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Presence in Dry Pea Cotyledons of Soluble Succinate Dehydrogenase That Is Assembled into the Mitochondrial Inner Membrane during Seed Imbibition

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ABSTRACT

Succinate dehydrogenase (succinate: phenazine methosulfate oxidoreductase, EC 1.3.99.1) activity in crude mitochondrial fraction from pea (var. Alaska) cotyledons increased during seed imbibition to reach a maximum after about 12 hours. The increase was not inhibited by either cycloheximide or D(-)-threo-chloramphenicol. The postmicrosomal fraction from dry cotyledons, but not that from fully imbibed ones, contained a soluble form of succinate dehydrogenase. The soluble enzyme was partially purified by ammonium sulfate fractionation and diethylaminoethy-cellulose and Sepharose 6B column chromatography. The enzyme showed no succinate-coenzyme Q oxidoreductase activity and had a molecular mass of about 100,000 daltons. The soluble enzyme seemed to differ only slightly from succinate dehydrogenase solubilized from the mitochondrial inner membrane from fully imbibed cotyledons by a detergent. It is proposed that the soluble succinate dehydrogenase is associated with an intact mitochondrial inner membrane in dry cotyledons to form an active one during seed imbibition.

A rapid development of mitochondria, i.e., the formation of active and cristae-rich mitochondria from inert ones with a few cristae, takes place in endosperm or cotyledons in the early stage of seed germination (2, 3, 16, 19, 22, 26). The development is accompanied by increases in mitochondrial protein and phospholipid content and in respiratory, SDHs, and Cyt c oxidase activities (9, 13, 16, 22).

Previous work in our laboratory showed that in pea cotyledons, neither CHI nor CAP inhibited mitochondrial development during seed imbibition, suggesting no involvement of de novo synthesis of mitochondrial protein in the development (17). It has been proposed that in dry seeds, there may be soluble proteins which await seed hydration to be assembled into a phospholipid-rich and inert mitochondrial inner membrane to form an active one (17, 20). There is, however, no direct evidence for the proposal.

Here, we show the presence of an SDH in a soluble form in dry pea cotyledons which seems to be associated with the mitochondrial inner membrane during seed imbibition. We also report a comparison between SDHs existing in a soluble form in dry cotyledons and solubilized from the mitochondrial inner membrane isolated from fully hydrated cotyledons.

MATERIALS AND METHODS

Plant Material. Pea seeds (Pisum sativum var. Alaska) were purchased from Watanabe Seed Co., Kogota, Miyagi, Japan. The surface-sterilized seeds were germinated in the dark at 25 C in Petri dishes containing deionized H2O or an antibiotic solution (0.5 mM CHI or 0.5 mM CAP). The cotyledons were taken at the appropriate age and washed with deionized H2O.

Cell Fractionation. Crude mitochondrial and postmitochondrial fractions were prepared from the cotyledons obtained from 5 g of dry seeds by differential centrifugation as described previously (18) except that BSA and cysteine were omitted from the grinding medium. The omission of BSA, which would be profitable for purification of SDH from postmitochondrial fraction, resulted in no significant change in SDH activity of the crude mitochondrial fraction. The postmitochondrial fraction was further centrifuged at 100,000g for 1 h, and the supernatant was designated postmicrosomal fraction.

Assays. The assay method of SDH activity was based on that of King (8) using PMS and DCIP as the electron acceptors. The reaction mixture contained 33 mM K-phosphate (pH 7.8), 1.0 mM KCN, 0.1% (w/v) BSA, 20 mM potassium succinate, 3 mM PMS, 0.07 to 0.12 mM DCIP, and an aliquot of enzyme preparation in a final volume of 0.3 ml. Unless otherwise indicated, the reaction was initiated by the addition of potassium succinate and followed by recording the decrease in A at 600 nm at 25 C. Since some enzyme preparations contained substances rapidly reducing DCIP with PMS as an electron transport mediator, the solution omitting potassium succinate from the reaction mixture was preincubated at 25 C for 5 min to oxidize almost all of the substances. Succinate-coenzyme Q oxidoreductase activity was assayed by the method of Hatefi and Stigall (6) with slight modifications. The reaction mixture contained 33 mM K-phosphate (pH 7.8), 20 mM potassium succinate, 2 mM EDTA, 1 mM KCN, 88 μM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone, a coenzyme Qs analog, 0.05 mM DCIP, and an aliquot of enzyme preparation in a final volume of 0.3 ml. The reaction was started by the addition of the coenzyme Qs analog in 10 μl of ethanol and followed as described for SDH activity assay. One unit of SDH or succinate-coenzyme Q oxidoreductase activity is defined as the amount of enzyme that oxidizes 1 μmol of succinate/min under the assay conditions. Protein was determined by the method of Lowry et al. (10) after precipitation with 10% (w/v) trichloroacetic acid.

Partial Purification of Soluble SDH. Dry seeds (50 g) were powdered with a motor-driven pulverizer, and the postmicrosomal fraction was prepared as described under "Cell Fractionation." Solid ammonium sulfate was added to the fraction to 30% satu-
ration, and the precipitate was removed by centrifugation. The supernatant was brought to 60% saturation with the slow addition of solid ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in 10 mM K-phosphate (pH 7.8).

The solution was passed through a Sephadex G-25 (coarse) column (30 x 425 mm) previously washed with 10 mM K-phosphate (pH 7.8), and the void volume fraction was applied to a DEAE-cellulose column (Whatman DE52, 44 x 110 mm) pre-equilibrated with the buffer described above. After washing the column with 150 ml of the same buffer containing 0.1 M KCl, the enzyme was eluted from the column with 300 ml of the buffer containing KCl in a gradient from 0.1 to 0.2 M. Active fractions were brought to 60% saturation with solid ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in the buffer described above.

The solution was dialyzed against 10 mM K-phosphate (pH 7.8) for 1 h and centrifuged at 10,000g for 20 min to remove insoluble materials. The supernatant fraction was applied to a Sepharose 6B column (16 x 750 mm) previously washed with the same buffer (pH 7.8) containing 0.1 M KCl, and the enzyme was eluted from the column with the buffer containing 0.1 M KCl.

All of the above procedures were carried out at 4 °C.

Solubilization of SDH from Mitochondrial Inner Membrane. Submitochondrial particles prepared from the cotyledons of pea seeds imbibed for 1 day as described before (15) were suspended in 10 mM K-phosphate (pH 7.8) containing 5% (w/v) sucrose. To the suspension (about 10 mg protein/ml) was added 10% (w/v) Emulgen 810 (a nonionic detergent from Kao Co. Ltd., Tokyo, Japan) to make a final concentration of 0.05% (w/v). The mixture was kept in an ice bath for 30 min, then centrifuged at 100,000g for 1 h. The supernatant was designated solubilized SDH fraction.

Polyacrylamide Gel Electrophoresis. Disc electrophoresis in a 7% (w/v) polyacrylamide gel (for a comparison of the mobility of soluble SDH from dry cotyledons and solubilized SDH from imbibed cotyledons) or in 5-9% (w/v) polyacrylamide gels (for determination of the mol wt of SDH) was performed on the basis of the method of Hedrick and Smith (7). After electrophoresis, the gels were stained with a solution containing 80 mM Na-phosphate (pH 7.8), 12 mM sodium succinate, 0.01% (w/v) PMS, and 0.01% (v/v) nitroblue tetrazolium to detect SDH. Catalase (240,000), hexokinase (99,000), BSA (67,000), and a-amylase (45,000) were used as mol wt markers.

Chemicals. 2,3-Dimethoxy-5-methyl-6-decyl-1,4-benzoquinone was generously provided by Dr. Karl Folkers (Institute for Biochemical Research, The University of Texas at Austin). PMS, DCIP, CHI, CAP, and nitroblue tetrazolium were products of Sigma Chemical Co., and BSA (fraction V) was of Reheis Chemical Co. Catalase and hexokinase were obtained from Boehringer Mannheim GmbH, and a-amylase was from Seikagaku Kogyo Co.

RESULTS

Change in SDH Activity in Crude Mitochondrial Fraction from Cotyledons during Seed Imbibition. The activity of SDH in crude mitochondrial fractions from cotyledons increased rapidly in the very early stage of germination to reach a maximum (two to three times the activity of the fraction from dry cotyledons) after about 12 h from the onset of seed imbibition (Fig. 1). Similar results have been reported by Solomos et al. (22) with P. sativum var. Homesteader and Morohashi and Shimokoriyama (13) with Phaseolus mungo. The profile of increase in the activity was very similar to that in Cyt c oxidase activity reported previously (16).

The increase in the activity of SDH was not inhibited by either CHI or CAP (Fig. 2). The increase was stimulated when seeds imbibed a CHI solution at a concentration (0.5 mM) high enough to inhibit the incorporation of [14C]leucine into mitochondrial proteins (cf. 17).

Existence of SDH in the Postmicrosomal Fraction from Dry Cotyledons. The inability of CHI to inhibit the activity increase suggests the presence of an SDH precursor in dry cotyledons. To test this, we conducted a series of experiments to determine whether there might be SDH in a soluble form in dry cotyledons which is assembled to the mitochondrial inner membrane during seed imbibition. If such a soluble SDH is present in dry cotyledons, it would be recovered in the postmicrosomal or the postmicrosomal fraction whether it exists in the cytosol or in the mitochondrial matrix, because mitochondria in dry seeds are very fragile and are fragmented during isolation (20).

Both postmitochondrial and postmicrosomal fractions from dry cotyledons contained large amounts of substances with high mol wt which rapidly reduced DCIP in the presence of PMS. Their reducing capacity was increased by heating the fractions at 100°C. Thus, it was impossible to assay SDH activity in the fractions by the usual method in which the reaction was initiated by the addition of PMS.
As shown in Figure 3, we could detect SDH activity in both postmitochondrial and postmicrosomal fractions from dry cotyledons by an improved assay method described under "Materials and Methods." About half of the SDH activity in a crude extract of dry cotyledons was recovered in the postmitochondrial fraction. Further, about half of the SDH activity in the postmitochondrial fraction remained unprecipitated even after centrifugation at 100,000g for 1 h. On the other hand, SDH activity in the postmitochondrial fraction from the cotyledons of seeds imbibed for 18 h was less than half of that from dry cotyledons, and no SDH activity was detected in the postmicrosomal fraction from fully imbibed cotyledons. Total SDH activity in the crude extract from fully imbibed cotyledons was significantly higher than that from dry cotyledons.

Partial Purification of Soluble SDH in Dry Cotyledons. Succinate dehydrogenase in the postmicrosomal fraction from dry seeds was partially purified by ammonium sulfate fractionation and DEAE-cellulose and Sepharose 6B column chromatography. Figures 4 and 5 show the elution profiles of the SDH from the DEAE-cellulose and Sepharose 6B columns, respectively, and the results of a typical purification are summarized in Table I. The enzyme was purified over 116-fold (231-fold in the case of a peak fraction after Sepharose 6B column chromatography) with a recovery of about 30%. Substances reducing DCP in the presence of PMS were eluted later than SDH in both column separations, but the final preparation still contained small amounts of the substances (data not shown).

Properties of Soluble SDH in Dry Cotyledons. A comparison was made between soluble SDH purified partially from postmicrosomal fraction from dry cotyledons and SDH solubilized from submitochondrial particles from fully imbibed cotyledons (Table II). There was no difference in the optimal pH between these SDH's, but the $K_m$ value of soluble SDH for succinate was about 20 times as large as that of solubilized SDH. The low $K_m$ value of solubilized SDH was not due to the presence of detergent in the reaction mixture, because the same value was obtained with a detergent-free solubilized SDH preparation (the detergent was removed from solubilized SDH fraction by DEAE-cellulose column chromatography). Solubilized SDH was activated by 30–80% by being preincubated with 20 mM succinate for 10 min at 25 C, while soluble SDH was not influenced by such preincubation. About 10-fold activation of SDH by the preincubation was observed with submitochondrial particles from fully imbibed cotyledons, but less than 10% activation occurred in the case of a solubilized SDH preparation partially purified by DEAE-cellulose column chromatography (data not shown). It has been proposed that activation of SDH by preincubation of the enzyme with succinate is due to a removal of oxaloacetate associated with the enzyme protein (1). The observed difference in the effect of

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TABLE I. Purification of SDH from Postmicrosomal Fraction from Dry Seeds

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units x 10^2</td>
<td>mg</td>
<td>units x 10^3/mg protein</td>
<td>% fold</td>
<td></td>
</tr>
<tr>
<td>Postmicrosomal fraction*</td>
<td>703</td>
<td>2990</td>
<td>0.23</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>(NH_4)_2SO_4 30-60% precipitate</td>
<td>1009</td>
<td>896</td>
<td>1.13</td>
<td>143</td>
<td>4.8</td>
</tr>
<tr>
<td>Eluted fraction from DEAE-cellulose column</td>
<td>563</td>
<td>128</td>
<td>4.40</td>
<td>80</td>
<td>18.7</td>
</tr>
<tr>
<td>(NH_4)_2SO_4 60% precipitate</td>
<td>284</td>
<td>11.8</td>
<td>24.1</td>
<td>40</td>
<td>102</td>
</tr>
<tr>
<td>Eluted fraction from Sepharose 6B column</td>
<td>197</td>
<td>7.2</td>
<td>27.4</td>
<td>28</td>
<td>116</td>
</tr>
<tr>
<td>Peak fraction from Sepharose 6B column</td>
<td>26.6</td>
<td>0.49</td>
<td>54.3</td>
<td>3.8</td>
<td>231</td>
</tr>
</tbody>
</table>

* The fraction was dialyzed against 10 mM K-phosphate (pH 7.8) before assays.
Table II. Comparison between Soluble and Solubilized SDHs

<table>
<thead>
<tr>
<th></th>
<th>Soluble SDHb</th>
<th>Solubilized SDHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m) (mm)</td>
<td>4.65</td>
<td>0.24</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>7.8</td>
<td>7.8</td>
</tr>
<tr>
<td>Activation by preincubation with succinatec (%)</td>
<td>0</td>
<td>28-78</td>
</tr>
<tr>
<td>Succinate-coenzyme Q oxidoreductase activity (units)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* A purified preparation (the peak fraction from Sepharose 6B column) from dry seeds.

* Solubilized SDH fraction.

The enzyme preparations were preincubated with 20 mM succinate for 5 min at 25°C.

**DISCUSSION**

Biochemical studies with microorganisms and animal cells have shown that SDH is synthesized on cytoplasmic ribosomes under the control of nuclear genes (11, 12, 25). Consequently, the fact that CHI did not inhibit an increase in SDH activity in crude mitochondrial fractions from pea cotyledons during seed imbibition (Fig. 2) suggests no involvement of *de novo* synthesis of SDH in the increase. This leads us to postulate the following possibilities: (a) An inactive form of SDH present in the mitochondrial inner membrane in dry cotyledons is activated during seed imbibition. (b) Alternatively, SDH in a soluble form present in dry cotyledons is assembled into the mitochondrial inner membrane during seed imbibition. The soluble SDH may be in the cytosol or may be in the mitochondrial matrix, because mitochondria in dry seeds are so fragile that they are broken down during isolation and proteins in their matrix are not recovered in the crude mitochondrial fraction (20). The latter possibility is more probable, since the mitochondrial inner membrane becomes rich in protein as seeds imbibe (16, 17, 20).

The present work shows that an SDH was present in the postmicrosomal fraction from dry pea cotyledons and could be partially purified without treatment with either detergents or chaotropic reagents. In general, SDH is too tightly associated with the mitochondrial inner membrane to be detached from the membrane without the treatment described above (5). SDH in the crude mitochondrial fraction from fully hydrated pea cotyledons is also in such a situation (14). The present work clearly indicates the presence of a soluble or easily solubilized SDH in dry pea cotyledons. The soluble SDH shows no succinate-coenzyme Q oxidoreductase activity and has a molecular mass of about 100,000 daltons, indicating that the SDH is in a form free from other proteins and not in a form of complex II. The molecular mass obtained is similar to that of beef heart SDH (97,000 daltons) (4). The soluble SDH seems to differ only slightly from SDH solubilized by a detergent from the mitochondrial inner membrane of fully imbibed cotyledons. Further detailed studies are required to draw a conclusion as to whether the soluble SDH differs from membrane-bound SDH in the molecular form. Especially, more accurate mol wt of soluble and solubilized SDHs must be estimated in combination with other techniques and with more purified preparations in order to make a conclusive answer to the above question.

No SDH activity was detected in the postmicrosomal fraction from fully imbibed cotyledons (Fig. 3). This suggests that soluble SDH present in dry cotyledons is associated with the mitochondrial inner membrane during seed imbibition. Since total SDH activity in crude extracts increased during seed imbibition (Fig. 3), we propose that the assembly of soluble SDH into the mitochondrial inner membrane is accompanied by activation of the SDH. In yeast cells, free F1 accumulates in the postbrowsable fraction when repressed cells are incubated in a low glucose medium in the presence of CAP, and the accumulated F1 is partially assembled into functional oligomycin-sensitive ATPase when the cells are further grown in the medium without CAP (23, 24). It has been proposed that at least in yeast cells, a steady-state pool of free F1 exists outside the mitochondria, and that the F1 is integrated into the mitochondrial inner membrane when some protein is synthesized within the mitochondria (21, 24). Considering this, there is a possibility that soluble SDH in dry pea cotyledons is an intermediate in the course of SDH synthesis, which awaits some structural alterations in either the SDH itself or the mitochondrial inner membrane (or both) before it is assembled into the membrane.

About half of the SDH activity in the postmitochondrial fraction from dry cotyledons was sedimented by centrifugation at 100,000g for 1 h, and the particulate SDH activity decreased only slightly after seed imbibition (Fig. 3). We need to characterize the sedimentable materials in order to interpret this finding more fully. The present work presents preliminary evidence for the proposal that mitochondrial development in pea cotyledons during seed imbibition is achieved by assembly of soluble proteins preexisting in dry cotyledons into the mitochondrial inner membrane. Further detailed studies on the assembly of soluble SDH into the membrane will give us important information about the biogenesis of mitochondria.
SOLUBLE SUCCINATE DEHYDROGENASE

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