Effect of the Electrochemical Proton Gradient and Anions on the ATPase Activity of Soybean Submitochondrial Particles

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ABSTRACT

Submitochondrial particles from soybean (Glycine max L. cv Jupiter) hypocotyls with an ATPase activity of 0.3 to 1.0 micromole per minute per milligram were prepared by sonication with Mg-ATP. The particles catalyzed ATP synthesis with NADH and succinate; the ratios of ATP/O with these substrates were 1.0 and 0.1, respectively. As monitored by oxonol VI, the particles built up and maintained a membrane potential that was higher with NADH than with succinate or Mg-ATP. The ATPase activity of the particles increased two to threefold by preincubation with 50 millimolar phosphate at a temperature of 38°C. The increase in ATPase activity became higher (five to sixfold) when particles were preincubated with Mg-ATP plus phosphate. Under the latter conditions, collapse of Δψ by carbonyl cyanide p-trifluoromethoxyphenylhydrazone prevented the activation. An increase in ATPase activity of the particles was also observed with NADH and succinate, although activation was lower with succinate. With these substrates, phosphate did not increase ATPase activation. When particles were preincubated with Mg-ATP, anions that stimulate ATP hydrolysis (malate, malonate, and bicarbonate) had an activating effect similar to that of phosphate. The data suggest that the soybean mitochondrion ATPase can be activated by Δψ but that this activation is increased by the binding of certain anions to a conformation of the enzyme that appears during hydrolytic cycles.

The H⁺-ATP synthases of energy-transducing membranes catalyze the synthesis of ATP from ADP and phosphate with the energy of electrochemical H⁺ gradients derived from electron transport (10, 25). The ATP synthases of several biological sources have been extensively studied and they are known to be formed by a membrane sector (F₅₋), whose function is to mediate transport of H⁺ to the catalytic moiety (F₅), responsible for the synthesis of ATP and, under some conditions, ATP hydrolysis (10, 25). The studies that have been carried out on the ATP synthases on plant mitochondria are not as numerous as those made using mammalian mitochondria. However, some studies (6, 8, 11, 12, 27) indicate that the ATPase activity of plant mitochondria is subject to regulatory mechanisms. The observation that the ATPase activity of plant submitochondrial particles is increased by trypsin (6, 8, 11, 27) suggests that the enzyme is regulated by an inhibitor protein similar to that described in H⁺-ATPases from other energy-transducing membranes (2, 19, 23, 24).

This work describes results indicating that the ATPase activity of submitochondrial particles from soybean hypocotyls, similar to that of mammalian mitochondria (4, 9, 22, 28), can be increased by the establishment of a Δψ derived from electron transport or ATP hydrolysis. However, it was also found that during hydrolysis of ATP, some anions that are known to stimulate ATP hydrolysis (3) induce a further activation of the enzyme. This suggests that these anions activate the ATPase of plant mitochondria by binding to an enzyme form that appears during the catalytic cycle.

MATERIALS AND METHODS

Soybean seeds (Glycine max L. cv Jupiter) were surface-sterilized for 15 min in a 1% sodium hypochlorite solution and washed several times with distilled water. The seeds were germinated at 30°C in the dark for 5 d in wet filter paper. The etiolated shoots (500–700 g) were cut and placed in 1.5 L of 0.5 M mannitol, 20 mM Hepes/KOH (pH 7.4), 1 mM EGTA, 4 mM cysteine, and 0.1% BSA (the pH of the solution was adjusted to 7.4 with KOH at 25°C). The shoots were homogenized with a Polytron disintegrator (probe 9) on batches of 40 ml for 4 s. The homogenate was passed through four layers of cheesecloth, the pH adjusted to 7.0 with KOH, and was centrifuged at 1,400g for 10 min to eliminate unbroken cells and the cell wall fraction. The supernatant was centrifuged at 11,000g for 20 min. The mitochondrial pellet was washed two times with 100 ml of a second medium containing 0.3 M mannitol, 10 mM Hepes, 0.1% BSA, 4 mM cysteine, and 0.1 mM EGTA at pH 7.0 and centrifuged as before. Finally, the pellet was washed once in 50 ml of 0.25 M sucrose, 10 mM Hepes (pH 7.0), and centrifuged at 8,800g for 20 min. The resulting pellet was resuspended in the latter medium. For the preparation of submitochondrial particles, the mitochondrial suspension (50–70 mg/ml) was adjusted to a concentration of 10 to 15 mg/ml with ice-cold 0.25 M sucrose, 10 mM Hepes (pH 7.0), and 6 mM Mg-ATP. The suspension was sonicated in an ice bath for two 30-s periods, separated by a 1 min cooling period. The sonicate was centrifuged for 10 min at 27,000g at 4°C in order to remove unbroken mitochondria. The supernatant was centrifuged for 30 min at 100,000g at 4°C; the pellet was suspended in 5 ml of 0.25 M sucrose and 10 mM Hepes (pH 7.0) and centrifuged again. The resulting pellet was finally suspended in the last medium to a concentration of 20 to 30 mg protein/ml and stored in 0.2 ml aliquots at –70°C for times that did not exceed 3 weeks. The total amount of protein obtained varied.
between 10 to 20 mg, which was about 30 to 35% of the total mitochondrial protein. The entire procedure was carried out at 4°C.

Protein was estimated by the method of Lowry et al. (14).

O₂ uptake was determined polarographically using a Clark-type electrode in 1.75 ml of a medium containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, and 5 mM KH₂PO₄, brought to pH 7.2 with KOH at 30°C. Other additions are specified under "Results."

ATP synthesis was measured by uptake of ³²Pi. The assay medium in a final volume of 1.4 ml contained 0.25 M sucrose, 20 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 10 mM ³²Pi (3.3 Ci/mol), 25 mM glucose, 1 mM ADP, 12 units of hexokinase, and either 15 mM NADH or 20 mM succinate. Changes in this assay medium are described under "Results." The reaction was started by the addition of 1.4 mg protein and arrested after 10 min with 6% trichloracetic acid, final concentration. ³²Pi incorporation was determined as described elsewhere (1).

A double beam spectrophotometer was used to estimate the membrane potential of the submitochondrial particles by following the absorbance changes of oxonol-VI (18) at 603 to 630 nm. The assay was carried out at 30°C in a 1 ml system containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 6 μM oxonol.

ATPase activity was measured spectrophotometrically by NADH oxidation at 30°C following changes in absorbance at 340 nm in an ATP regenerating system as described previously (1). The medium contained 150 mM sucrose, 50 mM Tris-acetate (pH 8.0), 30 mM K-acetate, 3 mM Mg-acetate, 3 mM ATP, 1 μM FCCP, 3 μM rotenone, 0.1 mM phosphoenolpyruvate, 10 units pyruvate kinase, 8 units lactate dehydrogenase, and 0.2 mM NADH in a final volume of 3.0 ml. After the addition of the particles, the oxidation of NADH was recorded. Submitochondrial particles from soybean hypocotyls exhibited a rotenone-insensitive oxidation of NADH. This activity was determined by measurement of NADH oxidation in the same mixture that was used for assay of ATPase activity, except for the addition of ATP. This value was approximately 100 nmol min⁻¹ mg⁻¹ and was subtracted from the value of NADH oxidation observed in the presence of ATP. The difference represented the value of ATP hydrolysis. When particles were preincubated with Mg-ATP or ADP, sufficient time was allowed for exhaustion of ADP carried from the preincubation media before ATPase activity was calculated.

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Abbreviation: FCCP: carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ΔψH, proton motive force.

RESULTS

General Characteristics of Soybean Submitochondrial Particles. Oxonol-VI has been used to monitor the membrane potential of plant submitochondrial particles (18). In agreement with these findings, it was found that submitochondrial particles from soybean hypocotyls were able to build up and maintain a membrane potential with either NADH, succinate, or ATP (Fig. 1). The highest potential was observed with NADH. The potential formed with ATP, NADH, or succinate was collapsed by oligomycin, rotenone, and FCCP, respectively (Fig. 1). In agreement with these findings, it was found that submitochondrial particles catalyzed a higher rate of synthesis of ATP with NADH than with succinate (Table I). In agreement with many reports (for review, see Ref. 17), it was found that a significant portion of the aerobic oxidation of NADH was rotenone insensitive. For the rotenone-sensitive respiration, a P:O ratio of 1.0 was calculated. The values obtained are in agreement with those reported for pea cotyledon submitochondrial particles (5). In soybean mitochondria prepared as described here, higher P:O ratios were found (16), which indicates that preparation of the particles results in partial uncoupling of oxidative phosphorylation.

Phosphate Induced Increase of ATPase Activity of Submitochondrial Particles from Soybean. In many different preparations of soybean submitochondrial particles, an ATPase activity that ranged between 0.3 and 1.0 μmol min⁻¹ mg⁻¹ was observed. In heart submitochondrial particles, it has been reported (1) that high concentrations of phosphate relieve the inhibitory action of the natural ATPase inhibitor protein on ATP hydrolysis in a temperature-dependent process. Experiments were thus carried out to see if phosphate could induce the same activating effect in the ATPase of soybean particles. The data of Figure 2 show that, indeed, the preincubation of particles with phosphate induced a significant activation of the ATPase. The activation was higher at 38°C than at 20°C (Fig. 2A). At 50°C the activation was faster than at 38°C, but at this temperature the activity declined with time. The rate of activation also depended on pH (Fig. 2B); activation was faster at pH 8.0 than at pH 7.5 and 7.0. These latter findings are in agreement with those of Iwasaki and Asahi (11). However, we observed that after maximal activity was reached at pH 8.0 and 7.5, the activity started to diminish. Therefore, pH 7.0 and a temperature of 38°C were chosen to study the characteristics of the activation process. It is necessary to point out that regardless of the initial ATPase activity of the submitochondrial particles (average of 17 preparations, 0.7 ± 0.2 μmol min⁻¹ mg⁻¹), incubation with phosphate at pH 7.0 and 38°C always brought about activation of hydrolysis. The average of these latter values in 10 experiments was 1.8 ± 0.4 μmol min⁻¹ mg⁻¹.
Table I. ATP Synthesis and Oxygen Uptake by Soybean Submitochondrial Particles

The uptake of $^{32}$Pi and O$_2$ consumption by the particles was followed for 10 min at 30°C in the presence of NADH (15 mM) or succinate (20 mM) under the assay conditions described in “Materials and Methods.” Rotenone-sensitive ATP synthesis and O$_2$ uptake, using NADH as substrate, were calculated from the difference between the values obtained with NADH and those observed after the addition of 5 μM rotenone.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ATP Synthesis</th>
<th>O$_2$ uptake</th>
<th>P/O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol·min$^{-1}$·mg$^{-1}$</td>
<td>natom·min$^{-1}$·mg$^{-1}$</td>
<td>ratio</td>
</tr>
<tr>
<td>NADH</td>
<td>61</td>
<td>161</td>
<td>0.4</td>
</tr>
<tr>
<td>NADH (rotenone-sensitive)</td>
<td>48</td>
<td>48</td>
<td>1.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>10</td>
<td>104</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Fig. 2. Activation of soybean submitochondrial particle ATPase by phosphate: effect of temperature and pH. Soybean submitochondrial particles (1 mg/ml) were preincubated in 0.5 ml of 0.25 M sucrose and 50 mM potassium-phosphate A, pH adjusted to 7.0 and incubation at the temperatures indicated; (■), activity of particles incubated at 38°C without phosphate; B, incubation at 38°C at the indicated pH with 50 mM phosphate. At the time shown, aliquots were withdrawn and added to a 3 ml cuvette for the spectrophotometric assay of ATP hydrolysis (see “Materials and Methods”).

Fig. 3. Activation of soybean mitochondrial ATPase by Δψ; effect of phosphate. Particles (1 mg/ml) were preincubated at 38°C in 0.5 ml of 0.25 M sucrose, 20 mM Heps-KOH (pH 7.0), and 10 mM succinate (A), 10 mM NADH (B), or 6 mM Mg-ATP (C) without (open symbols) or with (closed symbols) 50 mM potassium-phosphate. At the time shown, aliquots were withdrawn and assayed spectrophotometrically for ATP hydrolysis.

**Activation of ATPase Activity by Establishment of Δψ and Phosphate.** The ATPase activity of particles from mammalian mitochondria can be increased by incubation with oxidizable substrates (4, 9, 28) or Mg-ATP (22); this is due to abolition of the inhibitory action of the inhibitor protein. Similar to mammalian submitochondrial particles, the ATPase activity of the soybean particles was activated by preincubation with NADH, succinate, or Mg-ATP (Fig. 3). NADH induced the highest increase in ATPase activity. As NADH induced the highest membrane potential (Fig. 1), there would seem to exist a correlation...
between the activation of the ATPase and the magnitude of the membrane potential. A possible correlation between $\Delta p$H and activation of hydrolysis was not evaluated. It has been reported (9) that $\Delta p$H is the main factor involved in the relief of the inhibitory action of the inhibitor protein. However, other authors (4) have suggested that both $\Delta p$H and $\Delta p$H are involved in the process, but the latter to a lower extent.

It was considered of interest to explore whether the activation of the ATPase, as induced by incubation of particles with succinate, NADH, and Mg-ATP, could be further increased by phosphate. As shown in Figure 3, A and B, the inclusion of phosphate in the preincubation medium that contained NADH or succinate did not significantly affect the activation of the enzyme. In contrast, when the particles were preincubated with Mg-ATP, the inclusion of phosphate in the preincubation medium brought about a significant increase in the ATPase activity of the particles (Fig. 3C). This was particularly marked in the first 30 min of preincubation.

The data in Table II illustrate some of the factors that may be involved in the activation of the ATPase by Mg-ATP and phosphate. In the presence of EDTA, ATP, and phosphate, the activation was much lower than when Mg$^{2+}$ was included in the preincubation medium. In addition, it was observed that in the presence of phosphate, Mg-ADP (plus P$^{32}$-P[adenosine-5'S]-pentaphosphate to prevent formation of ATP through adenylate kinase activity) provoked a lower activation of the ATPase. Thus, the findings suggest that hydrolysis, and not ATP per se or ADP as reaction product, is the factor involved in the activation of the ATPase. It is also noteworthy that FCCP prevented the activation induced by Mg-ATP and phosphate. This indicates that coupled hydrolysis is required for activation. The activated ATPase was about 90% sensitive to oligomycin (Table II). This indicates that the mitochondrial ATPase was the enzyme that was activated and not a contaminating ATPase. The sensitivity to oligomycin also indicated that at least 90% of the activity measured was due to F$_{1}$ bound to F$_{0}$

### Table II. Activation of ATP Hydrolysis of Soybean Submitochondrial Particles in Various Mixtures

The particles (0.25 mg) were preincubated in 0.25 ml of 0.25 m sucrose, 50 mM Tris-acetate (pH 8.0) and the additions indicated for 30 min at 38°C. After 60 min at 38°C ATP hydrolysis was assayed spectrophotometrically. Experiments 1 and 2 were made with different preparations of submitochondrial particles. The ATPase activity of particles at zero time (no preincubation) was 1.0 $\mu$mol $\cdot$ min$^{-1} \cdot$ mg$^{-1}$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>ATPase Activity $\mu$mol $\cdot$ min$^{-1} \cdot$ mg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 mM KH$_2$PO$_4$</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$ + 6 mM Mg-ATP</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$ + 1.5 mM EDTA + 6 mM ATP</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$ + 6 mM Mg-ADP + 200 $\mu$M AP5A$^+$</td>
<td>2.4</td>
</tr>
<tr>
<td>2</td>
<td>50 mM KH$_2$PO$_4$</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$ + 6 mM Mg-ATP</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$ + 6 mM ATP + 1 $\mu$M FCCP</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>50 mM KH$_2$PO$_4$ + 6 mM Mg ATP + 1 $\mu$g/ml oligomycin</td>
<td>0.3</td>
</tr>
</tbody>
</table>

$^+$P$^{32}$P[adenosine-5'S]-pentaphosphate.

### Table III. Stimulation of Soybean Mitochondrial ATPase. Effect of Anions

The particles (0.2 mg) were preincubated in 0.2 ml of 0.25 m sucrose, 50 mM Tris-acetate (pH 8.0) and the additions indicated for 30 min at 38°C. At the end of this time, an aliquot was withdrawn and assayed spectrophotometrically for ATP hydrolysis. The initial activity of the particles (no preincubation) was 0.6 $\mu$mol $\cdot$ min$^{-1} \cdot$ mg$^{-1}$.

<table>
<thead>
<tr>
<th>Additions</th>
<th>ATPase Activity $\mu$mol $\cdot$ min$^{-1} \cdot$ mg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mM Mg-ATP</td>
<td>1.3</td>
</tr>
<tr>
<td>6 mM Mg-ATP + 50 mM KH$_2$PO$_4$</td>
<td>2.5</td>
</tr>
<tr>
<td>6 mM Mg-ATP + 50 mM maleate</td>
<td>2.1</td>
</tr>
<tr>
<td>6 mM Mg-ATP + 50 mM malonate</td>
<td>2.3</td>
</tr>
<tr>
<td>6 mM Mg-ATP + 50 mM HCO$_3^-$</td>
<td>2.3</td>
</tr>
</tbody>
</table>

### DISCUSSION

In agreement with numerous reports on the ATPase activity of mammalian (1, 2, 4, 9, 23, 28) and plant (6, 8, 11, 12, 27) mitochondria, the present findings indicate that the ATPase activity of soybean submitochondrial particles is subject to similar regulatory mechanisms. The ATPase activity of plant submitochondrial particles has been shown to be increased by trypsin (6, 8, 11, 27), $\Delta p$H (Fig. 3), and phosphate (Fig. 2). Since these agents overcome the inhibitory action of the inhibitor protein in the ATPase activity of other types of mitochondria, it is probable that at least part of the regulation is mediated by an inhibitor protein with properties similar to those described in other energy-transducing membranes (1, 2, 4, 9, 19, 23, 24, 28). However, more direct evidence is needed to ascertain that indeed an inhibitor protein regulates the ATPase activity of plant mitochondria.

With respect to the regulation of ATPase activity in plant mitochondria, the effect of phosphate and other oxyanions is of particular interest. In the ATPase of mammalian (3, 21), yeast (26), and plant (6, 7, 15, 20, 29, 30) mitochondria, it has been shown that certain anions stimulate hydrolysis of ATP when they are included in the assay media. On the basis of the oxyanion specificity observed with mammalian F$_{1}$-ATPases, a specific
oxygen binding site on the enzyme has been proposed (3). However, in the soluble and particulate ATPase, the stimulatory effect of oxygen ions is quantitatively different, the stimulation being much higher with the soluble enzyme (3). The reason for this differential effect is not entirely known, but the findings suggest that different regulatory mechanisms operate in soluble and particulate ATPases. Here it was found that preincubation of soybean submitochondrial particles with oxygen ions increased their ATPase activity, and that maximal activation was achieved by preincubation with Mg-ATP plus phosphate (Table III). The activation depended on the existence of \( \Delta \mu H \) since FCCP prevented activation (Table II). In contrast, when \( \Delta \mu H \) was built up through electron transport, phosphate (Fig. 3) and the other oxygen ions failed to enhance activation.

Taken together, the data seem to indicate that the ATPase may exist in different conformations and that the different conformations have a distinct sensitivity to oxygen ions. Indeed, the observation that maximal activation was observed with oxygen ions in an enzyme undergoing repeated hydrolytic cycles suggests that a conformation of the enzyme that appears during the catalytic cycle is susceptible to the action of oxygen ions. In fact, this may account for the rather low rate of the activation process (the \( T_{0.5} \) for maximal activation was approximately 15 min). This suggests that the intermediate form of the enzyme with which oxygen ions interact has a short lifetime, and in consequence, the probability of interaction would be low. Collapse of \( \Delta \mu H \) by uncouplers may induce a further diminution of the lifetime of this intermediate and thus account for the inhibiting effect of FCCP on the activation process. However, this conclusion requires further experimental support since, at this stage, other alternatives, such as a differential affinity of the enzyme for phosphate (and perhaps other oxygen ions) under coupled and uncoupled conditions (13), may explain the activating action of phosphate and its prevention by FCCP.

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