ATPase in Lipid Body Membranes of Castor Bean Endosperm

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ABSTRACT

Lipid body membranes purified from castor seed endosperm of dry seeds and 4 d old seedlings were found to have an ATPase activity associated with them. This was confirmed by equilibrium density centrifugation of the membranes using acid lipase as a marker enzyme. The specific activity ranged from 45 to 200 nanomoles per milligram protein per minute. The pH optimum was 9.0 but at pH 7.5 nearly 40% of the maximum activity was retained. The apparent K_m for Mg-ATP was 0.5 millimolar. A divalent cation was required for activity and MgATP was the most effective. Other nucleoside triphosphates were also hydrolyzed but there was no hydrolysis of pyrophosphate or p-nitrophenylphosphate. The ATPase was not inhibited by oligomycin, vanadate, dicyclohexylcarbodiimide, or molybdate but was inhibited by sodium azide. Washing the membranes with increasing concentrations of NaCl removed up to 60% of the ATPase activity but none was removed by 3 millimolar ethylene-diaminetetraacetate.

The endosperm of castor seeds contains large quantities of storage lipid (triricinolein) which is contained in membrane bound lipid bodies (7). During early seedling growth the fat is hydrolyzed and the fatty acids are converted to sugars which support growth of the seedling. The lipid body membrane has a half-unit structure because of the hydrophobic nature of the organelle’s contents (16), though it is presumed to form bilayers when the triglyceride is extracted during membrane purification. EM and SDS-PAGE have shown that the membrane has several proteins associated with it (3) and acid lipase and antymycin A insensitive Cyt c reductase activities have been detected. The role of the acid lipase is still uncertain. It is incorporated into lipid bodies during seed development, is present at maximum activity in dry seeds, and declines in activity during germination and early growth (12). In all other oil seeds studied, lipase activity appears only during seed germination (9). The control of lipid hydrolysis during castor seed germination is still unclear (11) and it was during investigations into possible control mechanisms that an ATPase activity associated with the lipid body membrane was found. The enzyme kinetics were characterized and are described in this paper.

MATERIALS AND METHODS

Plant Material. Castor seeds (Ricinus communis L. cv Hale) were soaked in running tap water for 1 d and allowed to germinate in moist vermiculite in the dark at 30°C in a humidified growth chamber. All biochemicals were purchased from Sigma Chemical Co.

Membrane Preparation. Lipid bodies were isolated from the endosperm of 4 d old castor bean seedlings by slicing in ice cold grinding medium (13) with a razor blade for 10 min. The pulp was filtered through nylon mesh and made up to 80 ml with grinding medium before being centrifuged at 10,000g for 15 min. The fat pad, containing the lipid bodies, was removed from the surface of the supernatant with a spatula and washed in 40 ml of fresh medium by suspension and centrifugation. The washing was repeated twice. The lipid bodies were finally removed, placed in a screw top test tube with 3 ml of buffer and extracted three times with 10 ml diethyl ether to remove the triacyl glycerols. Trace amounts of ether were evaporated under a stream of N_2. The membrane fraction was then centrifuged at 100,000g for 1 h and the pellet resuspended by gentle grinding with a ground glass homogenizer in 1.2 ml of modified grinding medium, containing 10 mM Hepes-NaOH (pH 7.5) and 5 mM DTT and stored at −20°C.

ATPase Assay. MgATP-dependent ATP hydrolysis was determined by measuring the release of phosphate from ATP by a colorimetric method (8). Unless otherwise stated, aliquots of the membrane preparation (5–10 µg protein) were incubated in a medium containing 50 mM glycine-NaOH buffer (pH 9.0), 10 mM KCl, 1 mM MgCl_2, 1 mM EDTA, 2 mM DTT, and 2.5 mM MgATP. The samples were incubated at 37°C for 30 min.

Density Gradient Centrifugation. The membrane preparation was layered onto a sucrose density gradient consisting of a sucrose solution increasing linearly in concentration from 15 to 50% (w/w) over a 3 ml cushion of 50% sucrose. The gradient also contained the other constituents of the grinding medium. The gradients were centrifuged at 100,000g for 3 h in an SW 27-1 rotor in a Beckman L2-65B ultracentrifuge and 0.4 ml fractions were collected with an ISCO density gradient fractionator. All steps were carried out at 0 to 4°C. The sucrose concentration of each fraction was measured with a Bausch and Lomb refractometer.

Enzyme Assays. Acid lipase was measured by a fluorimetric method (13) and other enzyme activities were measured spectrophotometrically (fumarase (14), Cyt c oxidase (5) and phosphatase activity was measured with PNPP (5) as substrate with the same buffer as used for ATPase measurement).

Protein Assay. Protein was determined by the method of Lowry (10) after TCA precipitation.

RESULTS AND DISCUSSION

An ATPase was detected in lipid body membranes purified from castor bean endosperm of 4 d old seedlings. The specific activity ranged from 45 to 200 nmol mg⁻¹ protein min⁻¹ depending on the preparation. The rates were linear for at least 1 h at 37°C.

Although the fat pad was washed thoroughly and refloated by centrifugation three times before the membranes were extracted it was possible that ATP hydrolysis was being catalyzed by a
contaminating nonspecific phosphatase found in high activity in crude endosperm extracts. The addition of various amounts of sodium molybdate (an inhibitor of phosphatases) had very little effect on the rate of ATP hydrolysis (Table I). Furthermore, assays for phosphatase activity carried out using PNPP as the substrate showed very low activity (0.33 nmol Pi mg\(^{-1}\) protein min\(^{-1}\)) and this was totally inhibited by 1 mM sodium molybdate. Pi was cleaved from ADP at only 7% of the rate from ATP, and PPI hydrolysis was not detectable. Thus, the ATPase activity was not due to a nonspecific phosphatase. The effect of the pH of the medium on the ATPase activity is shown in Figure 1. Maximal activity occurred at pH 9.0 although the rate of ATP hydrolysis at pH 7.5, which would be a more likely pH in the cytoplasm, was nearly 40% of the maximum. Routine assays were carried out at pH 9.0. The ATPase activities of membranes prepared from plant cells are usually stimulated by divalent cations (6), and the ATPase associated with the lipid body membrane is no exception. Figure 2 shows the dependence of ATPase activity on the concentration of Mg\(^{2+}\), Mn\(^{2+}\), and Ca\(^{2+}\) in the presence of 100 mM NaCl. ATP hydrolysis was not detectable if EDTA was included in place of the divalent cation. Mg\(^{2+}\) was the most effective divalent cation giving maximal stimulation at 2.5 mM but then progressively limited ATPase activity so that at 20 mM Mg\(^{2+}\) the rate of hydrolysis was only 33% that of the maximum. Mn\(^{2+}\) was most effective at 1 mM but the maximum rate was less than that achieved with Mg\(^{2+}\). CaCl\(_2\) was even less effective in stimulating ATPase activity. If NaCl was omitted from the medium the effect of Mg\(^{2+}\) was reduced by a factor of 2 to 3 depending on the Mg\(^{2+}\) concentration. The omission of NaCl only caused a 20% decrease in activity when Ca\(^{2+}\) was the divalent cation present. Similar interactions in the effects of monovalent and divalent cations have been reported for other membrane associated ATPases (4, 15).

The effect of substrate concentration (Mg-ATP) was measured and a Lineweaver-Burk plot of the data is shown in Figure 3. From this the apparent \(K_p\) for Mg-ATP was calculated to be 0.5 mM. In calculating the \(K_p\), the points for concentrations above 4 mM Mg-ATP were ignored since the enzyme became slightly inhibited. Again such inhibition has been reported for other ATPases, e.g. that of the oat root plasma membrane (1).

Substrate specificity was tested by incubating aliquots of lipid body membrane preparation with other nucleoside triphosphates and pyrophosphate (Table II). It was found that Pi was cleaved from both GTP and ITP at a faster rate than from ATP. UTP and CTP were less efficiently hydrolyzed and cleavage of PPI was not detectable.

Acid lipase was used as the marker enzyme for the lipid body membrane in the density gradient experiments (12). The apparent equilibrium density of the membrane was found to be 1.19 g cm\(^{-1}\) (Fig. 4) which is somewhat higher than that reported by Moreau et al. (12) who found it to be 1.12 g cm\(^{-1}\). The density of the membranes was not changed if the sucrose gradient contained only 1 mM EDTA (as was used previously (12)). It has been reported (9) that the amount of lipase removed from corn lipid body membranes during neutral lipid extraction can vary between 50 to 80% of the total. Since membrane density is a function of the phospholipid to protein ratio it is possible that different extraction conditions during other treatment in these experiments caused relatively less protein loss and hence a greater equilibrium density. Lipid body membranes were also prepared from the endosperm of dry seeds and these too had an apparent

Table 1. Effect of various Inhibitors on the Lipid Body ATPase

| Inhibitor Concentration | ATPase Activity
|-------------------------|-----------------|
| Control                 | 100 % of control
| Sodium azide, 1 mM      | 21 %            |
| Oligomycin, 5 µg/ml     | 100 %           |
| Sodium vanadate, 10 µM  | 109 %           |
| DCCD, 10 µM             | 113 %           |
| Sodium molybdate, 1 mM  | 106 %           |
| Dinitrophenol, 100 µM   | 118 %           |

Fig. 1. Effect of pH on the rate of ATP hydrolysis. The buffer contained a mixture of imidazole-tricine-glycine (30 mM each) adjusted to the required pH values with NaOH. The other constituents were as in “Materials and Methods.”

Fig. 2. Effect of divalent cations on the rate of ATP hydrolysis. MgCl\(_2\) (\(\bullet\)), MnCl\(_2\) (\(\circ\)), CaCl\(_2\) (\(\mathbf{■}\)). Assay buffer contained 50 mM glycine-NaOH (pH 9.0), 2 mM DTT, 2.5 mM ATP, 100 mM NaCl.
equilibrium density of 1.19 g cm⁻¹ (data not shown). ATPase activity was measured and it can be seen in Figure 4 that the peak of activity coincided with the acid lipase. The same was found for lipid body membranes from dry seeds. The ATPase was clearly associated with the lipid body membrane and to assess whether the enzyme was a 'peripheral' protein, aliquots of membrane preparation were incubated for 30 min on ice with various concentrations of NaCl or 3 mM EDTA followed by centrifugation at 100,000g for 1 h. Figure 5 shows that the acid lipase was not removed by salt washing but 60% of the ATPase activity was removed by 0.5 M NaCl. EDTA did not cause any loss of ATPase or lipase activity. It appears that at least a portion of the ATPase is relatively loosely bound to the lipid body membranes by ionic interaction but since the EDTA wash had no effect on ATPase activity it is unlikely that there is a strong ionic bond. More efficient washing of peripheral proteins from membranes is often achieved with 100 mM sodium carbonate at pH 11.5 (3). However, it was not possible to use this procedure because the sodium carbonate caused irreversible inhibition of the ATPase at this pH.

The ATPase was not inhibited by oligomycin or DCCD (Table 1) which are known to inhibit ATPases of plastids and mitochondria. Sodium vanadate, an inhibitor of plasma membrane ATPase was similarly ineffective. The ATPase was inhibited by NaN₃ which reduced the activity by 80% at 1 mM. Since mitochondrial

ATPase is strongly inhibited by azide this raised the possibility that the lipid body ATPase activity might be due to contamination by mitochondrial F₁, released during the chopping of the endosperm. This was thought to be unlikely for the following reasons (a) the lipid bodies were washed by flotation with fresh buffer three times during the preparation, (b) all of the ATPase remained with the membranes during the density gradient cen-

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**Table II. Substrate Specificity of the Lipid Body ATPase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ATPase Activity (nmol mg⁻¹ protein min⁻¹)</th>
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<tbody>
<tr>
<td>ATP</td>
<td>61</td>
</tr>
<tr>
<td>GTP</td>
<td>101</td>
</tr>
<tr>
<td>ITP</td>
<td>85</td>
</tr>
<tr>
<td>UTP</td>
<td>22</td>
</tr>
<tr>
<td>CTP</td>
<td>13</td>
</tr>
<tr>
<td>ADP⁺</td>
<td>3</td>
</tr>
<tr>
<td>PPI⁺</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PNPP⁺</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*Control rate of ATP hydrolysis was 45 nmol mg⁻¹ protein min⁻¹.

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**Figure 3.** Double reciprocal plot of the effect of substrate (Mg-ATP) concentration on rate of hydrolysis.

**Figure 4.** Sucrose density gradient containing lipid body membranes after centrifugation at 100,000g for 3 h: Acid lipase (○), ATPase (●).

**Figure 5.** Lipid body membranes were washed with varying concentrations of NaCl, pelleted (100,000g for 1 h) and assayed for acid lipase (○) and ATPase (●).
Table III. Changes in Mitochondrial Enzyme Activities during Seedling Development

Measurements made by standard spectrophotometric techniques described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Dry seed</th>
<th>4 Day</th>
<th>Activity increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol min⁻¹</td>
<td>endosperm⁻¹</td>
<td>-fold</td>
</tr>
<tr>
<td>Fumarase</td>
<td>0.02</td>
<td>2.0</td>
<td>100</td>
</tr>
<tr>
<td>Cyt c oxidase</td>
<td>0.01</td>
<td>1.5</td>
<td>150</td>
</tr>
<tr>
<td>ATPase</td>
<td>0.013</td>
<td>0.53</td>
<td>41</td>
</tr>
</tbody>
</table>

It is possible to assign a function to the lipid body ATPase on the present evidence. Many membranes contain ATPases and some of these have been shown to be coupled to ion pumping between cell compartments. Proton pumping by lipid body membrane preparations was not detected by quinacrine fluorescence quenching or by [¹⁴C]methylamine uptake (2). However, in lipid bodies it is unlikely that proton pumping across the membrane would occur due to the hydrophobic contents of the organelle.

trifugation and 33% of the activity remained with the membranes after treatment with 0.5 M NaCl, (c) the mitochondrial ATPase activity in 4 d seedlings was 40-fold higher than that in dry seeds and the number of lipid bodies and lipase activity decreases between 0 and 4 d of germination (Table III). Nevertheless, the lipase activity to ATPase activity ratios were quite similar in dry seeds and 4 d seedlings (2.6 and 1.7, respectively).