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Oxidation of Proline by Plant Mitochondria[1]

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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 O_2 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 O_2, but not NAD. The major ninhydrin-positive product formed via Δ^1-pyrroline-5-carboxylic acid was glutamate. Mitochondria were capable of further metabolism of glutamate, as radioactive CO_2, organic acids, and aspartate were recovered after [^{14}C]proline feeding experiments. These results demonstrate the mitochondrial association and O_2 dependence of plant proline metabolism.

In mammals, insects, yeasts, and bacteria, the metabolism of proline is begun by conversion to P5C[2] (5, 7, 8) or in some cases P2C (2, 8). This conversion is typically mitochondrial, and is catalyzed by proline oxidase, an O_2-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 $CaCl_2$. 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 (28,000*g*, 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 (17,500*g*, 18 min) with final suspension in 0.4 M sucrose. Mitochondrial protein was measured by the Lowry procedure (9).

O_2 Uptake Measurements. O_2 concentration was monitored with a Clark O_2 electrode (Yellow Springs Instrument Co.) in a stirred, temperature-controled (27 ± 0.2 C) 4-ml reaction volume. The reaction vial was fitted into the light path of a Bausch & Lomb spectronic 70 spectrophotometer, so that O_2 concentration and percentage of light transmittance through the reaction suspension (520 nm) could be simultaneously recorded. The reaction medium contained 0.2 M sucrose, 1 mg/ml BSA, 1 mM $MgSO_4$, 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. [^{14}C]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 $^{14}CO_2$ was desired, reactions were set up in vials inside a stoppered 500-ml Erlenmeyer flask containing a scintillation vial holding 1 ml of ethanolamine. 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 CO_2. The $^{14}CO_2$-containing ethanolamine 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 NH_4OH 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: Δ^1-pyrroline-5-carboxylic acid; P2C: Δ^1-pyrroline-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). This mixture was added to a Dowex 50-H^+ column (4 × 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 NH_4OH. 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 O_2 Uptake. In initial experiments, 5 mM L-proline supported O_2 uptake by mitochondria isolated from etiolated seedling tissues at the following rates [nmol of O_2/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 mitochondrial 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 O_2 electrode traces of O_2 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 O_2 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 O_2 uptake. The mitochondrial supernatant fraction exhibited a slow endogenous O_2 uptake that was not stimulated by the addition of proline.

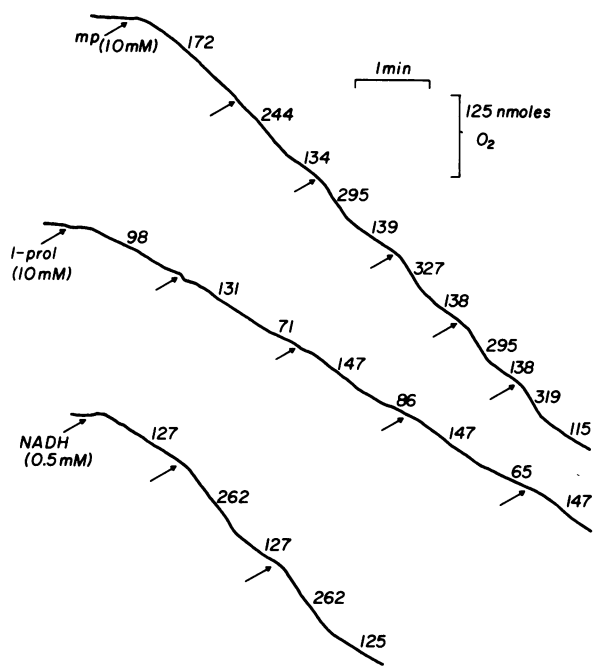

FIG. 1. O_2 uptake by isolated wheat mitochondria incubated with 10 mM malate + 10 mM pyruvate, 10 mM proline, or 0.5 mM NADH. Numbers give O_2 uptake rates in nmol of O_2 min^{-1}·mg of $protein^{-1}$. Unlabeled arrows indicate the addition of 300 nmol of ADP. Reaction media contained 0.2 M sucrose, 1 mg/ml BSA, 1 mM $MgSO_4$, 4 mM K-phosphate, and 10 mM TES (pH 7.6).

Other Amino Acids. L-Arginine (5 mM), L-serine (2 mM), L-glycine (2 mM) or L-alanine (2 mM) did not support O_2 uptake, nor did their presence influence the rate of proline oxidation. L-Glutamate (3 mM) affected a rate of O_2 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. O_2 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 K_m value for proline (Fig. 2).

Identification of Reaction Products. To verify that proline-dependent O_2 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-^{14}C]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-

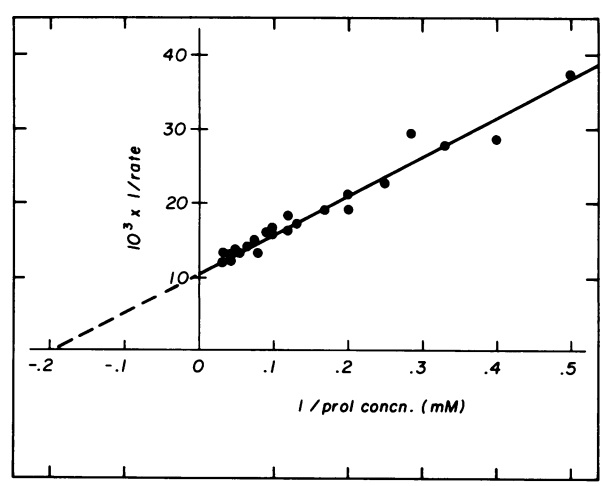

FIG. 2. Double reciprocal plot of the rate of state 4 O_2 uptake by isolated wheat mitochondria at various proline concentrations. Reaction media were as in Figure 1.

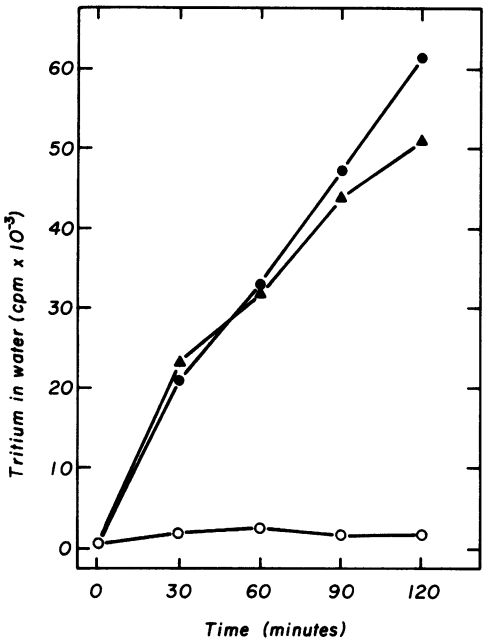

FIG. 3. Recovery of tritium in water after incubation of L-5T-proline with isolated wheat and corn mitochondria. The incubation mixture contained 0.5 ml of reaction medium, 0.5 ml of mitochondrial suspension (representing 4 g of wheat and 8 g of corn shoots), 5 mM L-proline, and 1.88×10^6 cpm L-5T-proline. 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 P5C after incubation of ^{14}C proline with isolated corn or wheat mitochondria

Material	Treatment	[a]Experiment 1: Dowex-1(acetate)eluate (0.1 N acetic acid)	[a]Experiment 1: P5C spot from chromatogram	[b]Experiment 2: OAB-P5C eluate from Dowex-50(H^+)
		cpm		
Corn mitochondria	-P5C	2600	450	250
Corn mitochondria	+P5C	-	-	600
Wheat mitochondria	-P5C	1500	250	800
Wheat mitochondria	+P5C	-	-	950

[a]Incubation mixture contained 0.5 ml reaction medium, 0.5 ml mitochondrial suspension in 0.4 M sucrose, 5 mM L-proline containing 1.38×10^6 cpm of L-U-^{14}C proline and water to give a total volume of 1.2 ml.

[b]Same incubation mixture as experiment 1 except that 2.23×10^6 cpm precursor was added; 0.95 μmol unlabeled P5C were added to the incubation mixture as indicated.

3,4T-proline. Tritium from this compound should be released only if the proline carbon skeleton were converted at least to fumarate, presuming the usual metabolic sequence (proline → glutamate → α-ketogluturate → 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-^{14}C]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 ^{14}C 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-^{14}C]proline was incubated with mitochondria under conditions allowing $^{14}CO_2$ to be measured. The reaction mixture was also 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 CO_2 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 O_2 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 $min^{-1} \cdot g$ fresh wt^{-1} for corn and wheat), less than 10% of that found in the supernatant (66 and 35 nmol $min^{-1} \cdot g$ fresh wt^{-1} for

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

Incubation mixture contained 0.5 ml reaction medium, 0.5 ml mitochondrial suspension in 0.4 M sucrose (representing 8 g fresh shoots in each case), 5 mM L-proline containing 6.26×10^5 cpm L-3, 4T-proline and 0.05 ml 3.3% Triton X-100 where indicated. Total volume was 1.25 ml.

Treatment	Radioactivity in water: 1 hr	2 hr
	cpm	
Corn mitochondria	9700	15,000
Corn mitochondria + Triton X-100 (0.1%)	0	0
Wheat mitochondria	5600	12,600
Wheat mitochondria + Triton X-100 (0.1%)	0	0

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

Incubation mixture contained 0.5 ml standard reaction medium, 0.5 ml mitochondrial suspension (representing approximately 6 g corn or 7 g wheat shoots), and 5 mM L-proline containing 2.9×10^6 dpm ^{14}C and 2.8×10^6 dpm tritium. Total volume was 1.2 ml.

Fraction	Radioactivity: Corn ^3H	Corn ^{14}C	Wheat ^3H	Wheat ^{14}C
	dpm			
Tritiated H_2O	25,900	-	14,400	-
0.5 N acetic acid eluate of Dowex-1 (acetate) column	150	21,800	0	10,800

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

Treatment[a]	Radioactivity per fraction: CO_2	Organic acids	Acidic amino acids
	cpm		
Corn mitochondria	2,900	21,400	19,300
Wheat mitochondria	7,100	51,900	77,400

[a]Incubation mixture contained 0.5 ml reaction medium, 0.5 ml mitochondrial suspension in 0.4 M sucrose (representing about 6 and 9 g fresh corn and wheat shoots, respectively) and 5 mM L-proline containing approx 1.6×10^6 cpm L-U-^{14}C proline.

Table V. Inhibition of proline metabolism by anaerobic conditions

Material	Treatment[a]	Radioactivity in acidic fraction[b] after 1 hour
Corn mitochondria	aerobic	12,400
Corn mitochondria	anaerobic	1,900
Corn mitochondria	anaerobic + NAD	300
Wheat mitochondria	aerobic	14,700
Wheat mitochondria	anaerobic	2,200
Wheat mitochondria	anaerobic + NAD	900

[a]Reactions were carried out in Thunberg tubes evacuated 5 times and refilled with air (aerobic) or N_2 gas (anaerobic) before tipping in proline to start the reaction. Reaction medium as in Table IV except for the inclusion of NAD (1.5 mM) as indicated.

[b]This fraction consists of material retained on Dowex-1 (acetate) and eluted with 0.5 N acetic acid. It contains glutamate, aspartate and organic acids.

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

O_2 uptake data show 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 CO_2. Thus, the O_2 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 O_2, 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 O_2 uptake or isotopically measured 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 Boggess *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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