Alkaline inorganic pyrophosphatase and Mg-ATPase are localized within the mitochondria of maize seedling mitochondria.

NaF inhibited the PPase activity, whereas oligomycin and dicyclohexylcarbodiimide inhibited the Mg-ATPase activity.

The mitoplast preparation synthesized PP\textsubscript{i} from P\textsubscript{i} under conditions excluding hydrolysis of endogenous ATP.

PP\textsubscript{i} synthesis was inhibited by ADP, antimycin A, NaCN and 2,4-dinitrophenol but not by oligomycin.

It is suggested that PP\textsubscript{i} synthesis in the maize seedling mitochondria proceeds at the expense of the energy of electron transport chain and is independent of the ATP synthesis.

Alkaline inorganic pyrophosphatase\textsuperscript{1} (EC 3.6.1.1) is widely distributed in animals (Wang & Strittmatter, 1975; Felix & Fleisch, 1975) and plants (Simmons & Butler, 1969; Bennett et al., 1973; Maslowski & Maslowska, 1976). It is postulated that this enzyme plays a role in the control of biosynthesis of proteins, carbohydrates, nucleic acids and fatty acids due to hydrolysis of PP\textsubscript{i} to inorganic phosphate (Kornberg, 1962).

The enzyme has attracted an additional interest when it was demonstrated that, in addition to normal hydrolysis of PP\textsubscript{i}, it can also catalyse transphosphorylation according to the formula (Brien et al., 1975)

\[
PP\textsubscript{i} + ROH \rightleftharpoons ROP + P\textsubscript{i}
\]

\textsuperscript{1} Abbreviations: PP\textsubscript{ase}, alkaline inorganic pyrophosphatase; PP\textsubscript{i}, pyrophosphate; DCCD, dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol.
This is of importance in terms of energy, because part of the pyrophosphate bond energy can be retained in the phosphate ester formed.

Recently it has been found that PPase participates also in biosynthesis of PP\textsubscript{i} from P\textsubscript{i} in the chromophores of photosynthesizing bacteria Rhodospirillum rubrum (Guillory & Fisher, 1972; Nashikawa et al., 1973) and in mitochondria of rat liver and bovine heart (Mansurova et al., 1977) at the expense of the energy of electron transport chain. It can be assumed that similar reactions may take place also in the mitochondrial membranes of higher plants.

In this paper we report on the submitochondrial localization of PPase and its role in the energy-linked PP\textsubscript{i} synthesis.

**MATERIAL AND METHODS**

*Isolation of mitochondria.* Mitochondria were isolated from 6-day-old etiolated maize seedlings by the modified method of Ikuma & Bonner (1967). Finely cut seedlings (800 g) were ground in an ice-cooled mortar lined with a fine nylon net, with 2 vol. of the homogenization medium containing 0.3 M-sucrose in 25 mM-Tris/HCl buffer, pH 8.3, 1 mM-EDTA, 0.1% bovine serum albumin and 0.05% cysteine. The suspension was squeezed through a double layer of cheese-cloth, the filtrate was adjusted to pH 7.2 and centrifuged at 1000 g for 15 min. The pellet was discarded and the supernatant was centrifuged at 10 000 g for 15 min. The sediment was washed with 0.4 M-sucrose in 10 mM-Tris/HCl buffer containing 0.1 mM-EDTA and 0.1% of bovine serum albumin (pH 7.2), and centrifuged for 10 min at 250 g. The pellet was discarded and the supernatant was recentrifuged for 15 min at 6000 g to obtain the preparation of crude mitochondria.

The washed mitochondria were purified by centrifugation in the following discontinuous sucrose density gradient in 10 mM-Tris/HCl buffer, pH 7.2 (Douce et al., 1972): 3.5 ml of 0.6 M, 3.5 ml of 0.9 M, 7.0 ml of 1.2 M and 14 ml of 1.4 M-sucrose. After equilibration of the gradient at 0°C for 16 h, 1-ml samples of washed mitochondria in 0.3 M-sucrose were layered on top of the gradient and centrifuged for 60 min at 40 000 g (horizontal rotor of VAC 601 ultracentrifuge). After centrifugation, 1-ml fractions were collected and cytochrome c oxidase activity, sucrose density and absorption at 520 nm were determined. Fractions with the highest cytochrome c oxidase activity were combined, diluted with 10 mM-Tris/HCl buffer, pH 7.2, to decrease sucrose concentration to 0.3 M, and centrifuged for 15 min at 10 000 g. The pellet consisted of purified mitochondria.

*Isolation of mitochondrial membranes.* Purified mitochondria were subjected to osmotic shock and were fractionated by centrifugation in discontinuous sucrose density gradient according to Parsons & Williams (1966) and Mannella & Bonner (1975). The mitochondrial suspension (1 ml) was rapidly pipetted into 50 ml of 10 mM-sucrose and was stirred for 20 min at 4°C. The suspension was layered on top of the 0.6 - 0.9 - 1.2 M-sucrose gradient and centrifuged for 60 min at 40 000 g. The pellet of mitoplast (inner membrane + matrix) and the fraction obtained at the 0.6 - 0.9 M-sucrose interface (outer membrane) were collected, diluted with 3 vol.
of 10 mM-Tris/HCl buffer, centrifuged for 90 min at 60,000 g, and their purity was estimated using electron microscopy and cytochrome c oxidase, NADH-cytochrome c reductase sensitive to antimycin A and Mg-ATPase as the markers for inner membrane, and NADH-cytochrome c reductase insensitive to antimycin A as a marker for outer membrane (Stenlid, 1970; Douce et al., 1972).

Determinations of the enzymes, protein and PPi. PPase and ATPase activities were determined by measuring liberation of P_i at 37°C in the medium containing 40 mM-Tris/HCl buffer, pH 8.7 (for PPase) or pH 7.5 (for ATPase), 5 mM-MgCl_2, 1 mM-Na_4P_2O_7 or 2 mM-ATP, respectively. The reaction was terminated by the addition of 10% trichloroacetic acid; the protein precipitate was removed by centrifugation and P_i was determined according to Fiske & Subbarow (1925).

Cytochrome c oxidase was assayed as described by Wharton & Tzagoloff (1967) and NADH-cytochrome c reductase (sensitive and insensitive to antimycin A) by the method of Douce et al. (1972).

Protein was assayed according to Lowry et al. (1951) with bovine serum albumin as a standard.

PP_i was determined according to Putnins & Yamada (1975).

Electron microscopy. Purified mitochondria and pellets of mitochondrial membranes were fixed in 2% glutaraldehyde in cacodylate buffer, pH 7.2, and 0.3 M-sucrose for 30 min and then in 1% OsO_4 in 0.2 M-cacodylate buffer, pH 7.2, and 0.3 M-sucrose for 1 h. A stepwise ethanol dehydration was followed by embedding in Epon. After polymerization, thin sections were cut with a LKB III 8800 ultramicrotome, stained with uranyl acetate and lead citrate, and examined using Tesla BS 613 electron microscope operating at 60 kV.

RESULTS AND DISCUSSION

Submitochondrial localization of PPase. On purification of mitochondria by centrifugation in sucrose density gradient (Fig. 1), the fraction obtained at the 1.2 - 1.4 M-sucrose interface exhibited the highest activity of cytochrome c oxidase (the mitochondrial inner membrane marker) and the maximum absorption at 520 nm. As it can be seen from Table I, all the activity of ATPase and most of the PPase activity (73%) were localized in the mitoplast preparation containing inner membranes.

Data from Table I and electron micrographs (Fig. 2) indicate the relatively high purity of the outer membrane preparation. It showed no activity of NADH-cytochrome c reductase sensitive to antimycin A, the marker of inner membrane. Mitoplasts were slightly contaminated with outer membranes: they contained 16% of the activity of NADH-cytochrome c reductase insensitive to antimycin A.

The incomplete recovery of PPase suggests that a part of this enzyme was removed from the membranes during the fractionation procedure, may be due to the relatively loose binding of PPase with the membranes. This suggestion is supported by the fact that 2 min sonication of membranes released PPase almost completely, whereas
ATPase only slightly (Table 2). PPase was also readily extracted from membranes of animal mitochondria (Schick & Butler, 1969; Batenburg & van der Bergh, 1972).

Properties of alkaline PPase. PPase from the maize seedling mitoplast exhibited the maximum activity at pH 8.7 - 9.0, similarly as it was previously shown for the purified PPase of the mitochondrial fraction of maize leaves (Maslowski et al., 1977).

![Graph showing purification of crude mitochondria from maize seedlings by centrifugation in discontinuous sucrose density gradient. Crude mitochondria were isolated by the modified method of Ikuma & Bonner (1967). ○, Cytochrome c oxidase; ○, A₅₂₀; □, sucrose concentration.]

The enzyme showed an absolute requirement for Mg²⁺. At 1 mM-PP₁ concentration, the maximum activity was observed at the Mg²⁺/PP₁ ratio of 5. Under these conditions, ATP at concentrations exceeding 1 mM inhibited PPase activity almost completely (Fig. 3). The inhibition by ATP was relieved effectively by increasing the concentration of Mg²⁺.

The comparative data on the effect of inhibitors on PPase and ATPase bound to mitochondrial membranes (Fig. 4) show that antimycin A (an inhibitor of electron transport) had practically no effect on the activity of either enzyme. Oligomycin and dicyclohexylcarbodiimide (inhibitors of ATP synthesis) inactivated ATPase (by 40 and 60%, respectively); sodium fluoride strongly inhibited PPase (by 88%) but had no effect on ATPase activity.

PP₁ synthesis by PPase. It has been shown that PPase participates in the energy-linked synthesis of PP₁ in bacterial and mammalian mitochondria (Baltscheffsky &
Fig. 2. Electron micrographs of submitochondrial fractions from maize seedling mitochondria.
A. Purified mitochondria; B, outer membrane; C, mitoplast.

P. Masłowski et al. (facing p. 178).
van Stedingk, 1966; Guillory & Fisher, 1972; Nashikawa et al., 1973). It could be assumed that the same reaction takes place in plant mitochondria. To check this assumption, the suspension of mitoplasts from maize seedlings (10 - 20 mg of protein)

Fig. 3. Effect of ATP and Mg²⁺ on pyrophosphatase activity in the maize seedling mitoplasts. The mitoplast preparation was obtained as described in Methods. PPase activity was assayed in the presence of increasing ATP concentrations and MgCl₂ at concentrations of: ●, 5 mM, and ○, 15 mM.

Fig. 4. Effect of inhibitors on PPase and ATPase activities in maize seedling mitoplasts. The mitoplast preparation was obtained as described in Methods. The inhibitors were applied at the following concentrations: Antimycin A (ATM), 10 µg/mg of protein; NaF, 2 mM; oligomycin (OgM), 10 µg/mg of protein; dicyclohexylcarbodiimide (DCCD), 1 mM.

was incubated for 15 min at 30°C in the reaction medium containing 10 mM-KH₂PO₄, 10 mM-Tris/HCl, 5 mM-MgCl₂, 0.1% of bovine serum albumin, and 35 mM-sodium succinate (pH 7.4). The reaction was stopped with 2 mM-HClO₄, and PP₁ was determined according to Putnins & Yamada (1975). Concentration of endogenous PP₁.
in the mitochondria deprived of outer membranes was about 5 μmoles/g of protein, and was increasing during 15 min incubation (Fig. 5A). However, PP₁ could be also formed on hydrolysis of ATP by ATP-pyrophosphatase, or by phosphorylation of various substrates by ATP. In this case, ATP concentration should be paralleled by an increase in PP₁ concentration.

Table 1

*Distribution of PPase and ATPase in submitochondrial fractions from maize seedling, mitochondria*

Cytochrome c oxidase, NADH-cytochrome c reductase sensitive and insensitive to antimycin A, the marker enzymes of mitochondrial membranes, were also assayed. The enzyme activities are expressed as μmoles/min.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein (mg)</th>
<th>Cytochrome c oxidase</th>
<th>NADH-cytochrome c oxidase</th>
<th>PPase</th>
<th>ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>activity</td>
<td>antymycin-sensitive activity</td>
<td>total specific</td>
<td>activity</td>
</tr>
<tr>
<td>Purified mitochondria</td>
<td>12.0 (100)</td>
<td>16.6 (100)</td>
<td>1.38 (100)</td>
<td>5.3 (100)</td>
<td>0.44 (100)</td>
</tr>
<tr>
<td>Mitoplast (inner membrane +)</td>
<td>8.8 (73)</td>
<td>13.8 (83.2)</td>
<td>1.57 (91.6)</td>
<td>4.8 (16.5)</td>
<td>0.54 (16.5)</td>
</tr>
<tr>
<td>Outer membranes</td>
<td>2.0 (17)</td>
<td>0.4 (2.4)</td>
<td>0.20 (0.0)</td>
<td>0.0 (0.0)</td>
<td>2.85 (49.5)</td>
</tr>
</tbody>
</table>

Table 2

*The effect of sonication on PPase and ATPase activity and PP₁ synthesis in maize seedling mitoplast*

After sonication the submitochondrial particles were centrifuged at 20000 g for 30 min, and the PPase and ATPase activities and PP₁ concentration were determined in the pellet.

<table>
<thead>
<tr>
<th>Time of sonication (min)</th>
<th>PPase μmoles/min per mg protein</th>
<th>ATPase μmoles/min per mg protein</th>
<th>PP₁ 10² μmoles/min per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>2.8</td>
<td>2.4</td>
<td>0.35</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>2.3</td>
<td>0.30</td>
</tr>
<tr>
<td>1.5</td>
<td>0.3</td>
<td>2.1</td>
<td>0.05</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0</td>
<td>1.9</td>
<td>0.01</td>
</tr>
</tbody>
</table>

To define the origin of PP₁, endogenous ATP was removed by incubation with hexokinase and glucose. The addition of P₁ after 10 min incubation resulted in a rapid synthesis of PP₁ (Fig. 5B). Inhibition of ATP synthesis by oligomycin had practically no effect on the amount of PP₁ formed. These data indicate that PP₁
formed in the submitochondrial particles of maize seedlings was derived directly from $P_i$, and its formation was not mediated by ATP.

To examine whether $P_i$ synthesis is associated with the functioning of the electron transport chain, antimycin A and NaCN (electron transport inhibitors) and 2,4-dinitrophenol (an uncoupler of oxidative phosphorylation) were added

![Graph](image)

Fig. 5. $P_i$ synthesis in the mitoplasts of maize seedling mitochondria (A), and the effect of oligomycin and removal of endogenous ATP (B). For details of preparation of mitoplasts and incubation conditions, see Methods.

A. The incubation mixture contained in 2 ml: 10-20 mg of protein, 10 mM-Tris/HCl buffer, pH 7.4, 10 mM-KH$_2$PO$_4$, 5 mM-MgCl$_2$, 0.1% bovine serum albumin and 35 mM-sodium succinate; ○, without ADP added; ○, supplemented with 4 mM-ADP.

B. ○, The incubation mixture containing $P_i$ and Mg$^{2+}$ (cf Fig. 5A) was supplemented with oligomycin (10 μg/ml). ○. The incubation mixture described in Methods (without $P_i$ and Mg$^{2+}$) contained 20 mM-glucose and 1 unit of hexokinase; after 10 min incubation, 10 mM-$P_i$ and 5 mM-MgCl$_2$ were added.

alternatively to the incubation mixture. All these compounds inhibited $P_i$ synthesis. Participation of PPase in the synthesis of $P_i$ was confirmed by the fact that NaF, a potent inhibitor of this enzyme, also inhibited $P_i$ synthesis by about 70% (Fig. 6). Moreover, submitochondrial particles subjected to 2 min sonication lost to the same extent (90%) both the PPase activity and the ability to synthesize $P_i$ (Table 2).

It seems that in the inner membranes of plant mitochondria, similarly as in animal mitochondria, the endergonic $P_i$ synthesis by PPase is coupled with the exergonic redox reaction of electron transport in the respiratory chain. This is testified to by the correlation between the PPase activity and synthesis of $P_i$. Thus, it could be assumed that alkaline inorganic pyrophosphatase might be a component
Fig. 6. Effect of inhibitors on PP₇ synthesis in mitoplasts from maize seedling mitochondria. The mitoplast preparation was obtained as described in Methods. The inhibitors were added at the following concentrations: NaCN, 1.5 mM; NaF, 2 mM; antimycin A (ATM), 10 μg/mg of protein; 2,4-dinitrophenol (DNP), 1.5 mM; ADP, 2 mM.

of the phosphorylation-coupling system. This is in agreement with the results of Racker (1962) who isolated from bovine heart mitochondria a coupling factor showing both the ATPase and PPase activities.

REFERENCES

SUBMITOCHONDRIALNA LOKALIZACJA I FUNKCJA ALKALICZNEJ NIEORGANICZNEJ PIROFOSFATAZY W SIEWKACH KUKURYDZY

Streszczenie

Alkaliczna nieorganiczna PPaza, podobnie jak Mg-ATPaza, występuje w mitoplastach mitocondriów siewek kukurydzy.

NaF hamował aktywność PPazy, a oligomycyna i dwucykloheksylokarbodium — aktywność Mg-ATPazy.

Preparat mitoplastu synetyzował PPi z Pi w warunkach wykluczających hydrolizę endogennego ATP. Reakcja syntetyzowała hamowana przez ADP, antymycynę A, NaCN i 2,4-dwinitrofenol, ale nie była hamowana przez oligomycynę.

Dane te sugerują, że syntaza PPi, w mitochondrialach kielków kukurydzy przebiega kosztem energii transportu elektronów w łańcuchu oddechowym niezależnie od syntety ATP.

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