Oligomycin-sensitive ATPase of Submitochondrial Particles from Corn

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ABSTRACT

To test the hypothesis (Plant Physiology 59: 155–157) that monocotyledons contain a unique oligomycin-insensitive ATPase, we prepared submitochondrial particles and a soluble fraction from sonicated corn mitochondria (Zea mays L. cv. EarliKing). Although the ATPase activity of the whole sonicate was relatively insensitive to oligomycin, the corn submitochondrial particles possessed an ATPase activity that was nearly completely inhibited by oligomycin, and was activated by trypsin. This ATPase is similar to that from other sources (plants, animals, and microorganisms). The soluble fraction also contained an active ATPase, which was inhibited by azide and stimulated by sodium chloride and trypsin. The soluble fraction differed from other F1-ATPases in that it was cold-stable.

Research into the mechanisms of oxidative and photosynthetic phosphorylation has shown that the energy-transducing ATPases (ATP: phosphohydrolase EC 3.6.1.3) from a wide variety of organisms are extremely similar. Well prepared preparations from chloroplasts, from yeast and mammalian mitochondria (11), from bacteria (1), and from pea mitochondria (4, 8) show similarities in many catalytic and structural properties. In particular, the mitochondrial ATPases in their membrane-bound state are noted for their sensitivity to the antibiotic oligomycin (10, 11). This sensitivity, conferred upon the F1-ATPase by integral membrane components, is lost when the F1-ATPase is solubilized (10). The soluble enzyme is noted for extreme cold lability (12) and a high mol wt (380,000 daltons) (10).

Recently, workers from two laboratories have reported that the ATPase activity of sonicated corn mitochondria is not inhibited by oligomycin (5, 15). In addition, the solubilized form of the corn ATPase was shown to be stable in the cold, and of low mol wt (40,000–60,000 daltons) as demonstrated by gel filtration (15). The workers suggested that mitochondria of corn, and of the other monocotyledons tested, may possess a unique energy-transducing ATPase system (15).

In this paper we report on the results of experiments designed to test this hypothesis. Submitochondrial particles, which are low in contaminating soluble enzymes, were prepared from sonicated corn mitochondria and were shown to be inhibited by oligomycin in the normal fashion; the remaining soluble fraction contained an ATPase that resembled F1-ATPase.

MATERIALS AND METHODS

Corn seeds (Zea mays L. cv. EarliKing) were surface-sterilized for 15 min in a 3% sodium hypochlorite solution and washed several times in running water. The seeds were then allowed to germinate in the dark for 3 days in Vermiculite in a growth chamber at 27 C. The etiolated shoots (100–200 g) were harvested and mitochondria isolated according to a differential centrifugation procedure that was described previously (14). A greenish layer around the brown mitochondrial pellet was removed by suction. The mitochondria were resuspended in 0.25 mol sucrose and repelleted at 20,000g for 8 min. The washed mitochondrial pellet was suspended in 20 to 30 ml of “SMP buffer” (0.25 mol sucrose and 50 mm TES brought to pH 7 at 25 C with Tris). The mitochondria were then sonicated at 5 C for two 1-min bursts at full power on an Artek Sonic Dismembrator. Unbroken mitochondria were removed by centrifugation at 20,000g for 8 min. The translucent supernatant layer (whole sonicate) was then centrifuged at 90,000g for 60 min at 4 C. The pellet (SMP) was resuspended in 2 ml of SMP buffer preparatory to assay. The supernatant layer (soluble fraction) was assayed directly.

Trypsin treatments were carried out in SMP buffer by the addition of 0.1 u g of trypsin for each 1 u g of protein. After 15 min at 30 C, 1.6 u g of trypsin inhibitor for each 1 u g trypsin protein was added to stop the reaction.

ATPase activity was assayed at 30 C by the method described previously (4, 8). The assay medium was as described in Table I. The reaction was started by the addition of the fraction to be assayed, and stopped after 15 min by the addition of 1 ml of ice-cold quench solution (1.8 M NaClO4, 0.12 M glycine, and 0.3 M HCl). Inorganic phosphate was measured on a portion of the quenched mixture by the Mozersky et al. method (9), which was modified as described previously (8). A unit of ATPase activity is defined as the amount of enzyme required to liberate 1 mol of phosphate in 1 min, under the above assay conditions.

Protein was measured by a very simple and fast dye-binding procedure (13). When defatted BSA was used as the standard, the technique gave good agreement with the Lowry method (7).

Tri-sodium ATP, TES, oligomycin, and lima bean trypsin inhibitor were obtained from Sigma. Pancreatic trypsin was from Calbiochem. All other chemicals were from Fisher, and were of the highest purity available.

RESULTS AND DISCUSSION

The whole sonicate from corn mitochondria showed an ATPase activity that was only partially inhibited by oligomycin (Table I, exp. 1). However, the particulate fraction (SMP) prepared from the sonicate showed an ATPase activity that was more than 95% inhibited by low levels of oligomycin. Since the mitochondrial ATPases are generally associated with a trypsin-sensitive inhibitor polypeptide (11), we tried treating corn SMP with trypsin. The treatment produced a 3-fold activation of the ATPase, which remained sensitive to oligomycin. Our experience with SMP prepared from peas (Grubmeyer and Spencer, manuscript in preparation) shows that they are also very similar to typical SMP from rat liver and yeast mitochondria, and to those from corn. They are inhibited by oligomycin when fully separated from soluble pro-
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Plant Physiol. Vol. 61, 1978

Table I. ATPase Activity of SMP and Soluble Fractions from Corn Mitochondria

<table>
<thead>
<tr>
<th>Material</th>
<th>Treatment</th>
<th>Rate of Pi release (Units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control + Oligomycin</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole sonicate</td>
<td>None</td>
<td>0.14</td>
</tr>
<tr>
<td>SMP</td>
<td>None</td>
<td>0.22</td>
</tr>
<tr>
<td>Trypsinized SMP</td>
<td>None</td>
<td>0.75</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>0.1 M NaCl</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>0.5 M NaF + 0.1 M NaCl</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>3 mM a-glycerophosphate as substrate</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole sonicate</td>
<td>None</td>
<td>0.74</td>
</tr>
<tr>
<td>SMP</td>
<td>None</td>
<td>0.96</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>None</td>
<td>0.41</td>
</tr>
<tr>
<td>Trypsinized soluble fraction</td>
<td>No pre-assay treatment</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>1 hr at 25 C pre-assay</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>1 hr at 0 C pre-assay</td>
<td>0.45</td>
</tr>
</tbody>
</table>

1NOTE: The standard assay mixture (in 2.0 ml final volume) was 0.3 M sucrose, 3 mM MgCl₂, 3 mM ATP, and 25 mM TES, brought to pH 8.0 at 25 C with tris. Oligomycin (1 µg) was added as noted. The reaction was started by the addition of the fraction to be assayed and continued for 15 min.

In Expt. 1 the fractions were prepared as described in Methods. Amounts of protein added were 16 - 25 µg. In Expt. 2 sonication and centrifugation were done at 20 C to prevent any possible cold degradation of F₁-ATPase that had been solubilized. Amount of protein added was 21 µg. Trypsinization and pre-assay treatments were carried out in SMP buffer. Other treatments were added to assay medium.

A blank space indicates test was not made. The entry n.d. means activity was not detectable (less than 5% of control rates). All assays were repeated several times and results were reproducible.

The soluble fraction remaining as a supernatant layer after the high speed centrifugation was also found to contain an ATPase activity. This activity could be a nonspecific phosphatase, an F₁-ATPase that was solubilized during sonication, or the novel low mol wt ATPase observed by Sperk and Tuppy (15). With 3 mM β-glycerophosphate as a substrate we were unable to detect any hydrolysis activity with the soluble fraction. This ruled out a typical nonspecific phosphatase as a component of the soluble fraction. We found that the ATPase activity of the soluble fraction was stimulated 2-fold by 0.1 M NaCl; this stimulation was similar to that for the soluble ATPase of pea mitochondria (4). To determine further whether the fraction was actually solubilized F₁-ATPase, we added 0.5 mM NaN₃ to the assay medium. Azide, which normally inhibits metallo-enzymes (3), is also an inhibitor of F₁-ATPases (10) including that from peas (4). The soluble fraction was about 90% inhibited by 0.5 mM NaN₃ in the presence of 0.1 M NaCl; this inhibition is similar to that of the soluble pea enzyme under the same conditions (4).

The soluble fraction was found to be stable to freezing at -40 C, and to prolonged exposure to cold treatments at 0 C. This finding is in direct contrast to that observed with the purified ATPase from pea mitochondria (4) and with other soluble F₁-ATPases (11), but it confirmed the experiments of Sperk and Tuppy (15) with the partially purified corn enzyme. A variety of compounds have been found to stabilize the mammalian F₁-ATPases at low temperature, including the F₁-ATPase inhibitor polypeptide, the protein-phospholipid complex F₀ and mitochondrial phospholipids (12). The partial inhibition by oligomycin of the ATPase activity of the soluble fraction suggests that some of the F₁-ATPase might have complexed with an F₀-type protein-phospholipid.

If inhibitor polypeptide was stabilizing the enzyme, then trypsin treatment should result in increased activity and the appearance of cold lability. Our results show that trypsin did increase ATPase activity, but did not enhance cold lability (Table I, expt. 2). In this experiment we carried out sonication and centrifugation at 20 C to minimize any loss of F₁-ATPase caused by cold. The high activity of the whole sonicable and soluble fractions is in agreement with our findings on pea SMP that ATPase activity of SMP is stimulated by being kept at temperatures above 0 C (Grubmeyer and Spencer, manuscript in preparation).

The soluble fraction, although it possessed some catalytic properties that were similar to those of F₁-ATPase, cannot be definitely classified. While it may be found to be a completely separate ATPase enzyme, it is likely either a complex of F₁-ATPase with some cold-protecting agent, or a depolymerized form of the enzyme which is either capable of rapid reassociation under assay conditions, or which has a catalytically active subunit.

Finally, we would like to comment on the widespread use of whole sonicable preparations to investigate plant mitochondrial ATPase (2, 5, 6, 15). These preparations can be misleading since they not only contain two distinct forms of the ATPase (soluble and membrane-bound), but also contain adenylate and nucleotide kinases, which confuse the results of substrate specificity studies. In addition they may contain other enzymes that react with nucleotides or assay components. SMP, which require little additional effort to prepare, are less subject to contamination and provide more informative results.

Acknowledgment—We are grateful to D. McIvor for editorial assistance.

LITERATURE CITED

ATPase OF CORN SUBMITOCHONDRIAL PARTICLES

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