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Proline Oxidation in Corn Mitochondria[1]

INVOLVEMENT OF NAD, RELATIONSHIP TO ORNITHINE METABOLISM, AND SIDEDNESS ON THE INNER MEMBRANE

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ABSTRACT

Proline-dependent oxygen uptake in corn mitochondria (*Zea mays* L. B73 × Mo17 or Mo17 × B73) occurs through a proline dehydrogenase (pH optimum around 7.2) bound to the matrix side of the inner mitochondrial membrane. Sidedness was established by determining the sensitivity of substrate-dependent ferricyanide reduction to antimycin and FCCP (*P*-trifluoromethoxycarbonylcyanide phenylhydrazone). Proline dehydrogenase activity did not involve nicotinamide adenine dinucleotide reduction, and thus electrons and protons from proline enter the respiratory chain directly. Δ^1-Pyrroline-5-carboxylate (P5C) derived from proline was oxidized by a P5C dehydrogenase (pH optimum approximately 6.4). This enzyme was found to be similar to proline dehydrogenase in that it was bound to the matrix side of the inner membrane and fed electrons and protons directly into the respiratory chain.

Ornithine-dependent oxygen uptake was measurable in corn mitochondria and resulted from an ornithine transaminase coupled with a P5C dehydrogenase. These enzymes existed as a complex bound to the matrix side of the inner membrane. P5C formed by ornithine transaminase was utilized directly by the associated P5C dehydrogenase and was not released into solution. Activity of this dehydrogenase involved the reduction of nicotinamide adenine dinucleotide.

The accumulation of proline in several species of plants is a well-established response to stress. Proline accumulation results primarily from stimulated synthesis and the concomitant inhibition of proline oxidation (16). The oxidation of proline occurs within the mitochondria and is more sensitive to inhibition by water stress than is the oxidation of other mitochondrial substrates (14). Inhibition of proline oxidation by stress is necessary to cause proline to accumulate, although it alone does not account for observed rates of accumulation. Accumulated proline may serve as a neutral osmoticum and as a reserve of nitrogen. Upon relief of stress, accumulated proline is rapidly oxidized, suggesting a role as an energy reserve (15, 16).

The enzymes involved with proline oxidation recently have been partially characterized as to their submitochondrial location and electron transport characteristics (1, 2, 7, 10, 17). The first enzyme in this process is proline DH,[2] which catalyzes the conversion of proline to P5C. This enzyme activity had previously been referred to as proline oxidase activity; however, proline DH is more appropriate (see "Conclusions").

The P5C thus formed is oxidized to glutamate by a P5C DH, with this reaction possibly involving the formation of the intermediate GSA. In the present study, we provide evidence for two distinct mitochondrial enzymes capable of oxidizing P5C. One of these P5C DH activities can only be measured in disrupted mitochondria and is involved in the oxidation of ornithine. Previous papers have shown that mitochondria contain ornithine transaminase activity (3, 12, 18). In this paper, we have further characterized the enzyme systems involved with the oxidation of proline and ornithine.

MATERIALS AND METHODS

Corn seedlings (*Zea mays* L. B73 × Mo17 or Mo17 × B73) and mung bean seedlings (*Vigna radiata* L.) were grown in the dark at 30 ± 2°C in moist vermiculite. Mitochondria were isolated from shoots of 3- to 4-d-old seedlings according to Day and Hanson (5). Protein was estimated by the method of Lowry *et al.* (11), using BSA (fraction V) as the standard. Assays were conducted at 26 ± 2°C unless otherwise indicated, and were initiated upon addition of substrate. FCCP (3.0 mM), antimycin A (0.15 mM), rotenone (3.0 mM), and SHAM (300 mM) were solubilized in 80% ethanol.

Oxygen Uptake. O_2 utilization was measured at 26 ± 1°C in 3.0 to 3.2 ml of medium using a Clark O_2 electrode (model 53, Yellow Springs Instrument Co.). ADP:O ratios, RCR, and O_2 content of air-saturated water were determined according to Estabrook (9).

Enzyme Assays. Substrate-dependent NAD reduction or NADH oxidation was followed by measuring changes in *A* at 340 nm. Ferricyanide reduction was measured by following decreasing *A* at 420 nm. The reduction of DCIP was followed as decreasing *A* at 600 nm. The following mM extinction coefficients were used: E_{340} = 6.22 mM^{-1} cm^{-1} for NADH, E_{420} = 1.03 mM^{-1} cm^{-1} for ferricyanide, E_{600} = 21.0 mM^{-1} cm^{-1} for DCIP, and E_{260} = 15.4 mM^{-1} cm^{-1} for ADP (7).

Preparation and Assay of P5C. P5C was synthesized according to the procedure of Williams and Frank (19), with the P5C

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[2] Abbreviations: DH, dehydrogenase; P5C, Δ^1-pyrroline-5-carboxylic acid; GSA, glutamic-γ-semialdehyde; α-KG, α-ketoglutaric acid; FCCP, *P*-trifluoromethoxycarbonylcyanide phenylhydrazone; SHAM, salicylhydroxamic acid; RCR, respiratory control ratio; DCIP, 2,6-dichloroindophenol; *o*AB, *o*-aminobenzaldehyde; EGTA, ethyleneglycol-bis(β-aminoethyl ether)*N,N'*-tetraacetic acid; OAA, oxaloacetic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; PMS, phenazine methosulfate; MTT, Mes-Tes-Tricine.

concentration determined using ninhydrin. It was concentrated by evaporation to dryness under an air stream (to remove the HCl) and redissolved in water. Purity of P5C preparations was evaluated by using partially purified P5C reductase from mung bean hypocotyls (no measurable P5C reductase was present in corn seedlings). Approximately 90% correlation between NADH oxidation and P5C utilization was found. This result corresponds closely with results of Williams and Frank, and indicates a purity of at least 90% after concentration. However, when using relatively high concentrations of P5C (around 10 mM), a contaminant with a maximum concentration of 1 mM is possible. Therefore, it was essential to correlate observed activity with P5C utilization.

P5C utilization was measured by using the ninhydrin technique of Chinard for proline and ornithine determination (4). P5C exhibits an absorption spectrum similar to proline in this assay, whereas glutamate does not react under the same conditions.

P5C formation was followed by reacting it with 20 mM *o*AB in 5% TCA/ethanol (w/v). One ml of the *o*AB solution was added to 2 ml samples, followed by centrifugation at 30,000*g* for 10 min. Absorbance at 443 nm of the clear supernatant was determined after 40 min total incubation. The *o*AB reagent was prepared immediately before use (19).

Isolation of P5C Reductase from Mung Bean Hypocotyls. Mung bean hypocotyls (50 g) were homogenized with a mortar and pestle in 100 ml of 0.4 M sucrose, 50 mM KH_2PO_4, and 5 mM EGTA (pH 7.6). All operations were conducted at 4°C. The resulting slurry was squeezed through 4 layers of cheesecloth and centrifuged (all centrifugations were at 30,000*g* for 10 min). The supernatant was fractionated by using ammonium sulfate, with the 40% to 50% pellet containing P5C reductase activity. This pellet was resuspended in 5 ml of 10 mM Tes (pH 7.2) and dialyzed against the same solution for 1 h. The suspension was then centrifuged, with the supernatant adjusted to 10% ammonium sulfate (to help stabilize the enzyme) and stored at −20°C. This procedure results in a 12-fold purification and a preparation that exhibits no interfering NADH oxidation. The enzyme preparation is relatively stable since 50% of the activity was still present after 8 months storage.

RESULTS AND DISCUSSION

Involvement of NAD in Proline and P5C Oxidation. To determine if NAD reduction is involved in the activities of proline DH and P5C DH, we suspended mitochondria in a medium of low osmolarity (10 mM each Mes, Tes, and Tricine), which allowed sufficient permeability of substrates into the mitochondrial matrix. In comparison to intact mitochondria (suspended in media with 250 mM sucrose), mitochondria suspended in media without sucrose are referred to as swollen mitochondria. In swollen mitochondria, the outer membrane has ruptured, as indicated by a 6-fold increase in succinate dependent Cyt *c* reduction over that found in intact mitochondria. In contrast, the inner membrane does not rupture since malate DH activity does not increase in the 30,000*g* supernatant of suspended swollen mitochondria as compared to intact (7).

Measurement of NAD reduction within swollen mitochondria, required inhibition of as much of the NADH DH activity as possible. This inhibition was achieved through the addition of 0.5 μM antimycin A, which left a residual rate of NADH oxidation of around 10 nmol/min·mg protein over a broad pH range (Fig. 1).

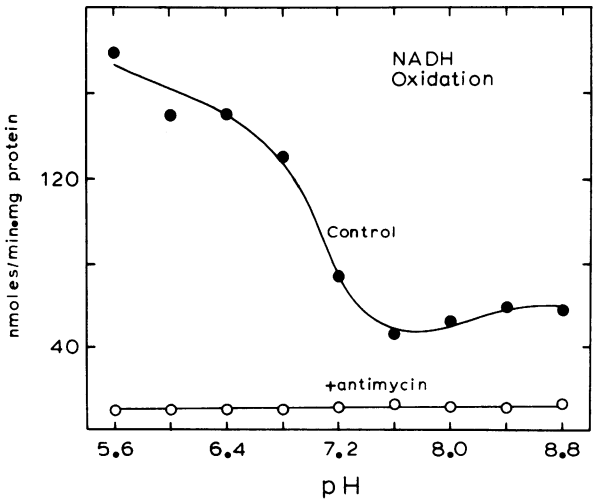

FIG. 1. Inhibition of NADH oxidation by 0.5 μM antimycin in swollen mitochondria. Assays were conducted in 3.0 ml of 30 mM MTT and were initiated upon addition of 100 μM NADH. Mitochondrial protein averaged 0.64 mg/assay. Data represent the mean of three separate experiments.

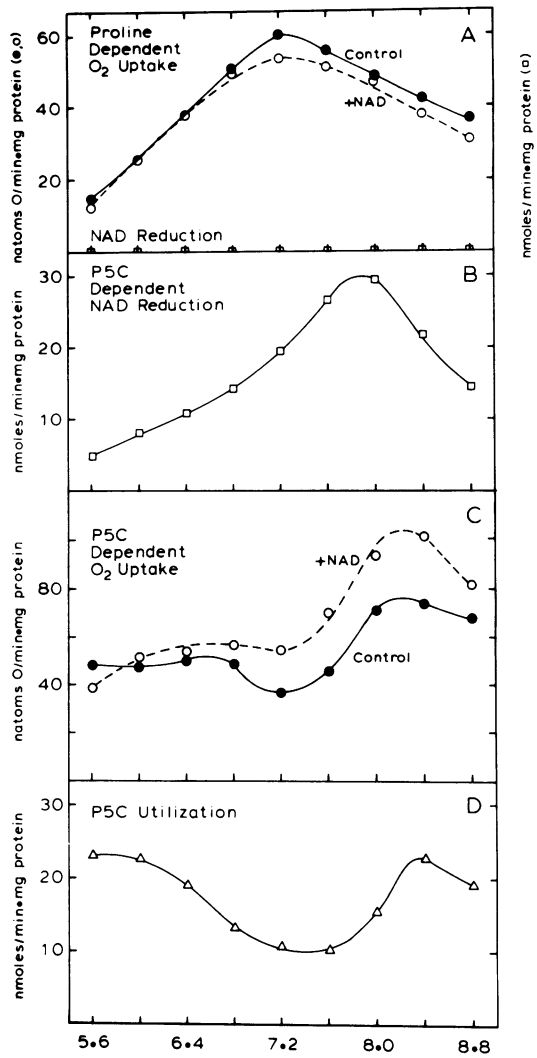

FIG. 2. Effects of pH on the oxidation of proline and P5C in swollen mitochondria. NAD reduction was assayed in 3.0 ml of 30 mM MTT containing 2 mM NAD, 0.5 μM antimycin, and either 10 mM L-proline or 6.5 mM DL-P5C. O_2 uptake was followed in 3.0 ml of 30 mM MTT containing 10 mM L-proline or 13 mM DL-P5C. O_2 uptake was followed in the presence and absence of 2 mM NAD. For the measurement of P5C utilization, mitochondria were incubated at 25 ± 1°C for 30 min in 2.5 ml of 30 mM MTT containing 1.44 mM DL-P5C (neutralized HCl solution). The reaction was terminated by addition of 2.5 ml glacial acetic acid. P5C content was determined by using ninhydrin (4). Mitochondrial protein averaged 1.38 mg/assay (A), 0.67 mg/assay (B), 1.36 mg/assay (C), and 1.44 mg/assay (D). All data represent the mean of three experiments.

Higher concentrations of antimycin (1 μM) or inclusion of other inhibitors (10 μM rotenone, 1 mM NaN_3, or 1 mM SHAM) resulted in little additional inhibition. This residual NADH oxidation is sometimes attributable to contaminating ER in the mitochondrial preparation.

To ensure that we could follow NAD reduction within the matrix of swollen mitochondria, we measured the activities of malate and glutamate DH. Malate-dependent NAD reduction was measurable (32.2 nmol/min·mg protein with 10 mM malate) and required the presence of a small amount of glutamate (0.5 mM), presumably for the removal of OAA by transamination. Glutamate-dependent NAD reduction was also measurable (7.45 nmol/min·mg protein with 10 mM L-Glu). The rates of NAD reduction obtained underestimate the actual rates by the amount of residual NADH oxidation (Fig. 1). We also followed NADH oxidation by these enzymes and found activity with both malate DH (7,320 nmol/min·mg protein with 250 μM OAA) and glutamate DH (19.9 nmol/min·mg protein with 10 mM α-KG and 5 mM NH_4Cl). NADH oxidation by a particular enzyme was measured as an increase in oxidation over the residual rate, and thus represents the actual rate. Therefore, by using swollen mitochondria, it was possible to measure both NAD reduction and NADH oxidation by enzymes within the matrix.

If NAD reduction is involved in the activity of proline and P5C DH *in vivo*, there should be a correlation between substrate-dependent O_2 uptake and NAD reduction. We followed these activities over a broad pH range in swollen mitochondria (Fig. 2). Proline-dependent O_2 uptake (Fig. 2A) was found to exhibit a pH optimum of 7.2 as previously reported for intact mitochondria (7). This activity was not stimulated by NAD, and NAD reduction could not be measured under the same conditions. In addition, no P5C-dependent NADH oxidation (reversal of proline-dependent NAD reduction) could be measured. This result clearly establishes that proline DH activity does not involve the reduction of NAD, and thus electrons and protons are fed directly into the respiratory chain. Indications that proline DH is flavin-linked are consistent with this result (10). In corn mitochondria, we could find no stimulation of O_2 uptake upon addition of 0.5 mM FAD, although we did observe some stimulation (26%) with 0.5 mM FMN. Proline DH activity measured as DCIP reduction in swollen mitochondria (60 μM DCIP with 0.5 μM antimycin) also exhibited a pH optimum of 7.2 in either the presence or absence of 1 mM PMS. The rates in the presence of PMS were decreased considerably. Proline-dependent O_2 uptake was stimulated by $MgCl_2$ (40% with 5 mM) as previously reported (10).

When P5C-dependent NAD reduction was measured (Fig. 2B), an optimum of around pH 8 was observed. This is similar to that previously reported for crude mitochondrial isolations from several tissues (17) and to that obtained from detergent-solubilized mitochondria (2), although the rates are considerably higher than those previously reported. However, it is markedly different from the optimum of pH 6.1 for P5C-dependent O_2 uptake in intact mitochondria (7). Therefore, P5C-dependent O_2 uptake was measured in swollen mitochondria (Fig. 2C). Two optima were observed, one near pH 6.4 that was not stimulated by NAD, and another around pH 8 that was. When P5C utilization was measured (Fig. 2D), two corresponding activities were obtained. Therefore, under these conditions, two enzymes are involved in the oxidation of P5C. P5C-dependent O_2 uptake at pH 6.4 was not stimulated by 0.5 mM FMN, and was inhibited somewhat (27%) by 0.5 mM FAD. The pH 6.4 activity was stimulated 17% by 5 mM $MgCl_2$. P5C and $MgCl_2$ (as low as 1 mM) were found to react above pH 7.2, with visible precipitation occurring around pH 8. This reaction explains $MgCl_2$ inhibition of P5C DH observed by Boggess *et al.* (2).

Reversal of P5C-dependent NAD reduction (glutamate-dependent NADH oxidation) was not measurable over the pH range.

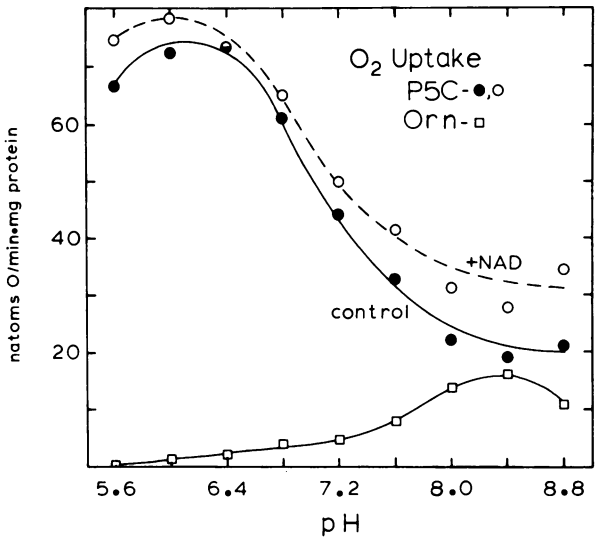

FIG. 3. Effects of pH on the oxidation of P5C and ornithine in intact mitochondria. O_2 uptake was followed in 3.0 ml of 250 mM sucrose, 5 mM KH_2PO_4, and 30 mM MTT containing 10 mM DL-P5C (or 10 mM L-ornithine in the presence of 10 mM α-KG). P5C-dependent O_2 uptake was followed in the presence and absence of 2 mM NAD. Ornithine-dependent O_2 uptake was measured as an increase in the rate above that with α-KG. Mitochondrial protein averaged 1.55 mg/assay. Data represent the mean of three separate experiments.

The inability to measure such activity suggests that the pH 8-P5C DH may not be significantly reversible. However, this could occur as a result of an equilibrium greatly toward glutamate formation (analogous to malate DH). Thus, reversibility possibly could be measured, if the P5C is removed as it is formed. All attempts to demonstrate that this occurs have failed. These include trying to trap the P5C with 1 mM *o*AB, and attempts to remove the P5C using partially purified P5C reductase from mung bean hypocotyls. Reversibility using added P5C reductase was tried with intact mitochondria and with mitochondria solubilized in 0.030% Triton X-100 (7). In each, considerable P5C DH activity (measured as NAD reduction in the mitochondria) and considerable P5C reductase activity (P5C-dependent NADH oxidation) were present in the reaction medium. No reversibility of P5C DH could be measured (either as glutamate-dependent NADH oxidation or [^{14}C]proline production from [^{14}C]glutamate), indicating that the failure to measure reversibility of P5C DH under these conditions is not merely a result of a simple equilibrium reaction. It was also possible that additional cofactors may be necessary for reversal. Inclusion of $MgCl_2$ and ATP (1 mM each) was tried, inasmuch as these are known to be involved in glutamate to P5C conversion during proline synthesis (13); however, no reversibility was observed.

Ornithine-Dependent O_2 Uptake and P5C DH Activities. P5C-dependent NAD reduction cannot be measured in intact mitochondria (2, 7). Consistent with this, the associated P5C-dependent O_2 uptake activity (optimum near pH 8) is not measurable (Fig. 3). A possible explanation of this is that the P5C, NAD, or both, are not reaching the enzyme active site in intact mitochondria around pH 8. Upon swelling of the mitochondria, this inaccessibility is relieved. Inaccessibility could result from P5C not being able to penetrate the inner membrane (assuming the active site is exposed to the matrix), or from this enzyme being part of an enzyme complex. Since P5C-dependent O_2 uptake can be measured due to the other P5C DH (compare Figs. 2C and 3), P5C evidently can penetrate the membrane (refer to the section on sidedness).

If the pH 8-P5C DH is part of an enzyme complex, this complex would be involved in the degradation of proline or ornithine,

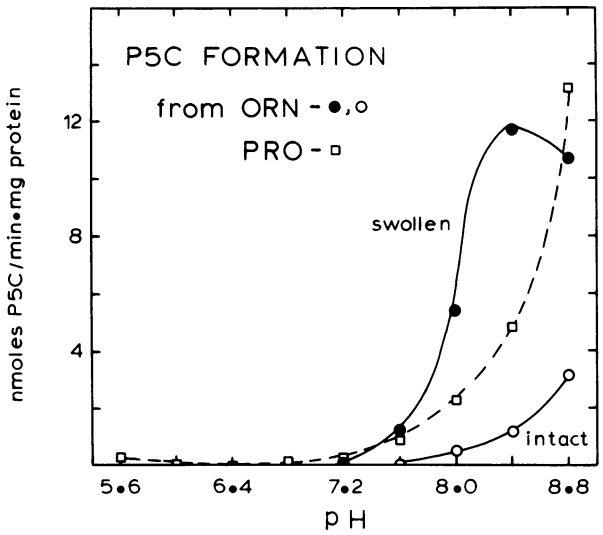

FIG. 4. P5C formation from proline and ornithine. Assays were conducted in 2.0 ml 30 mM MTT and 5 mM KH_2PO_4 (±250 mM sucrose). Ornithine-dependent P5C formation was followed by using 10 mM L-ornithine and 10 mM α-KG in the presence of 0.5 μM antimycin. Proline-dependent P5C formation was determined in the presence of sucrose, using 10 mM L-proline. Samples were incubated with substrate for 30 min at 26 ± 1°C. The reaction was terminated by addition of the *o*AB solution. Mitochondrial protein averaged 0.42 mg/assay. Data represent the mean of three experiments.

inasmuch as these two processes are the only such degradative processes known to involve P5C as an intermediate. Since ornithine transaminase activity is known to be present within the mitochondria (3, 12, 18), we attempted to measure ornithine-dependent O_2 uptake in intact mitochondria. Activity was present and had a pH optimum of around 8.4 (Fig. 3). The presence of α-KG was essential for measurement of ornithine-dependent O_2 uptake, presumably as an amino acceptor for ornithine transaminase activity. Ornithine-dependent O_2 uptake decreases upon mitochondrial swelling (by 60%), correlating with the appearance of measurable P5C-dependent NAD reduction (and the associated P5C-dependent O_2 uptake). These results suggest that these enzymes are complexed.

Further evidence to support the association of ornithine transaminase with the pH 8-P5C DH was obtained by measuring both proline and ornithine-dependent P5C production (Fig. 4). Proline-dependent P5C formation in intact mitochondria was measurable only above pH 7.2. Thus, P5C derived from proline is utilized by the pH 6.4-P5C DH, whereas the pH 8-P5C DH activity (only measurable in swollen mitochondria) is not involved. This pH response explains the stoichiometric production of P5C from proline at pH 8.5, as measured by Huang and Cavalieri (10). In addition, those experiments were conducted in the presence of $MgCl_2$ which effectively removes P5C from solution at that pH. Earlier attempts to demonstrate reasonable P5C production from proline failed since the mitochondria were incubated at pH 7.6 (1).

When P5C production from ornithine was followed with intact mitochondria (in the presence of antimycin), very little P5C formation was observed. However, considerable P5C formation occurred when this same activity was followed in swollen mitochondria. This activity (ornithine transaminase) had a pH optimum of 8.4. Thus, P5C derived from ornithine is normally not released into solution *in vivo*, but is utilized directly by the pH 8-P5C DH. Consistent with this, P5C in solution does not have access to the active site of this enzyme in intact mitochondria. Upon swelling the mitochondria and disrupting the complex, P5C derived from ornithine is released into solution (thus measurable),

Table I. *Comparison of NADH and P5C-Dependent O_2 Uptake in Swollen Mitochondria at pH 8*

Assays were conducted in 3.0 ml of 5 mM KH_2PO_4 and 30 mM MTT (pH 8). Mitochondrial protein averaged 1.47 mg/assay. Data represent the mean of three experiments.

Substrate	Average Control Rate	10 μM Rotenone	0.5 μM Antimycin	1 mM NaN_3	1 mM SHAM
	natoms O/min·mg protein	*% inhibition*			
10 mM DL-P5C	109	76	75	19	78
0.5 mM NADH	239	4	85	33	+22

and P5C in solution gains access to the active site of this enzyme, resulting in measurable P5C-dependent NAD reduction and the associated P5C-dependent O_2 uptake. Thus, it is clear that ornithine transaminase and the pH 8-P5C DH are complexed. This P5C DH is associated with the inner mitochondrial membrane (7).

Ornithine-dependent O_2 uptake in corn mitochondria has a very low specific activity. This activity is not significantly stimulated by exogenous NAD or higher concentrations of ornithine (in intact or swollen mitochondria). In addition, ornithine-dependent NAD and ferricyanide reduction cannot be measured. Thus, to characterize ornithine-dependent O_2 uptake further, a tissue with higher activity must be used. Arginine-dependent O_2 uptake (10 mM L-arginine and 10 mM α-KG) was also measurable in intact mitochondria with a rate of around 8 natoms O/min·mg protein at pH 8. This activity was measured as an increase in the rate above that with α-KG.

Inhibitor Sensitivities and Measurable ADP:O Ratios. The oxidation of proline is known to be sensitive to inhibition by rotenone, antimycin, and azide (7). Thus, electrons and protons from proline enter the respiratory chain before the rotenone sensitive iron-sulfur proteins. Further, proline oxidation has a measurable ADP:O ratio similar to that of malate + pyruvate (1, 7). The ADP:O and inhibitor sensitivities previously published for P5C oxidation are similar to those for proline oxidation and are for the pH 6.4 P5C DH activity (7). Inasmuch as the pH 8 P5C DH activity is not measurable in intact mitochondria, ADP:O ratios cannot be determined. With ornithine, ADP:O ratios could not be accurately determined because of the low rates of ornithine-dependent O_2 uptake and interference from α-KG (which itself exhibits an ADP:O ratio).

Inhibitor sensitivities of ornithine oxidation could not be determined due to low specific activity in corn mitochondria. When substrate oxidation occurs at a rate considerably below the potential capacity of the electron transport chain, inhibitor studies are not relevant because electron flow occurs primarily through the remaining uninhibited capacity of the system. A similar response can be obtained with substrates that are oxidized with a high specific activity if the rates are determined under adverse conditions such as insufficient cofactor availability or measurement at pH values considerably different than the optimum.

P5C-dependent O_2 uptake (near pH 8 in swollen mitochondria) results primarily from NADH oxidation since glutamate oxidation is too slow under similar conditions. Thus, a comparison was made between the inhibitor sensitivities of exogenously added NADH and the oxidation of P5C (Table I). As can be seen, the oxidation of P5C was very sensitive to rotenone and SHAM in comparison with the oxidation of exogenously added NADH. Thus, electrons and protons from NADH produced by this P5C DH enter the respiratory chain through the endogenous NADH DH. In corn mitochondria, we consistently find that the oxidation of substrates having rotenone sensitivity show similar sensitivity

Table II. *Sidedness of Various Enzyme Active Sites on the Inner Mitochondrial Membrane*

Assays were conducted in 3.0 ml of 30 mM MTT (pH 8) containing 250 mM sucrose, 5 mM KH_2PO_4, 1 mM NaN_3, and 0.7 mM $K_3Fe(CN)_6$. Mitochondrial protein averaged 0.60 mg/assay. Data presented are the means of three separate experiments.

Substrate	Ferricyanide Reduction					
	Control Rate	0.5 μM Antimycin Sensitive	Inhibition	Control Rate	10 μM FCCP Sensitive	Inhibition
	nmol/min·mg protein		*%*	*nmol/min·mg protein*		*%*
10 mM Malate + 0.5 mM Glu	144	81.0	56	122	80.7	66
10 mM Succinate	228	189	83	270	159	59
10 mM L-Proline	159	151	95	159	80.9	51
10 mM DL-P5C (pH 6.4)	331	122	37	369	107	29

to SHAM. This includes the oxidation of malate, proline, and P5C (pH 6.4).

Sidedness on the Inner Mitochondrial Membrane. Since proline DH and the associated pH 6.4-P5C DH feed electrons and protons directly into the respiratory chain, we wanted to determine their sidedness on the inner mitochondrial membrane. To do this, we followed the sensitivity of substrate-dependent ferricyanide reduction to antimycin inhibition. Ferricyanide is an electron acceptor that cannot permeate the inner mitochondrial membrane. Thus, it can only accept electrons from enzymes exposed to the intermembrane region or from Cyt *c*, which is located within the outer phase of the inner membrane (6). Electrons that would normally flow to O_2 were shunted to ferricyanide by blocking Cyt oxidase with 1 mM NaN_3 (P5C interferes with KCN inhibition [7]). With enzymes exposed to the intermembrane region, ferricyanide reduction would be relatively insensitive to antimycin inhibition, inasmuch as these enzymes can reduce ferricyanide directly. Antimycin inhibits electron flow through the respiratory chain before Cyt *c* reduction. However, with enzymes exposed to the matrix, such as succinate and malate DH, ferricyanide reduction is sensitive to antimycin inhibition since this reduction can occur only by electron flow through Cyt *c*.

Therefore, the sidedness of an enzyme can be indicated by the amount of substrate-dependent ferricyanide reduction that is sensitive to antimycin inhibition (Table II). As with the oxidation of malate or succinate, we found that proline and P5C-dependent ferricyanide reduction showed considerable sensitivity to antimycin. This indicates that the active sites of proline DH and the pH 6.4-P5C DH are exposed to the matrix. If percent inhibition is compared between substrates, P5C-dependent ferricyanide reduction appears to be relatively insensitive to antimycin. This does not result from less inhibition by antimycin, but from a high control rate.

The use of this technique assumes that the enzyme is capable of reacting directly with ferricyanide and that antimycin only inhibits electron transport. This assumption is usually verified by disrupting the mitochondria to relieve the antimycin inhibition. We found that swelling the mitochondria (in the absence of antimycin) increased the rate of malate oxidation, but decreased the rates for the other substrates in Table II. When following the relief of antimycin inhibition by swelling, we observed increased rates of antimycin insensitive oxidation for all the substrates. However, since it was not possible to distinguish between rate changes resulting from swelling and those due to relief of antimycin inhibition, these data were not included. Instead, an independent technique using uncouplers was used to help establish the sidedness of these enzymes.

If a substrate is oxidized on the matrix side of the inner membrane, and if transport of that substrate requires the energy of a proton gradient, the substrate oxidation will be sensitive to FCCP (or other uncouplers). However, if the substrate is oxidized on the outer surface of the inner membrane (or if a proton gradient is not required for uptake), the addition of FCCP will have little effect (20). The results with FCCP correlate with those obtained using antimycin (Table II). Both proline and P5C-dependent ferricyanide reduction showed considerable sensitivity to FCCP. The percent inhibition with P5C again was influenced by the high control rate, and not by lack of inhibition by FCCP.

Ferricyanide reduction was not measurable in intact corn mitochondria with ornithine or P5C (pH 8) as substrates. Thus, these techniques could not be used to determine the sidedness of these enzymes.

CONCLUSIONS

The enzyme previously referred to as proline oxidase is bound to the inner mitochondrial membrane and is exposed to the matrix. Electrons and protons from proline enter the respiratory chain before the rotenone sensitive iron-sulfur proteins, with proline-dependent O_2 uptake, thus having an ADP:O ratio similar to that of malate + pyruvate. Proline-dependent O_2 uptake has a pH optimum of 7.2. Activity does not involve the reduction of soluble NAD, and therefore electrons and protons enter the respiratory chain directly, possibly through a flavoprotein. It is clear that proline-dependent O_2 uptake occurs through a proline dehydrogenase functionally linked to the respiratory chain, and not through a proline oxidase. Therefore, this activity should be referred to as proline dehydrogenase activity (8).

P5C derived from proline oxidation is oxidized via a P5C DH that has a pH optimum of 6.4. This P5C DH feeds electrons and protons directly into the respiratory chain and thus is bound to the inner mitochondrial membrane. Its active site is exposed to the matrix. This activity has an ADP:O ratio and inhibitor sensitivities similar to those of proline oxidation.

Ornithine-dependent O_2 uptake is measurable in corn mitochondria and occurs through an enzyme complex consisting of ornithine transaminase (pH optimum 8.4) and the pH 8-P5C DH. This complex is facing the matrix because NADH produced by this P5C DH is preferentially oxidized by the endogenous NADH DH.

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