Oxidation of Proline by Plant Mitochondria

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ABSTRACT

Mitochondria isolated from etiolated shoots of corn (Zea mays), wheat (Triticum aestivum), barley (Hordeum vulgare), soybean (Glycine max L. Merr.), and mung bean (Phaseolus aureus) exhibited a proline-dependent O2 uptake subject to respiratory control. ADP/O ratios with proline as substrate were intermediate between ratios obtained with exogenous NADH and malate + pyruvate as substrates. Isotope studies showed proline metabolism to be dependent on O2, but not NAD. The major ninhydrin-positive product formed via Δ1-pyroline-5-carboxylic acid was glutamate. Mitochondria were capable of further metabolism of glutamate, as radioactive CO2, organic acids, and aspartate were recovered after [14C]proline feeding experiments. These results demonstrate the mitochondrial association and O2 dependence of plant proline metabolism.

In mammals, insects, yeasts, and bacteria, the metabolism of proline is begun by conversion to P5C2 (5, 7, 8) or in some cases P2C (2, 8). This conversion is typically mitochondrial, and is catalyzed by proline oxidase, an O2-dependent flavoprotein (5, 7, 8, 21). Activity of this enzyme apparently has not been demonstrated in higher plants. Instead, several workers have reported the presence of proline dehydrogenase, a nonparticulate, NAD-linked enzyme which has been suggested, but not proved, to catalyze P5C formation in vivo (10–12, 19). Doubt that proline dehydrogenase is the in vivo catalyst for proline oxidation has arisen from the necessity to assay it at high pH (above pH 10, refs. 10 and 12) and from the observation that it co-purifies with P5C reductase (10), the NADH-linked proline biosynthetic enzyme that is typically stable and present in relatively high activity in a variety of tissues (6, 10, 12, 16, 17). These considerations led us to look for proline oxidation by mitochondria isolated from higher plants.

MATERIALS AND METHODS

Plant Material. Mitochondria were isolated from 3-day etiolated shoots of corn (Zea mays cv. WF9[N] × B37) and wheat (Triticum aestivum cv. Abe), from 4-day etiolated shoots of barley (Hordeum vulgare cv. Prior) and from 4-day etiolated hypocotyls of soybean (Glycine max L. Merr. cv. Amsoy 71) and mung bean (Phaseolus aureus). Corn seedlings were grown at 30 °C on moist paper towels saturated with 0.1 mM CaCl2. The other seedlings were grown in moist Vermiculite at room temperature (24–28 °C).

Mitochondrial Preparation. The procedure was the same as previously described for corn mitochondria (13). Briefly, shoots were clipped, ground in a mortar and pestle, and the homogenate filtered through cheesecloth. After a low speed centrifugation to remove debris, mitochondria were pelleted (28,000g, 6 min), resuspended in 0.4 M sucrose, and the suspension clarified by centrifugation at low speed. Mitochondria were then centrifuged through a 0.6 M sucrose cushion (17,500g, 18 min) with final suspension in 0.4 M sucrose. Mitochondrial protein was measured by the Lowry procedure (9).

O2 Uptake Measurements. O2 concentration was monitored with a Clark O2 electrode (Yellow Springs Instrument Co.) in a stirred, temperature-controlled (27 ± 0.2 °C) 4-ml reaction volume. The reaction vial was fitted into the light path of a Bausch & Lomb electronic 70 spectrophotometer, so that O2 concentration and percentage of light transmittance through the reaction suspension (520 nm) could be simultaneously recorded. The reaction medium contained 0.2 mM succrose, 1 mg/ml BSA, 1 mM MgSO4, 4 mM K-phosphate, and 10 mM TES (pH 7.6).

Radioisotope Experiments. Radiochemicals were obtained from Amersham/Searle. l-5T-Proline was purified, dried, and redissolved before use to remove tritiated water. [14C]Proline was purified by passing through a Dowex 1-acetate column. Glutamic acid was identified by its retention on Dowex 1-acetate, elution with 0.5 M acetic acid, and subsequent TLC (4) with standards. The method of Mitra et al. (14) was used to assay the appearance of tritium in water after incubation of mitochondria with tritiated proline.

When assay of 4CO2 was desired, reactions were set up in vials inside a stoppered 500-ml Erlenmeyer flask containing a scintillation vial holding 1 ml of ethanalamine. The reaction was terminated by adding 0.1 ml of 1 N HCl to the reaction mixture. The 500-ml flask was then shaken for 1 hr to allow equilibration of CO2. The 14CO2-containing ethanalamine was counted by liquid scintillation. When further fractionation of the reaction medium was required, it was diluted 5-fold and added to a Dowex 50-H+ column (4 × 0.7 cm), allowing the column effluent to pass through Dowex 1-formate as described by Wang (22). The formate column was eluted with 4 N formic acid to give an organic acid fraction. The Dowex 50-H+ column was eluted with 2 N NH4OH to give the amino acid fraction. After evaporating to remove ammonia, this fraction was added to a column of Dowex 1-acetate. The neutral amino acid fraction (containing proline) passed through this column, while the acidic amino acid fraction (containing glutamate) was eluted from the column with 0.5 N acetic acid.

Two-dimensional chromatography and autoradiography of amino acids was on thin layers of cellulose as described previously (4).

Two methods were used to isolate P5C. We have observed that P5C adheres to Dowex 1-acetate at pH 7.5, and can be eluted with 0.1 N acetic acid. Thus, reaction mixtures were added to Dowex 1-acetate columns, which had been washed with water to remove proline, then with 0.1 N acetic acid to elute P5C. After evaporation, the P5C-containing eluate was chromatographed in 1-butanol-acetic acid-water (12:3:5, v/v/v) along with standard P5C. In this

1 This work was supported by the Illinois Agricultural Experiment Station and the Iowa State University Graduate College.

2 Abbreviations: P5C: Δ1-pyroline-5-carboxylic acid; P2C: Δ1-pyroline-2-carboxylic acid; OAB: o-aminobenzaldehyde.
solvent P5C migrates ahead of glutamate, aspartate, P2C, and organic acids, and is recognized by its pink ninhydrin reaction. A modification of the assay of Phang et al. (18) was also used to identify P5C. This reaction was stopped by adding 2 ml of OAB (0.3%) in acidic ethanol (12 n HCl-95% ethanol, 1/7, v/v/v). This mixture was added to a Dowex 50-H+ column (4 x 0.7 cm) which was washed free of radioactivity with 2 N HCl. The column was then washed with water to remove excess HCl, and the P5C-OAB complex was eluted with 2 N NH4OH. The eluate was evaporated and chromatographed in 1-butanol-acetic acid-water (12/3/5, v/v/v). The yellow complex was observed on the chromatogram.

Proline Dehydrogenase Assay. The assay cuvettes contained 200 mM carbonate buffer (pH 10.5), 0.67 mM NAD, 10 mM L-proline, and 0.1 ml of resuspended mitochondrial pellet or 0.3 ml of supernatant. Total volume was 3 ml. Activity was measured as an increase in A at 340 nm.

RESULTS

Proline-dependent O2 Uptake. In initial experiments, 5 mM L-proline supported O2 uptake by mitochondria isolated from etiolated seedling tissues at the following rates [nmol of O2/min·mg of protein]: barley [30], corn [53], mung bean [24], soybean [32], and wheat [50]. Since wheat mitochondria showed the most consistent activity in preliminary tests, the more detailed studies reported here were carried out with mitochondrion isolations from 3-day-old etiolated wheat shoots. At this stage of development 50 to 80 g fresh wt of shoots yielded 6 to 10 mg of mitochondrial protein. Figure 1 shows O2 electrode traces of O2 uptake by wheat mitochondria dependent on exogenous NADH, malate + pyruvate, and proline. The addition of ADP stimulated proline oxidation and reduced light transmittance by the mitochondrial suspension in a manner similar to the state 3-state 4 transitions with the other substrates. ADP/O ratios in our best preparations with proline as substrate were around 2, a ratio intermediate between those observed with exogenous NADH (1.4–1.5) and malate + pyruvate (2.2–2.6). Proline-dependent O2 uptake was not influenced by exogenous NAD (1 mM) but state 3 oxidation was reduced 75% by antimycin A (0.3 µM) and almost completely inhibited by KCN (1 mM). DNP addition (state 4, 0.4 mM) resulted in an increased rate of O2 uptake. The mitochondrial supernatant fraction exhibited a slow endogenous O2 uptake that was not stimulated by the addition of proline.

Other Amino Acids. L-Arginine (5 mM), L-serine (2 mM), L-glycine (2 mM) or L-alanine (2 mM) did not support O2 uptake, nor did their presence influence the rate of proline oxidation. L-Glutamate (3 mM) affected a rate of O2 uptake (stimulated by ADP) approximately one-third to one-half that observed after the addition of 5 mM proline. Hydroxy-L-proline (5 mM) was oxidized at a low rate (<10% of the 5 mM proline oxidation rate). A slight inhibition of proline oxidation was observed after the additive addition of 5 mM hydroxy-L-proline.

Substrate Kinetics. O2 uptake was found to reach a maximum at 20 to 30 mM L-proline in the reaction medium. Reciprocal plots of the data from several experiments gave 5 mM as the Km value for proline (Fig. 2).

Identification of Reaction Products. To verify that proline-dependent O2 uptake represented proline metabolism, and to identify the products, isolated mitochondria were incubated with radioactive proline. Mitra et al. (14) have established a simple assay for proline oxidation in which proline labeled with tritium in the 5 position is used as precursor. Proline oxidation is indicated if tritium is transferred to water during incubation with a test tissue. Figure 3 indicates that tritium from carbon 5 appeared in the medium as tritiated water when L-5T-proline was incubated with either isolated corn or wheat mitochondria; a boiled control was inactive. This experiment shows that isolated mitochondria oxidized proline; the pathway via P5C is implied, as conversion to P2C should not release tritium from the 5 position.

The data in Table I show that a small amount of label can be recovered in P5C. When isolated corn or wheat mitochondria were incubated 1 hr with L-[U-14C]proline a small amount of label was recovered in P5C (Table I). In the first experiment presented in Table I P5C was identified by its pink reaction with ninhydrin after column and TLC; in the second, the relatively unstable compound was recovered as its yellow OAB derivative. The increase in recovered radioactivity on addition of carrier P5C in experiment 2 gives further evidence that P5C was an intermediate in the proline to glutamate conversion. However, the small amount of radioactivity recovered in P5C as compared to glutamate and organic acids (about 30,000 cpm) indicates that P5C was oxidized to glutamate about as rapidly as it was produced.

Further information was obtained from experiments with L-
Fig. 3. Recovery of tritium in water after incubation of L-5T-proline with isolated wheat and corn mitochondria. The incubation mixture contained 0.5 ml of reaction medium, 0.5 ml of mitochondrial suspension (representing 4 g of wheat and 8 g of corn shoots), 5 mM L-proline, and 1.88 x 10^8 cpm L-5T-proline. Reaction volume was 1.2 ml. At intervals, 0.1-ml aliquots were removed, water was sublimed into scintillation vials, and radioactivity was determined by liquid scintillation counting (17). (○—○): corn; (△—△): wheat; (□—□): boiled control.

Table I. Radioactivity in fractions containing PSC after incubation of L-[U-14C]proline with isolated corn or wheat mitochondria

<table>
<thead>
<tr>
<th>Material</th>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dowex-1(acetate) eluate</td>
<td>OA-PS eluate from Dowex-50(H)</td>
</tr>
<tr>
<td>Corn mitochondria</td>
<td>-PSC</td>
<td>2600</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>+PSC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Corn mitochondria</td>
<td>-PSC</td>
<td>1500</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>+PSC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wheat mitochondria</td>
<td>-PSC</td>
<td>1500</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>+PSC</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubation mixture contained 5 ml reaction medium, 0.5 ml mitochondrial suspension in 0.4 M sucrose, 5 mM L-proline containing 1.38 x 10^8 cpm of L-14C-proline and water to give a total volume of 1.2 ml.

Table II. Recovery of tritium in water after incubation of L-3, 4T-proline with plant mitochondria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity in water</th>
<th>1 hr</th>
<th>2 hr</th>
</tr>
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<tbody>
<tr>
<td>Corn mitochondria</td>
<td>9700</td>
<td>15,000</td>
<td>0</td>
</tr>
<tr>
<td>Corn mitochondria + Triton X-100</td>
<td>(0.1%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wheat mitochondria</td>
<td>5600</td>
<td>12,600</td>
<td>0</td>
</tr>
<tr>
<td>Wheat mitochondria + Triton X-100</td>
<td>(0.1%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table III. Distribution of radioactivity after 2 hr incubation of L-[U-14C]proline and L-5T-proline with corn and wheat mitochondria

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity</th>
<th>14CO2</th>
<th>5T-proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>25,900</td>
<td>14,400</td>
<td></td>
</tr>
<tr>
<td>0.5 M acetic acid eluate</td>
<td>150</td>
<td>21,800</td>
<td>0 10,800</td>
</tr>
</tbody>
</table>

Table IV. Radioactivity in various fractions after 2 hr incubation of plant mitochondria with L-[U-14C]proline

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity per fraction</th>
<th>14CO2</th>
<th>14C-proline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn mitochondria</td>
<td>2,900</td>
<td>21,400</td>
<td>19,300</td>
</tr>
<tr>
<td>Wheat mitochondria</td>
<td>7,100</td>
<td>51,900</td>
<td>77,400</td>
</tr>
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</table>

3.4T-proline. Tritium from this compound should be released only if the proline carbon skeleton were converted at least to fumarate, preserving the usual metabolic sequence (proline → glutamate → α-ketoglutarate → succinate → fumarate). Table II shows that significant radioactivity from the 3 and 4 positions was recovered in water after 1 and 2 hr incubation. Disruption of the mitochondria with 0.1% Triton 100 completely prevented tritium release from the precursor.

To identify specific products, L-[U-14C]proline was included in the reaction mixture along with L-5T-proline. The data in Table III indicate that the appearance of tritium in water was roughly equal to 14C in the acidic fraction, which contained organic acids and acidic amino acids such as glutamate and aspartate. While the acidic fraction was almost completely lacking in tritium, chromatography of the acidic fraction showed glutamate to be the major radioactive ninhydrin-positive compound containing appreciable radioactivity. Aspartate, the only other radioactive amino acid, contained 15 to 20% as much radioactivity as glutamate. These results establish that glutamate is a major product of proline oxidation by plant mitochondria and verify the previous result (Fig. 3) in suggesting that the conversion proceeds almost entirely by a pathway that labilizes tritium at the 5 position. Consistent with the result in Table II, glutamate accounted for only a part of the radioactivity in the acid fraction. The rest of the radioactivity was associated with aspartate and ninhydrin-negative compounds, which would arise from further metabolism of glutamate.

Additional evidence that glutamate produced from proline was further metabolized was obtained in an experiment in which L-[U-14C]proline was incubated with mitochondria under conditions allowing 14CO2 to be measured. The reaction mixture was divided into fractions designated organic acids, acidic amino acids (containing glutamate), and neutral amino acids (containing proline). Table IV shows that appreciable radioactivity was recovered in CO2 and that the ninhydrin-negative organic acid fraction was again heavily labeled.

Involvement of Proline Dehydrogenase. Metabolism of proline as demonstrated by the preceding results could have been initiated by either proline oxidase or proline dehydrogenase, although the mitochondrial nature of the activity and the failure of NAD to stimulate O2 uptake both suggest that proline dehydrogenase was not involved. Further evidence was provided by measurement of proline dehydrogenase activity in mitochondrial and supernatant fractions of corn and wheat homogenates. The mitochondrial suspension contained relatively little activity (0.4 and 3 nmol min ^-1 g fresh wt ^-1 for corn and wheat), less than 10% of that found in the supernatant (66 and 35 nmol min ^-1 g fresh wt ^-1 for corn and wheat).
corn and wheat). This confirms the report of Rena and Splittstoesser (19) that the dehydrogenase is nonmitochondrial. This point is amplified by data presented in Table V, indicating that proline metabolism was drastically reduced under anaerobic conditions, and that the anaerobic reduction in activity was not restored by the addition of NAD.

**DISCUSSION**

O₂ uptake data studied that proline metabolism by the tissue homogenates studied is linked to energy conversion by isolated mitochondria. Radiotracer experiments make it clear, however, that proline metabolism by isolated mitochondria is not limited to glutamate formation, but that proline can be broken down to form CO₂. Thus, the O₂ uptake results reflect a series of reactions and do not tell us whether the electron transfer at the first step of proline oxidation is directly to O₂ or to an intermediate carrier. Further work with the isolated enzyme will be required to clarify this point.

The absence of proline dehydrogenase in mitochondrial preparations, the failure of NAD to stimulate either proline-dependent O₂ uptake or isotope-sampled proline oxidation, and the inhibition of proline oxidation by anaerobic conditions all tend to rule out the possibility that mitochondrial proline metabolism is catalyzed by proline dehydrogenase. On the other hand, all evidence presented here is consistent with the operation of a mitochondrial proline oxidase, perhaps similar to the enzyme reported in rat liver (7, 21). Our findings do not negate the possibility that proline dehydrogenase may catalyze proline metabolism in other tissues (10–12, 19), or in the cytoplasm of the species examined in this study.

Localization of proline oxidation in the mitochondrion could account for the separation of proline synthesis and breakdown implied by the isotope experiments of Bogess et al. (3), as proline biosynthesis apparently takes place in the cytoplasm (6, 15, 17). A close connection between proline oxidation and mitochondrial energy conversion is also consistent with the suggested beneficial effect of accumulated proline in providing a ready source of energy, reducing power and amino nitrogen to the plant during recovery from water stress (1). Finally, the findings of this study provide a basis for more detailed work on the inhibitory effect of water stress on proline oxidation (20).

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