Reconstitution of Oxidative Phosphorylation and of Oligomycin-Sensitive ATPase by Five- and Six-Subunit Forms of Pea Mitochondrial F1-ATPase

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ABSTRACT

Five- and six-subunit forms of F1-ATPase were purified from pea (Pisum sativum L. cv Homesteader) cotyledon submitochondrial particles. Apart from the usual complement of five subunits, the six-subunit enzyme contained an additional 26,500-dalton protein. Both forms of the F1-ATPase were used to reconstitute oxidative phosphorylation in F1-depleted (ASU) as well as in F1, and oligomycin-sensitivity conferring protein (OSCP) deplet ed (ASUA) bovine mitochondrial membranes. The six-subunit enzyme was considerably more efficient in reconstituting the ATP synthesis than the five-subunit enzyme. Both forms of the enzyme were also able to reconstitute the ATPase activity in ASU as well as in ASUA particles. There were substantial differences, however, in the oligomycin sensitivity of the ATPase bound to the ASUA particles: 20 and 60% inhibition by oligomycin was obtained in the case of the five-subunit and six-subunit enzyme, respectively. We conclude that the 26,500-dalton protein present in the six-subunit F1-ATPase is responsible for the increase in oligomycin sensitivity of the bound enzyme and functions, therefore, as the plant OSCP.

A couple of years ago, we described the purification of the pea cotyledon mitochondrial F1-ATPase (5). We have shown that instead of the usual complement of five subunits, the purified enzyme contained six subunits with mol wt of 57,000 (α), 55000 (β), 36500 (γ), 26500 (δ), 22500 (δ′) and 8000 (ε). We have further reported (6), that the six-subunit F1-ATPase exhibited a considerable cold stability. Bovine heart mitochondrial F1-ATPase (composed of five subunits) is rapidly inactivated by exposure to cold (19), yet when the enzyme is combined with the OSCP; the resulting complex is much more cold stable (10). We were able to dissociate one of the subunits (26,500 D protein) from the remaining complex using sedimentation in a linear sucrose gradient in the presence of (NH4)2SO4 and deoxycholate (6). The same conditions were used to dissociate OSCP from beef heart mitochondrial F1-ATPase (26). In our latest paper (8) we have described the reconstitution of oxidative phosphorylation using a purified six-subunit pea mitochondrial F1-ATPase and F1-depleted bovine heart mitochondrial membranes. The pea enzyme was found to be more efficient in reconstitution than the F1-containing extract of bovine heart mitochondria. Analogous observations were reported by Vallejos et al. (25), who isolated a six-subunit F1-ATPase from bovine heart mitochondria (F1-X complex) and found it had higher coupling activity than a five-subunit F1. The X component was later identified as OSCP (26).

Thus, our previous observations present indirect evidence that the 26,500 D protein found in the preparation of the pea mitochondrial F1-ATPase is a plant counterpart of the mammalian OSCP protein. In this paper we present the most direct evidence obtained so far, that this is, indeed, the case.

MATERIALS AND METHODS

Preparation of Six-Subunit and Five-Subunit Pea Cotyledon Mitochondrial F1-ATPases

Purification of the six-subunit F1-ATPase was carried out exactly as described before (5). The purified enzyme (specific activity approximately 20 units/mg protein) was stored at −80°C in a buffer containing 300 mM sucrose, 2 mM EDTA, 2 mM ATP, 20 mM Tris-H2SO4 (pH 7.4), and 10% methanol. One unit of ATPase activity is defined as the amount of enzyme which released 1 μmol Pi/min under the assay conditions.

For preparation of the five-subunit F1-ATPase, pea (Pisum sativum L. cv Homesteader) cotyledon mitochondria were prepared and sonicated as described previously (5). However, instead of a low-ionic strength wash (5), the submitochondrial particles were subjected to further extensive sonication for 7 min at 45°C, followed by a 10 min period at 52 to 55°C (9, 14). The preparation was allowed to cool to room temperature and was centrifuged at 100,000g for 60 min at 20°C. The five-subunit F1-ATPase was purified from the supernatant by chromatography on DEAE-cellulose followed by sucrose density gradient centrifugation as in the case of the six-subunit enzyme (5). The purified enzyme (specific activity approximately 30 units/mg protein) was stored at −80°C in the same buffer as used above for the six-subunit enzyme.

Preparation of Bovine Heart Mitochondrial ASU- and ASUA-Particles

For the preparation of the ASU-particles, the published procedures (2, 4, 21) were followed exactly as described pre-
RESULTS AND DISCUSSION

Preparation of Five- and Six-Subunit Forms of the Pea Cotyledon Mitochondrial F<sub>1</sub>-ATPase

The enzyme released from the pea mitochondrial membranes by extensive sonication at elevated temperatures as described in "Materials and Methods" contained five subunits. The subunits had an apparent mol wt of 57,000, 55,000, 36,500, 22,500, and 8,000 on SDS-PAGE. The F<sub>1</sub>-ATPase obtained by a low-ionic strength wash of membranes (5) contains an additional subunit of 26,500 D (5, 6), which has been removed from the five-subunit preparation during the high temperature sonication step (22, 25).

Reconstitution of Oxidative Phosphorylation

Plant mitochondrial membranes seem to be more fragile and lose their capacity for oxidative phosphorylation much more readily than mitochondrial membranes prepared from other sources (7). We have, therefore, used bovine heart mitochondrial membranes to obtain F<sub>1</sub>-depleted (ASU-) as well as F<sub>1</sub> and OSCP-depleted (ASUA-) particles.

Table I shows the reconstitution of oxidative phosphorylation in bovine heart ASU- and ASUA-particles using purified five-subunit and six-subunit pea cotyledon mitochondrial F<sub>1</sub>-ATPases. The five-subunit enzyme was unable to stimulate ATP synthesis in the ASU-membranes to any significant extent unless oligomycin was also added to seal the remaining open proton channels (18). In the OSCP-depleted ASUA-particles, the five-subunit enzyme was incapable of stimulating ATP synthesis to any significant amount even in the presence of oligomycin since OSCP is required for ATP synthesis to take place (3).

It could be further seen from Table I that addition of the six-subunit F<sub>1</sub>-ATPase to the ASU- or ASUA-particles results in a substantial increase in oxidative phosphorylation. ATP synthesis is only slightly increased when oligomycin is also included. Apart from the regular five subunits, the six-subunit pea mitochondrial F<sub>1</sub>-ATPase contains an extra 26,500 D protein, which, we believe, is a plant equivalent of mamma-
The particles (600 μg of ASU or ASUA) were reconstituted with the purified pea mitochondrial F1-ATPase, the amounts of which (75–150 μg) were equivalent to 2.8 units of ATPase activity in case of the five-subunit as well as the six-subunit enzyme. Incubations in the presence of oligomycin were carried out as described in "Materials and Methods."

### Table II. Reconstitution of the Oligomycin-Sensitive ATPase in Bovine Heart ASU- and ASUA-Particles with Purified Five- and Six-Subunit Pea Cotyledon Mitochondrial F1-ATPase

<table>
<thead>
<tr>
<th>Additions to Particles</th>
<th>ASU ATPase</th>
<th>Oligomycin inhibition</th>
<th>ASUA ATPase</th>
<th>Oligomycin inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg of particle protein</td>
<td>%</td>
<td>units/mg of particle protein</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5-subunit F1</td>
<td>2.73 (2.5–2.8)*</td>
<td>60</td>
<td>2.42 (2.2–2.6)</td>
<td>20</td>
</tr>
<tr>
<td>5-subunit F1 + oligomycin</td>
<td>1.10 (1.1–1.1)</td>
<td>1.93 (1.7–2.2)</td>
<td>1.37 (2.3–3.9)</td>
<td>57</td>
</tr>
<tr>
<td>6-subunit F1</td>
<td>3.53 (3.2–3.7)</td>
<td>60</td>
<td>3.77 (3.2–3.9)</td>
<td>57</td>
</tr>
<tr>
<td>6-subunit F1 + oligomycin</td>
<td>1.57 (1.3–1.6)</td>
<td>56</td>
<td>1.62 (1.4–1.6)</td>
<td>57</td>
</tr>
</tbody>
</table>

* Numbers in parentheses show the range of values obtained in three reconstitution experiments.

The six-subunit enzyme brings along its own OSCP (6, 8). Thus, the six-subunit enzyme brings along its own OSCP and does not need the membrane supplied OSCP for tight and proper association with the membrane that allows the F1 portion to utilize the proton gradient and synthesize ATP (1). The plant OSCP is therefore capable of securing the link between plant F1 and mammalian F0 in a way that results in a functional ATP synthase complex.

**Reconstitution of Oligomycin-Sensitive ATPase in Bovine Heart ASU- and ASUA-Particles**

The OSCP activity is defined as an ability of this protein to confer oligomycin sensitivity on the F1-F0 complex (13, 24). In the absence of OSCP, F1 can still bind to the F0 portion; however, the resulting complex is not oligomycin sensitive (1, 13). Table II shows that both five-subunit as well as six-subunit F1-ATPase binds to the ASU-particles. As expected, the six-subunit enzyme binds more efficiently than the five-subunit enzyme. The oligomycin sensitivity of the resulting complex is, however, the same (about 60%) in both enzyme species as the ASU-particles contain relatively large amounts of endogenous OSCP (15) that obscure the effect of any exogenous OSCP contributed by the six-subunit pea F1. Similar sensitivity to oligomycin was observed byNorling et al. (16) on cross-reconstitution of potato F1-ATPase with F1-depleted bovine submitochondrial particles. Both forms of the F1-ATPase also bind to the ASUA-particles with the six-subunit enzyme again being more efficient (Table II). However, contrary to the ASU-particles, there is a great difference in the oligomycin sensitivity conferred onto the bound F1-ATPase. In the case of the five-subunit F1-ATPase, 20% of the bound enzyme is sensitive to oligomycin, which can be explained by the absence of any residual endogenous OSCP still bound to the ASU-particles (1). With the six-subunit enzyme, the oligomycin sensitivity increases to almost 60%. Thus, the presence of the 26,500 D protein increased the oligomycin sensitivity of the bound F1-ATPase from 20 to 60%. This is the most direct evidence so far, that the 26,500 D protein in our preparation of pea cotyledon mitochondrial F1-ATPase is the plant OSCP. Similarly as in case of the oxidative phosphorylation (Table I), the plant OSCP works well in conjunction with bovine heart mitochondrial membranes, suggesting a close structural relationship between OSCPs from vastly diverse species. The mol wt of pea mitochondrial OSCP (26,500) obtained from SDS-polyacrylamide gel electrophoresis is the same as that of rat liver OSCP (11) and close to the molecular mass of 20,967 reported for bovine heart OSCP (17). The more precise comparison awaits isolation in a pure form of the pea cotyledon OSCP, which, in view of limiting amounts of starting material, is not an easy task.

**ACKNOWLEDGMENT**

We would like to thank Mr. I. Duncan for technical assistance.

**LITERATURE CITED**