Solubilization of a Proline Dehydrogenase from Maize (Zea mays L.) Mitochondria

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ABSTRACT

L-Proline is oxidized to pyrroline-5-carboxylic acid in intact plant mitochondria by a proline dehydrogenase (EC 1.4.3) that is bound to the matrix side of the inner mitochondrial membrane (TE Ethion, CR Stewart [1981] Plant Physiol 67: 780–784). This investigation reports the first solubilization of the L-proline dehydrogenase (PDH) from plant mitochondria. The supernatant from NP-40-treated etiolated shoot mitochondria of maize, Zea mays L., reduced iodonitrotetrazolium violet in a proline dependent manner. The pH optimum for this activity was 8. The apparent Km for proline was 8.6 millimolar. When supplied with proline, this solubilized PDH activity also synthesized pyrroline-5-carboxylic acid. The PDH activity was inhibited in vitro by 300 millimolar potassium chloride but not by 300 millimolar potassium acetate. The PDH activity had a molecular mass that was greater than 150 kilodaltons. Mitochondria were prepared from etiolated shoots grown in 100% water-saturated vermiculite (control) and 16% water-saturated vermiculite (stress). The specific activity of solubilized PDH from the stress treatment was 11% of the same activity from the control treatment. Oxygen uptake in the presence of proline and ADP (state 3 proline oxidation) by mitochondria from the stress treatment was 25% of the same rate by mitochondria from the control treatment. Mitochondria were also prepared 16 hours after rewatering the seedlings growing in the stress treatment. Both the solubilized PDH specific activity and state 3 proline oxidation returned to the control levels. The specific activities of the NAD+-dependent pyrroline-5-carboxylic acid dehydrogenase and cytochrome c oxidase in the solubilized preparations were unaffected by these stress and recovery treatments. Oxygen uptake rates by intact mitochondria in the presence of ADP and NADH, succinate or malate-pyruvate were also unaffected by these treatments.

Proline accumulation in plants exposed to water deficit is a well-known stress response (9). The response results from a stimulation of proline biosynthesis as well as an inhibition of its utilization (11). Proline accumulation is usually accompanied by inhibition of growth (8). The oxidation of proline into the respiratory pathways is inhibited during proline accumulation (11).

Proline oxidative metabolism in plants has been described using intact mitochondria or mitochondrial particles and assayed by oxygen uptake measurements or spectrophotometrically (5, 6, 9). The inhibitory effect of water stress on proline oxidation has been investigated at the mitochondria level by Sells and Koepp (9). Because proline dehydrogenase activity has not been measured in solubilized preparations, there has been no characterization of the enzyme nor have there been any characterizations of the effects on the enzyme of stress or other treatments that inhibit proline oxidation. The role of proline transport in regulating proline oxidation is unclear.

PDH (EC 1.4.3) is a mitochondrial flavoprotein enzyme located on the matrix side of the inner membrane (4). Various kinds of evidence indicate that PDH donates electrons directly to the respiratory electron transport system in a manner similar to other inner membrane-bound enzymes, e.g., NADH- and succinate-dehydrogenases (5, 6).

This paper is the first report of solubilized PDH activity from plant mitochondria. The effect on the activity of growth under stress conditions was also examined and compared with the effects on other mitochondrial activities.

MATERIALS AND METHODS

Growth Conditions

Seeds of maize, Zea mays cv B73, were germinated between paper towels moistened with deionized water for 30 h at 30°C in a darkened incubator. After radicle emergence, the sprouted seeds were transferred to vermiculite that was saturated with water (560 mL water/100 g vermiculite) for optimal growth or 16% water-saturated vermiculite (100 g water/100 g vermiculite) for growth under water deficit. For the rewatered treatment, sufficient water was added to the 16% water-saturated vermiculite to saturate it. Vermiculite and seeds were incubated in closed vegetable crispers at 30°C in a darkened incubator for 60 h for the water saturated treatments and for 88 h in the dry vermiculite. The longer growth period was required for seedlings in the dry vermiculite to reach the same developmental stage as those in the well-watered treatment. For the rewatered treatment, the seedlings were watered after 72 h growth in the dry vermiculite.

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Abbreviations: PDH: proline dehydrogenase; INT: 2-p-iodo-3-p-nitro-5-phenyl tetrazolium chloride; MMT, Mops, Tricine; PSC, Δ′-pyrroline-5-carboxylic acid; RETS, respiratory electron transport system.
Table I. Solubilization of PDH

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min⁻¹ mL⁻¹</td>
<td>mg</td>
<td>pmol mg⁻¹ protein⁻¹</td>
<td>%</td>
</tr>
<tr>
<td>Crude</td>
<td>1.3</td>
<td>4.5</td>
<td>0.3</td>
<td>100</td>
</tr>
<tr>
<td>10,000g pellet</td>
<td>6.5</td>
<td>0.4</td>
<td>15.5</td>
<td>76</td>
</tr>
<tr>
<td>105,000g supernatant</td>
<td>4.9</td>
<td>0.4</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
<td>5</td>
</tr>
</tbody>
</table>

Mitochondrial Respiration

Mitochondria were prepared by a previously described method (4). Fifty to 100 g of shoots were used in each extraction. Oxygen uptake was measured with an oxygen electrode. Protein was determined by the Bradford method after precipitation with trichloroacetic acid (4).

Proline Dehydrogenase Solubilization

The mitochondrial pellet was resuspended in 5 mL of 0.4 M sucrose. The nonionic detergent, NP-40 (Sigma Chemical Co.), was used at a final concentration of 0.1% (w/v) to solubilize membrane bound molecules. After addition of detergent, the preparation was allowed to equilibrate at 4°C for 15 min before centrifugation at 105,000g in a Beckman Ti 70.1 rotor. This supernatant was passed through a 2.5 x 15 cm Sephadex G-100 column that had been equilibrated with 50 mM Mops, pH 7.5 at 4°C.

Enzyme Assays

Proline dehydrogenase was assayed by the method of Blake et al. (2) except that NaN₃ was substituted for NaCN and NP-40 was substituted for Triton X-100. Menadione was consistently used. NAD⁺-dependent P5C-dehydrogenase and Cyt c oxidase were measured as previously described (4). The extinction coefficients are 11.5 x 10⁵ M⁻¹ cm⁻¹ for INT (2) and 21,000 M⁻¹ cm⁻¹ for Cyt c (4).

Water Potential

Shoot water potentials were measured with a pressure chamber.

RESULTS

Solubilized PDH Activity

PDH activity was measured in cell-free preparations of corn shoots in the presence of detergent and oil-soluble vitamin-

Table II. Comparison of Proline-Dependent Iodonitrotetrazolium Reduction P5C Synthesis

<table>
<thead>
<tr>
<th>Source</th>
<th>INT Reduction</th>
<th>P5C Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min⁻¹ mg protein⁻¹</td>
<td>nmol min⁻¹ mg</td>
</tr>
<tr>
<td>Mitochondrial pellet</td>
<td>15.5 (4.5)</td>
<td>8.5 (0.8)</td>
</tr>
<tr>
<td>Sephadex G-100 void</td>
<td>0.4 (0.2)</td>
<td>6.4 (2.5)</td>
</tr>
</tbody>
</table>

* Mean (se) of three replicate assays.

K₃ (menadione). Vitamin-K₃ bisulfite did not support the activity. Sodium azide was not required for full activity but was included in the assay mixture to prevent electrons from being transferred to oxygen by Cyt oxidase. PDH was measurable in crude extracts and crude mitochondrial preparations of corn shoots (Table I). The activity was solubilized from maize mitochondria with 0.1% (w/v) NP-40. This solubilized activity passed though Sephadex G-100 in the void volume with relatively poor recovery (Table I).

The solubilized activity also synthesized P5C (Table II). The pH optimum for the activity in MMT buffer mixture was 8 when the column treated fraction was used (Figure 1) as well as when the high speed supernatant was used (data not shown). Assays of the column treated fraction gave an apparent Kₘ for L-proline of 6.6 mM. Solubilized activity was inhibited by 300 mM KCl but not by 300 mM K-acetate (Table III).

PDH activity was measured in extracts from maize shoots grown at low water potentials. Shoot water potentials were −0.1 and −0.7 MPa for well-watered and water-stressed plants. Rerwatered stressed plants recovered to the water potentials of the well-watered plants by 16 h after rewatering. Solubilized PDH activity from the water-stressed shoots was 11% of that from well-watered shoots (Table IV). PDH activity from water-stressed but rewatered shoots recovered to the level measured from well-watered shoots. These stress and

Table III. Effects of Chloride on Solubilized PDH Activity*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min⁻¹ mg protein⁻¹</td>
</tr>
<tr>
<td>Control</td>
<td>137 (7)</td>
</tr>
<tr>
<td>+ 300 mM KOAc</td>
<td>107 (7)</td>
</tr>
<tr>
<td>+ 300 mM KCl</td>
<td>9 (8)</td>
</tr>
</tbody>
</table>

* This preparation was solubilized by the standard procedure and eluted in the void volume of a Sephadex G-100 column.  b Mean (SE). Each value represents the mean of three assays.
Table IV. Comparison of PDH Activity Extracted from Mitochondria Isolated from Maize Seedlings Grown Well-Watered, Water-Stressed, and Stressed-but-Rewatered Treatments

<table>
<thead>
<tr>
<th>Vermiculite Treatment</th>
<th>PDH</th>
<th>Cyt c Oxidase</th>
<th>Shoot Water Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min⁻¹ mg protein⁻¹</td>
<td>nmol min⁻¹ mg protein⁻¹</td>
<td>MPa</td>
</tr>
<tr>
<td>100% H₂O saturated</td>
<td>25.9 (1.2)*</td>
<td>2.62 (0.1)</td>
<td>-0.1 (0.05)</td>
</tr>
<tr>
<td>16% H₂O saturated</td>
<td>2.9 (0.1)</td>
<td>2.95 (0.2)</td>
<td>-0.7 (0.1)</td>
</tr>
<tr>
<td>16% saturated but rewatered</td>
<td>39.9 (1.4)</td>
<td>2.08 (0.1)</td>
<td>-0.1 (0.05)</td>
</tr>
</tbody>
</table>

* Mean (SE). Each value represents the mean of three assays.

recovery treatments did not affect the activities of Cyt oxidase nor P5C-dehydrogenase in the same solubilized preparations.

Oxygen Uptake by Isolated Mitochondria

Mitochondria from each treatment were used to determine state 3 and state 4 respiration rates (Table V). State 3 respiration is the rate of oxygen uptake in the presence of a substrate and ADP. State 4 respiration is the rate of oxygen uptake in the presence of substrate but in the absence of ADP. The water-stress treatment did not affect the rates of oxygen uptake supported by NADH or succinate. When malate-pyruvate was the substrate, state 3 rates of mitochondria from water-stressed shoots were 60% of those from the well-watered controls. This activity did not completely recover to the control level upon rewattering. With proline as a substrate, state 3 respiration of mitochondria from water-stressed shoots decreased to 25% of the controls. The proline-dependent oxygen uptake returned to the control level in mitochondria from the rewartered shoots. The proline-dependent state 4 rate decreased to 50% of the control rate in the water-stressed shoots and returned to the control rate upon rewattering. The oxygen uptake by mitochondria from water-stressed seedlings was stimulated by ADP when NADH, succinate and malate-pyruvate were substrates, i.e. mitochondrial oxygen uptake was coupled to phosphorylation. This coupling was not observed when proline was supplied to mitochondria from water-stressed seedlings. Thus, the state 3 and state 4 rates were nearly the same (Table V).

Potassium chloride did not inhibit oxygen uptake by intact mitochondria when measured immediately after addition (Table VI). The mitochondria were less stable in the presence of salt and the activity declined as the mitochondria generally deteriorated. There was no apparent effect of KCl on the mitochondria that was similar to the effect of KCl on the solubilized PDH activity.

DISCUSSION

Solubilization of PDH

Maize PDH has been successfully solubilized and assayed by a procedure previously used for mouse PDH (2). As with mouse PDH, maize PDH activity could only be detected with the oil-soluble menadione (vitamin-K₃). PDH activity was not detectable with vitamin-K₃ bisulfite. This observation is consistent with the hypothesis that PDH donates electrons directly to the RETS. Vitamin-K₃ is an analog of ubiquinone, which is an endogenous electron transport component of the inner mitochondrial membrane.

The measured rate of P5C synthesis by the solubilized PDH preparation was greater than the proline dependent INT reduction rate. Our interpretation of this result is that the INT is being reoxidized during the assay. The pH optimum of 8 compared with the pH optimum of 7.2 reported for proline oxidation by intact maize mitochondria (5) is interpreted on the basis of the matrix pH being relatively high when RETS is functioning. The pH optimum for proline oxidation by intact spinach mitochondria was 8.0 to 8.5 (6). The Kₘ for proline of spinach mitochondria was 28 mm (6) which is about fourfold higher than our value of 6.6 mm for the solubilized maize PDH. Proline oxidation in spinach required magnesium ions and flavin-adenine (6). Our solubilized PDH activity was not influenced by magnesium ions, flavin-adenine, or riboflavin 5'-phosphate (data not shown). Thus, solubilized PDH displays some of its in vivo characteristics in vitro. The observation that solubilized PDH activity appears in the void volume of a Sephadex G-100 column indicates that the molecular mass of the enzyme is greater than 150 kD.

Effect of Chloride Ions on PDH

PDH activity in vitro was inhibited by chloride ions in the same manner that P5C dehydrogenase activity was inhibited by chloride ion. Boggess et al. (1) suggested that chloride ion
concentration might regulate the rate of proline oxidation. However, the inhibitory concentration of KCl on solubilized PDH activity did not have an immediate effect upon proline-dependent oxygen uptake by intact mitochondria. After the mitochondria had been exposed to salt for a few minutes, their integrity declined and the rates of oxygen uptake supported by all substrates were inhibited. The osmotic potential of 300 mM KCl or potassium acetate is approximately −1.5 MPa (approximating them as ideal solutions). Since solubilized PDH activity was not changed by 300 mM potassium acetate, osmotic potential does not appear to regulate this enzyme. This conclusion is consistent with the observation of others (6) that proline oxidation by spinach mitochondria was not affected by osmotic potentials as low as −2.0 MPa when sucrose, mannitol or polyethylene glycol was the osmoticum. Others (5, 6) have reported that concentrations of Triton X-100 that do not destroy mitochondrial structure, can inhibit proline-dependent oxygen uptake. Our results explain this observation as the solubilization of the extrinsic PDH.

Effects of the Water-Stress Treatment

Retarded growth at low water potential was accompanied by an 89% decrease in extractable PDH activity. Proline-dependent oxygen uptake was accompanied by a similar decrease. There was no stimulation of proline dependent oxygen uptake by ADP in the mitochondria from stressed seedlings. We interpret this result as indicating that PDH activity was limiting oxygen uptake. Malate-pyruvate dependent oxygen uptake was decreased by water stress to a lesser extent than was proline dependent oxygen uptake. Succinate-and NADH-dependent oxygen uptake was not affected by the water stress treatment and the rate was stimulated by ADP similarly to the well-watered controls. Stimulation of the oxygen uptake rate by ADP was observed when malate-pyruvate was the substrate. Thus, the water stress inhibition of proline-dependent oxygen uptake by mitochondria isolated from stressed seedlings is similar to the observed decrease in solubilized PDH activity due to the same treatment.

Sells and Koepp (9) reported a similar effect of water stress on proline oxidation by mitochondria from 3-d-old etiolated maize seedlings that were dehydrated for 12, 16, and 36 h at 27°C and 20% relative humidity resulting in shoot water potentials of −1.0, −1.7, and −2.3 MPa, respectively. Oxidation of standard substrates decreases with these treatments but proline oxidation decreased to a much greater degree. They, too, observed no ADP stimulation of proline-dependent oxygen uptake but ADP did stimulate oxygen uptake that was dependent upon NADH, succinate and malate-pyruvate.

The method of growing stressed maize seedlings used in our study resulted in shoot water potentials of −0.7 MPa, which falls within the mild water stress category (9). Shoot elongation rate in the stress treatment was one-half that of the control treatment. Our water-stress treatment method was that of Sharp et al. (10) which does not cause proline accumulation in maize seedling shoots. Proline accumulation in maize seedlings was reported only for shoots with water potentials lower than −2.0 MPa (9). At this water potential ultrastructural membrane damage in the mitochondria was reported.

The decrease in extractable PDH activity from shoots grown in water-deficit is consistent with our and Sells and Koepp’s observation (9) of the specific decrease in proline oxidation by mitochondria isolated from water-stressed shoots. Sells and Koepp (9) concluded that the observed decrease in proline oxidation was caused by a specific inner membrane alteration unique to proline oxidation. Their hypothesis was that small decreases in water potential decrease the activity of a proline transporter which causes the decrease in proline oxidative capacity. Our results show that water-stress decreases proline oxidation by decreasing PDH activity. Because PDH is a membrane bound component, the specific effect of water stress on PDH could be Sells and Koepp’s unique membrane alteration. The alteration could be in the presence of active PDH polypeptides rather than a change in the phospholipid content.

Sells and Koepp (9) also reported that the decrease in proline oxidation caused by water deficit was reversed 12 h after rewatering of the mildly and moderately dehydrated seedlings. Our results with both the intact mitochondria oxygen uptake measurements and with the extractable PDH activity are in agreement with their observation.

Wood (12) reported that the membrane association of PDH in Escherichia coli is redox dependent. It may be possible that PDH was present in the matrix but not membrane associated in the mitochondria from water-stressed shoots. If so, its activity should still have been detected in the 105,000g supernatant because this fraction contained membrane solubilized and matrix enzymes. Thus, if PDH was present in the mitochondria from water-stressed shoots, it was not in its active

### Table VI. Effect of 300 mM KCl on State 4 Oxidation of Proline by Mitochondria Isolated from Maize Seedlings Grown in Well-Watered, Water-Stressed, and Stressed but Rewatered Treatments

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Well-watered</th>
<th>Water-stressed</th>
<th>Rewatered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + KCl</td>
<td>Control + KCl</td>
<td>Control + KCl</td>
<td>Control + KCl</td>
</tr>
<tr>
<td>NADH</td>
<td>116 (4)*</td>
<td>227 (26)</td>
<td>133 (7)</td>
<td>191 (13)</td>
</tr>
<tr>
<td>Succinate</td>
<td>166 (24)</td>
<td>196 (32)</td>
<td>190 (10)</td>
<td>183 (9)</td>
</tr>
<tr>
<td>Malate-pyruvate</td>
<td>47 (6)</td>
<td>57 (7)</td>
<td>39 (5)</td>
<td>31 (3)</td>
</tr>
<tr>
<td>Proline</td>
<td>60 (6)</td>
<td>67 (6)</td>
<td>30 (9)</td>
<td>24 (4)</td>
</tr>
</tbody>
</table>

*Mean (SE). Each value represents the mean of three respiration measurements.
form. PDH from *E. coli* has been shown to contain a DNA binding and operon regulatory activity (12). However, such capabilities have not been reported for the PDH from *Saccharomyces cerevisiae* (3). Therefore, the PDH from prokaryotes and eukaryotes possess different characteristics.

**Metabolic Function of PDH**

Phang (7) describes a redox shuttle in mammalian cells that involves PDH in mitochondria and P5C reductase in the cytosol. This shuttle functions to transfer reducing equivalents from the cytosol to the mitochondrion and to regenerate NADP⁺ in the cytosol. The purpose of the NADP⁺ regeneration is Phang’s shuttle model is to drive the pentose phosphate pathway, which produces the phosphorylated ribose required for nucleotide synthesis. Phang concludes that the proline-P5C shuttle functions to coordinate the metabolism of amino acids and ribonucleotides by controlling the NADP⁺/NADPH ratio in the cytosol. If this model applies to growing maize seedlings which also require nucleotide biosynthesis, then the following hypotheses should be valid: (a) A P5C reductase that has a greater affinity for NADPH than NADH should be present in the cytosol of maize shoot cells. (b) A P5C exporter should be present in the inner mitochondrial membrane. (c) The decrease in PDH activity should be accompanied by a decrease in the functioning of the pentose phosphate pathway. The observation that solubilized PDH activity reported herein and proline oxidation by mitochondria are both alkaline while the pH optimum for the mitochondrial P5C dehydrogenase is acidic (5) suggests that PDH and P5C dehydrogenase do not act sequentially when RETS is coupled to oxidative phosphorylation. This observation is consistent with Phang’s model. Because PDH and P5C dehydrogenase both display less than optimal activity at pH 7.0, it is possible that proline is oxidized to glutamic acid in mitochondria where respiration is uncoupled.

If PDH is nuclearly encoded and cytosolically translated in maize as it is in *Saccharomyces* (3), then the change in extractable PDH activity during growth under water deficit represents a unique cytosol-mitochondria interaction during growth that is sensitive to small changes in water potential. Our observations of extractable PDH activity from maize are consistent with the previous observations in many plant species that proline oxidation is inhibited by water deficit. Our observations also support the hypothesis that proline oxidation is required for growth. If so, then proline accumulation is a symptom of metabolic dysfunction in suboptimal plant growth environments.

**LITERATURE CITED**