Properties of a Membrane-bound Phosphatase from the Thylakoids of Spinach Chloroplasts

Received for publication May 5, 1980 and in revised form July 18, 1980

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ABSTRACT

A 3-phosphoglycerate phosphatase activity of about 2 micromoles per minute per milligram chlorophyll is associated with the thylakoid membranes of spinach chloroplasts. The $K_m$ for 3-phosphoglycerate is 3 millimolar. The enzyme can be solubilized from thylakoid membranes by treatment with 0.33 molar MgCl₂ or sodium deoxycholate. The activity is not stimulated by sulfhydryl reagents or the addition of 10 millimolar MgCl₂. The enzymic activity is insensitive to ethylenediaminetetraacetate. The pH optimum is broad, between 5.5 to 7.5. Although the substrate specificity is broad, 3-phosphoglycerate is the best substrate of those tested at neutral pH. However, p-nitrophenyl phosphate was a more effective substrate at pH 5.5. The enzyme exhibits the general characteristics of an acid phosphatase. The name 3-PGA phosphatase is used to indicate the best substrate observed at neutral pH. A preliminary report of these results has been published (13).

MATERIALS AND METHODS

Chloroplasts were prepared according to several different methods during the investigation reported here. The procedure in each case will be referred to by literature citation or by the letter for the following three procedures.

Procedure A. Leaves from greenhouse-grown spinach (Spinacia oleracea L. var. Viroflay 99 M.R.) were washed with distilled H₂O and blotted dry, and midribs were removed. All subsequent steps were performed at 0 to 4 C. The leaves were homogenized for 10 s in a Waring Blender on high speed. The grinding medium consisted of 50 mM Tris-maleate buffer (pH 6.9), 1 mM EDTA, 2% (w/v) polyvinylpolypyrrolidone, and 10 mM 2-mercaptoethanol. The homogenate was squeezed through eight layers of cheesecloth and centrifuged at 12,000 g for 10 min. The pellet was washed twice by resuspension and centrifugation in 50 mM Tris-maleate (pH 6.9). This pellet was used as a crude source of thylakoids.

Procedure B. Chloroplast thylakoids were prepared as in procedure A, except the grinding medium was modified by substituting 50 mM Mes (pH 6.2) for the Tris-maleate buffer and adding 0.33 M sorbitol. These chloroplasts were lyed by resuspension in 50 mM Tris-maleate (pH 6.9) and washed twice by centrifugation.

Procedure C. Whole chloroplasts were prepared by grinding spinach leaves with a mortar and pestle. The homogenate was squeezed through eight layers of cheesecloth and centrifuged at 5,000g for 5 min. Grindng medium was as described for procedure B except 0.8 M sucrose was substituted for the 0.33 M sorbitol osmoticum.

3-PGA phosphatase was solubilized from chloroplast thylakoids by incubation of the thylakoplast suspension in 50 mM Tris-maleate buffer (pH 6.9) with 0.33 M MgCl₂ for 30 min at 0 C. The thylakoids were removed by centrifugation at 27,000g for 30 min. The supernatant solution was decanted and the centrifugation procedure was repeated to remove membranous material further. 3-PGA and PNP phosphatase activities were assayed with 20 mM substrate in 25 mM Tris-maleate (pH 6.9) or in 15 mM sodium cacodylate (pH 5.9), as described by Randall et al. (14). Phosphatase activity was determined by following the progress of a reaction mixture or by endpoint assay. Reactions were terminated by the addition of trichloroacetic acid to a final concentration of 2.5% (w/v). The enzymic rate was calculated from the amount of phosphate released after adjustment for the Pi in a control. Con-
trols contained all of the components of the assay, but trichloroacetic acid was added before the substrate. Linearity was demonstrated over the course of the endpoint assay.

Phosphate was measured by the method of Fiske and Subbarow (6) or by the modified microdetermination method of Chen et al. (5). The A of the reduced P-molybdate complex was measured at 800 nm when the method of Chen et al. (5) was used.

Protein was determined by the modified Lowry procedure of Bensadoun and Weinstein (3). Total Chl was determined by the A in 80% acetone (10).

RESULTS

Association of 3-PGA Phosphatase with Thylakoid Membrane.

In preliminary experiments, whole chloroplasts (prepared by procedure C) were observed to retain 25% of the total 3-PGA phosphatase activity observed in the crude homogenate. The nature of the phosphatase activity remaining in the supernatant, 75% of the total, was not examined. Differential centrifugation of osmotically lysed chloroplasts resulted in a pellet of starch particles and thylakoids with 90% recovery of the 3-PGA phosphatase activity observed in the whole chloroplast preparation. After centrifugation of the lysed chloroplasts, the activity of 3-PGA phosphatase remained constant at approximately 2 μmol min⁻¹ mg⁻¹ Chl in both the pellet and supernatant solution. These results suggested that the activity of 3-PGA phosphatase in the chloroplast was associated with the thylakoids.

Chloroplasts and starch particles were prepared and purified from spinach leaves (variety Viroflay 99 M.R.) on a discontinuous sucrose density gradient by the procedure of Tolbert et al. (17). The 3-PGA phosphatase activity was associated predominantly with the chloroplast fractions. The phosphatase activity observed with the starch particles was only 2% of the phosphatase activity in the chloroplast fractions. Randall et al. (16), using the spinach variety Long Standing Bloomsdale, had observed 3-PGA phosphatase activity associated with the starch particles and essentially none with the thylakoids. We repeated this experiment with Long Standing Bloomsdale spinach leaves and the 3-PGA phosphatase activity was observed predominantly with the chloroplast fractions, although somewhat more activity, approximately 15% of the total chloroplast activity, was associated with the starch particles. The reasons that Randall et al. (16) did not observe the enzyme in the chloroplast fractions are not understood.

Inasmuch as most of the enzymes responsible for carbon metabolism are soluble in the stroma, the membrane association of this protein seemed unusual and was characterized further. Various methods for the solubilization of membrane-bound proteins were tested (Table I). Greatest solubilization of the phosphatase activity was obtained with detergents or with salts at high ionic strength. The dependence of enzyme solubilization on MgCl₂ concentration was examined (Fig. 1). The optimum concentration of MgCl₂ for solubilizing the phosphatase was 0.33 M (ionic strength = 1). About one-half of the activity was solubilized at 80 mM MgCl₂ (ionic strength = 0.25). Sodium thiocyanate, a chlo-

<table>
<thead>
<tr>
<th>Additions to Suspension</th>
<th>Solubilized 3-PGA Phosphatase Activity</th>
<th>Specific Activity μmol min⁻¹ mg⁻¹ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (centrifugation omitted)</td>
<td>100*</td>
<td>0.3</td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>0.4</td>
</tr>
<tr>
<td>1.0 M NaCl</td>
<td>104</td>
<td>4.6</td>
</tr>
<tr>
<td>0.33 M MgCl₂</td>
<td>118</td>
<td>6.8</td>
</tr>
<tr>
<td>0.5% Sodium deoxycholate</td>
<td>72</td>
<td>0.4</td>
</tr>
<tr>
<td>0.5% Triton X-100</td>
<td>57</td>
<td>0.2</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Percentage of activity in thylakoids.

from the thylakoids. These results indicate that 3-PGA phosphatase is not associated with the coupling factor.

Enzyme Characterization. Initially, the phosphatase from spinach thylakoids was characterized with 3-PGA as the substrate. A broad pH optimum ranging from pH 6 to 7 was observed with the thylakoids or with the preparation solubilized by 0.33 M MgCl₂. The addition of 10 mM MgCl₂ to thylakoids did not change the activity or pH optimum of the 3-PGA phosphatase activity. The addition of 10 mM EDTA usually resulted in a minor stimulation of the phosphatase activity. The presence of 1 mM DTT did not affect the 3-PGA phosphatase activity. Addition of 10 mM reduced or oxidized glutathione had no effect on the enzyme activity. This response is unlike the non-chloroplastic acid phosphatase studied by Buchanan et al. (4).

The specificity of phosphate ester hydrolysis by the enzyme solubilized with 0.33 M MgCl₂ is shown in Table II. Similar results
Table II. Substrate Specificity of 3-PGA Phosphatase

The enzyme from spinach thylakoids (prepared by procedure A) was solubilized with 0.33 M MgCl₂ and assayed with 3.3 mM substrate in 25 mM Tris-maleate (pH 6.9). Relative activity is expressed as the percentage of phosphate released with 3-PGA as the substrate, except in the case of P-hydroxyacceptrate. The Vₘₐₓ for P-hydroxyacceptrate and 3-PGA were determined by kinetic plots and the relative activity with P-hydroxyacceptrate is expressed as the percentage of the Vₘₐₓ observed with 3-PGA.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PGA</td>
<td>100b</td>
</tr>
<tr>
<td>P-hydroxyaccepturate</td>
<td>80</td>
</tr>
<tr>
<td>Xylitol-1,5-bisP</td>
<td>70</td>
</tr>
<tr>
<td>p-Nitrophenyl-P</td>
<td>55</td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>51</td>
</tr>
<tr>
<td>Ribulose-1,5-bisP</td>
<td>30</td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>20</td>
</tr>
<tr>
<td>NADPH</td>
<td>17</td>
</tr>
<tr>
<td>ADP</td>
<td>17</td>
</tr>
<tr>
<td>P-glycolate</td>
<td>15</td>
</tr>
<tr>
<td>AMP</td>
<td>10</td>
</tr>
</tbody>
</table>

* The following substrates were hydrolyzed at a relative rate of less than 10%: 2-PGA, P-serine, pyrophosphate, 2,3-bis-PGA, α-glycerol-P, glucose-6-P, phytate, β-glycerol-P, fructose-1,6-bisP, P-choline, ATP, ribose-5-P, P-ethanolamine.

b 0.04 μmol Pi released/min.

were obtained with the enzyme bound to the thylakoids. Similar results were also obtained by Randall et al. (14, 16) and by Villareal et al. (20). Vₘₐₓ and Kᵡ values were determined for 3-PGA and P-hydroxyacceptrate. The Kᵡ value obtained for each substrate was about 3 mM.

The study of the specificity of the phosphatase from spinach thylakoids was broadened to include phosphoproteins and phospho-amino acids. The examination was initiated because of a report of a phosphoprotein phosphatase which was bound to thylakoid membranes (2). Little phosphoprotein phosphatase activity was observed with the thylakoid suspension. Casein, a phosphoprotein with P-serine residues, and the phospho-amino acids, P-serine and P-threonine, were each hydrolyzed at a relative rate of less than 10% of the rate observed with 3-PGA (2 μmol min⁻¹ mg⁻¹ Chl). Phosvitin, a phosphoprotein with P-serine residues, was hydrolyzed a little more rapidly at about 14% of the rate observed with 3-PGA.

Comparison with Acid Phosphatase. The relative hydrolytic activities of Sigma type I wheat germ acid phosphatase and the enzyme bound to spinach thylakoids were compared over a pH range with two phosphate esters, 3-PGA and PNP (Fig. 2). Addition of both substrates simultaneously to the spinach thylakoid enzyme resulted in little change in the phosphohydrolase activity, indicating that the phosphatase activity was the result of a single enzyme. At pH 5.5, PNP was the better substrate for each enzyme. The phosphatase bound to spinach thylakoids and wheat germ acid phosphatase exhibited similar responses to pH with each substrate. In the ratio of each enzyme’s hydrolytic activity toward the two phosphate esters changed with pH. As a result, the substrate specificity observed at pH 6.9 in Table II with the spinach thylakoid enzyme would have appeared different at pH 5.5.

Kinetics of Phosphatase from Spinach Thylakoids. The kinetics of 3-PGA hydrolysis by the phosphatase from spinach thylakoids have been examined using the thylakoid suspension, the preparation solubilized with 0.33 M MgCl₂, and the salt-solubilized enzyme dialyzed against 0.05% sodium deoxycholate. The latter two preparations consistently exhibit typical Michaelis-Menten kinetics with an apparent Kᵡ for 3-PGA between 2 and 3 mM (Fig. 2).

DISCUSSION

The phosphatase described here best hydrolyzes 3-PGA among many substrates tested by the authors and previously by Randall and Tolbert (14). The Kᵡ for 3-PGA is a relatively high value of 3 mM, which is obtainable in the chloroplast during photosynthesis. The Vₘₐₓ of the phosphatase bound to the thylakoids was about 2 μmol min⁻¹ mg⁻¹ Chl, which is less than the rate of CO₂ fixation during photosynthesis, but nevertheless significant. A
phosphatase that hydrolyzes 3-PGA, the first product of photosynthetic CO₂ fixation, which may relate to the thylakoid location. Hydrolysis of 3-PGA to glyceraldehyde may initiate a metabolic pathway to serine, which may operate in the dark, but which would not be necessary during glycerate and serine synthesis in the light by photorespiration.

The association of this phosphatase with the thylakoid membrane is unusual since most of the enzymes associated with carbon metabolism in the chloroplast are soluble in the stroma. The enzyme was not soluble in the stroma because it was not lost when chloroplasts were ruptured. We do not believe the enzyme to have been associated with the outer chloroplast membranes since 90% of the phosphatase activity in the chloroplast fraction remained with the thylakoids when the chloroplasts were lysed and removed from the outer membrane fraction by differential centrifugation. The thylakoids inside the intact chloroplasts should not have been exposed to the cytosolic proteins after homogenization to adsorb protein. The high salt concentration necessary for solubilization of this phosphatase indicates that it is quite tightly bound to the thylakoid membrane. The fact that this protein may be solubilized with salt suggests that it is a peripheral membrane protein, rather than an integral membrane protein, which generally require more drastic treatment (detergent) for solubilization.

The phosphatases which have been reported as 3-PGA phosphatase by Randall et al. (14–16), Villereal et al. (20), and Mulligan and Tolbert (13) exhibit the characteristics of an acid phosphatase. Acid phosphatases typically exhibit broad substrate specificity and an acidic pH optimum. Randall et al. (16) noted that at pH 6.3 the hydrolysis of PNP occurred at the same rate as the hydrolysis of 3-PGA. A high rate of 3-PGA hydrolysis at neutral pH, but more rapid hydrolysis of PNP at a lower pH, has been observed here (Fig. 2). This response is exhibited by the enzyme from spinach thylakoids and wheat germ acid phosphatase. The absence of Mg⁶⁺ requirement or an effect of EDTA on P-hydrolase activity is not unusual for an acid phosphatase (9). It is concluded that the 3-PGA phosphatase activity of spinach thylakoids is the result of an enzyme with the characteristics of an acid phosphatase.

Wheat germ acid phosphatase and the enzyme from spinach thylakoids exhibit different pH optima with 3-PGA and PNP. An acid phosphatase would be expected to bind and catalyze the hydrolysis of the monoanion species of the phosphate ester. Since the p-nitro group of PNP is intensely electron withdrawing, the second pKₐ of this phosphatase ester is 5.1. The second pKₐ of 3-PGA is 6.5 and, therefore, 3-PGA will be present as the monoaonion species at higher pH than PNP. Thus, the dissociation of the second proton from the phosphate ester may manifest itself in the pH optima observed for these substrates.

Regulation of the enzyme activity may result from the acid pH optimum of the phosphatase. The pH of the chloroplast is known to be alkaline in the light, about pH 8, and essentially neutral in the dark (7). This phosphatase has been shown to be active in the hydrolysis of phosphate esters at neutral pH but less active at higher pH. Diurnal fluctuations in pH, which depend on incident light, may result in the regulation of this phosphatase activity in vivo.

The presence of a thylakoid-bound phosphoprotein phosphatase has been suggested by Bennett (2). The substrate for the phosphatase is probably membrane-bound P-proteins of the chloroplast, such as the light-harvesting Chl protein complex. Although little phosphoprotein phosphatase activity was observed with the spinach thylakoid-bound phosphatase preparations here, the P-serine proteins tested may be poor substrate analogs of chloroplast membrane P-proteins.

The function of this enzyme in the biochemistry of the chloroplast is not clear. The phosphatase has a different substrate specificity and location than the acid phosphatase studied by Buchanan et al. (4). It seems unlikely that the physiological role of this chloroplast enzyme is related to that of vacuolar acid phosphatase. Since 3-PGA was the best substrate for the enzyme at pH values between 6 and 7, it has been assumed in previous publications that it is the physiological substrate. Although this seems to be the case, the possibility of other substrates exists.

LITERATURE CITED


Fig. 4. Kinetics of 3-PGA hydrolysis by the thylakoid-bound phosphatase. Spinach thylakoids were prepared according to procedure A and incubated with 2 to 33 mm 3-PGA in 25 mm Tris-maleate buffer (pH 6.9). The rate of phosphate ester hydrolysis was determined by sampling the reaction at different time points. Major axes show velocity (v) versus substrate concentration. Inset shows a Hill plot with a Hill coefficient of 1.3.