose 1-phosphate (G1P) and inositol hexaphosphoric acid (IHP). Results with glucose-6-phosphate (G6P) were of variable nature. In this context it is interesting to note that G6P is hydrolyzed by microsomal enzyme which has a pH optimum of 6.5 22; incubation at pH 5 for 10 min is known to inactivate the microsomal glucose-6-phosphatase2.

Of the three nucleotides tested highest and lowest activities were seen respectively with ATP and AMP; activity with ADP was intermediate.

In view of the results in Table 1 the substrate kinetics parameters with PNPP, BGP, FBP and the three adenine nucleotides were investigated. The substrate concentration range employed for these studies was 0.1-10 mM.

The typical substrate saturation curves for these substrates are shown in Fig. 1. As is evident characteristic substrate saturation plots were seen for all the substrates except AMP, where maximum activity was noted at 1 mM concentration. Concentrations of AMP beyond 1 mM were inhibitory (eg. see Fig 4). Therefore separate experiments were carried out where maximum AMP

<p>| Table 1 — Activity of rat liver lysosomal acid phosphatase (nmolPi/hr/mg protein) with synthetic and natural substrates. [Values are mean ± SE of the number of independent observations given in parentheses] |
|---|---|---|</p>
<table>
<thead>
<tr>
<th></th>
<th>Substrate</th>
<th>Enzyme activity</th>
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<tbody>
<tr>
<td></td>
<td>PNPP</td>
<td>2237.0 ± 177.49 (8)</td>
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<tr>
<td></td>
<td>BGP</td>
<td>1173.9 ± 113.10 (8)</td>
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<tr>
<td>Carbohydrates</td>
<td>FBP</td>
<td>763.8 ± 270.3 (5)</td>
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<tr>
<td></td>
<td>G1P</td>
<td>526.3 ± 40.4 (6)</td>
</tr>
<tr>
<td></td>
<td>IHP</td>
<td>246.5 ± 49.2 (7)</td>
</tr>
<tr>
<td></td>
<td>G6P</td>
<td>variable results (8)</td>
</tr>
<tr>
<td>Nucleotides</td>
<td>ATP</td>
<td>2321.4 ± 162.5 (10)</td>
</tr>
<tr>
<td></td>
<td>ADP</td>
<td>1804.0 ± 210.3 (8)</td>
</tr>
<tr>
<td></td>
<td>AMP</td>
<td>682.7 ± 69.7 (5)</td>
</tr>
<tr>
<td>Vitamin derivatives</td>
<td>TPP</td>
<td>84.3 ± 18.8 (8)</td>
</tr>
<tr>
<td></td>
<td>FMN</td>
<td>variable results (8)</td>
</tr>
</tbody>
</table>
concentration was 1 mM and data points were increased by including additional nine substrate concentrations over the 1 mM range.

The $K_m$ and $V_{max}$ values were determined from the corresponding Lineweaver-Burk and Eadie-Hofstee plots. For sake of brevity only the typical Eadie-Hofstee plots are shown in Fig. 2.

The inhibitory effect of higher concentration by AMP was analyzed in terms of Murray plot (Fig. 4) which is described separately.

As can be seen from the typical Eadie-Hofstee plots, for all the substrates tested the acid phosphatase activity resolved in two components. Component I had low $K_m$ and $V_{max}$ and Component II had high $K_m$ and $V_{max}$. The $K_m$ and $V_{max}$ values calculated from the Lineweaver-Burk and Eadie-Hofstee plots were in close agreement and these values were averaged. The mean of averaged values of $K_m$ and $V_{max}$ are given in Table 2.

As can be noted (Table 2), the values of $V_{max}$ was about 3.5 times higher with PNPP than that obtained with $\beta$GP. FBP was the poorest substrate with lowest value of $V_{max}$. The adenine nucleotides were good substrates and the extent of hydrolysis of the adenine nucleotides increased with phosphorylation.

The value of $K_m$ was relatively high and about comparable for PNPP and $\beta$GP as substrates. $K_m$ for FBP was also in the similar range. By contrast, the $K_m$ values for the three adenine nucleotide substrates were much lower indicating high affinity. Amongst the three nucleotides, the lowest $K_m$ was noted for AMP while the highest was noted for ADP; the value of $K_m$ for ATP was in the intermediate range.

The $V_{max}$ was about one and half times higher with PNPP as substrate compared with $\beta$GP. For the three nucleotides the value of $V_{max}$ decreased with the extent of phosphorylation. Paradoxically, however, the highest $V_{max}$ was seen for FBP. It may, however, be

![Fig. 1 — Typical substrate saturation curves for rat liver lysosomal acid phosphatase using different substrates. The enzyme activity (v) is plotted on ordinate against the corresponding substrate concentration on abscissa. The experimental details are as given in the text. A. PNPP, B. $\beta$GP, C. ATP, D. ADP, E. AMP and F. FBP.](image1)

![Fig. 2 — Typical Eadie-Hofstee plots for rat liver lysosomal acid phosphatase using different substrates. The enzyme activity (v) is plotted on ordinate against the corresponding values of v/[S] on abscissa. The experimental details are as given in the text and in Fig. 1. A. PNPP, B. $\beta$GP, C. ATP, D. ADP, E. AMP and F. FBP.](image2)
borne in mind that the $K_{m2}$ for FBP was also so very high (8.5 mM; eg. see Table 2); physiologically one could never expect to reach such high concentration of FBP (in the range of 50 to 60 mM i.e. 6 to 8 times $K_{m2}$) which will saturate component II to give the high $V_{max}$ value. Besides, FBP is metabolized cytoso-

cally. Hence, the observed high rate of hydrolysis of FBP by the lysosomal enzyme is unlikely to occur under physiologic conditions. The $K_{m2}$ values were comparable for PNPP, BGP, ATP and ADP but were the highest for FBP as pointed out above, once again emphasizing the fact that FBP was a poor substrate. The $K_{m2}$ values were lowest for AMP.

Since the acid phosphatase activity with all the substrates tested resolved in two components differing in their $K_{m}$ and $V_{max}$ values (Table 2, Fig. 2) it was of interest to find out whether the affinity of the enzyme for the substrates was concentration dependent. To evaluate this possibility Hill plot analysis was carried out. Typical Hill plots for the various substrates are shown in Fig. 3. As can be seen the Hill plots for all the substrates were biphasic. From the Hill plots the Hill coefficients $n_1$ and $n_2$ respectively for the low and high substrate concentration ranges were calculated. The concentration of substrate at which the transition in substrate binding pattern occurred was also computed. These data are given in Table 3 from which it may be noted that for all the substrates studied the value of $n_1$ was close to 1 indicating that in the lower substrate concentration range cooperative interaction was not involved. However, at higher substrate concentrations, except in the case of ADP, the value of $n_2$ increased indicating positive cooperativity. The transition point was in the range of 1 mM for all the substrates except for AMP which was in the range of 0.1 mM, a value lower by a magnitude. This is consistent with the substrate saturation kinetics described earlier (Fig. 2, Table 2). However, for AMP the substrate concentration effects were of dual nature; concentrations up to 1 mM activated the enzyme whereas higher concentrations i.e. above 1 mM inhibited the enzyme (eg. see Fig. 4).

Fig. 3—Typical Hill plots for rat liver acid phosphatase using different substrates. The value of log $v/V_o$ on ordinate is plotted against log[S] on abscissa for each substrate. The experimental details are as given in the text. A. PNPP, B. BGP, C. ATP, D. ADP, E. AMP and F. FBP.

Table 2—Kinetic properties of acid phosphatase with synthetic and natural substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Component I</th>
<th>Component II</th>
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<tbody>
<tr>
<td></td>
<td>$K_{m1}$</td>
<td>$V_{max1}$</td>
</tr>
<tr>
<td>PNPP (9)</td>
<td>0.331 ± 0.034</td>
<td>1577.3 ± 163.1</td>
</tr>
<tr>
<td>BGP (8)</td>
<td>0.243 ± 0.030</td>
<td>466.8 ± 65.4</td>
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<tr>
<td>ATP (8)</td>
<td>0.088 ± 0.010</td>
<td>1293.6 ± 126.4</td>
</tr>
<tr>
<td>ADP (5)</td>
<td>0.156 ± 0.022</td>
<td>1245.0 ± 161.2</td>
</tr>
<tr>
<td>AMP (8)</td>
<td>0.048 ± 0.007</td>
<td>835.0 ± 63.4</td>
</tr>
<tr>
<td>FBP (7)</td>
<td>0.361 ± 0.056</td>
<td>433.7 ± 49.9</td>
</tr>
</tbody>
</table>

$K_{m1}$ and $K_{m2}$ represent the Michaelis Menten constants and $V_{max1}$ and $V_{max2}$ represent the maximum velocity for the respective $K_{m}$.

$K_{m} = \text{mM}$, $V_{max} = \text{nmols/hr/mg protein}$
Inhibition of enzyme by higher concentration of AMP was analyzed in terms of Murray plot. The typical Murray plot is shown in Fig. 4, from which the Ki for AMP was found to be in the range of 10 mM. The averaged value of Ki from 9 experiments was 10.52 ± 0.46 mM. Physiologically, however, such high AMP concentration are unlikely to be present. Hence the significance of the inhibition of the enzyme by higher AMP concentrations remains obscure.

Nevertheless, the results of the present studies have clearly shown that the nucleotides are the preferred substrate for the lysosomal acid phosphatase with AMP showing the highest affinity i.e. low Kₐ values. It may hence be suggested that in situ the enzyme may preferentially act towards hydrolysis of mononucleotide such as AMP. Although experiments using other purine and pyrimidine nucleotides were not carried out. It is possible that even with these substrates, the mononucleotides could be the preferred substrates.

It was interesting to note that the acid phosphatase activities with all the substrate tested resolved in two components. This observation warrants some discussion. The increase in the enzyme activity with increasing substrate concentration would suggest positive cooperative interaction. The data from Hill plot analysis (Fig. 3 and Table 3) also suggest that in general high concentration range the number of substrate molecules bound increased. Nevertheless, as is apparent from Fig. 2, the substrate saturation curves were not sigmoidal implying that allosteric regulation was not involved. This was also substantiated by the Lineweaver-Burk plot (data not shown) and Eadie-Hofstee plots (Fig. 2); as is well recognized the allosteric data can not be analyzed by Lineweaver-Burk and Eadie-Hofstee plots. Therefore, the alternate explanation seems to be that the enzyme has an in-built ability to respond to varying concentrations of substrate. Thus the enzyme can hydrolyze the substrate with lesser efficacy if the substrate concentration is low and can act more efficiently if the substrate level is higher. This in-built ability will ensure complete breakdown of phosphate esters from phagocytosed macromolecules of cellular origin or from invading microorganisms, irrespective of the resulting concentration of phosphate esters. Multicomponent behavior is not unique to acid phosphatase but has also been noted previously for mitochondrial FoF₁-ATPase as well as mitochondrial Na⁺/K⁺ATPase by us and other researchers. Also, the in-built ability of the enzyme to act on monoo-, di-, and trinucleotide substrates i.e. AMP, ADP and ATP becomes additional handle for dephosphorylation.

The present preliminary studies were carried out using the nuclei-free homogenate as the source of the enzyme. Nevertheless, these studies have clearly shown that in situ the nucleotides may be preferred substrates of acid phosphatase. The results of the present studies also suggest scope for further experiments using lysosomes or lysosomes-rich fraction as the source of enzyme which can shed further light on the in situ function of acid phosphatase.

References


9 http://www.fpmoetbook.com/HEM63.htm


