Oxidative and Phosphorylative Activities of Mitochondria Isolated from Pea Root Tissues[1, 2]

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Introduction

The isolation of plant mitochondria which demonstrate respiratory control has been reported only recently (15, 16). Mitochondria have been isolated from root tissue of peas (13); however, these particulates have not been characterized with respect to their response to the phosphate acceptor system or to the metabolism of glutamate. This paper describes the properties of pea root mitochondria such as respiratory control and cofactor requirements as well as the probable pathway of glutamate oxidation.

Materials and Methods

Plant Material. Seeds of *Pisum sativum* L. var. Little Marvel, obtained locally, were surface sterilized by soaking 30 minutes in a 10 % Chlorox solution containing a pinch of Dreft detergent. The seeds were rinsed thoroughly and soaked 24 hours in water with aeration. Germination was carried out in the dark for 9 days at 22 ± 2° by placing the seeds on a perforated aluminum sheet supported above the surface of water in a stainless steel pan. Metal parts were sprayed with Krylon acrylic spray.

Isolation of Mitochondria. Approximately 120 g of roots was ground for 2 to 3 minutes in a chilled mortar with 10 g sand and 65 ml extraction medium containing 0.4 M sucrose, 5 mM EDTA, 6 mM $MgCl_2$, 4 mM cysteine, and 1 mM Tris, pH 7.2.

The brei was strained through muslin and the residue washed with an additional 130 ml of medium. The combined filtrates (200 ml) were centrifuged at 500 × *g* for 10 minutes. The supernatant fraction was centrifuged at 10,000 × *g* for 10 minutes. The mitochondrial pellet was washed twice by resuspension, with the aid of a loose-fitting Potter-Elvenjem homogenizer, in 40-ml medium containing 0.4 M sucrose, 6 mM $MgCl_2$, and 1 % bovine serum albumin, followed by recentrifugation at 10,000 × *g* for 10 minutes. The washed mitochondrial pellet was suspended in 6.5 ml of 0.4 M sucrose containing 6 mM $MgCl_2$ and 1 % bovine serum albumin. The preparation was carried out at 0 to 5°. The final mitochondrial suspension contained 0.36 to 0.71 mg mitochondrial nitrogen per ml. Mitochondria prepared in this manner stained supravitally with Janus Green B and appeared as typical plant mitochondria as observed with the phase microscope.

Nitrogen Analysis. An aliquot of the mitochondrial suspension was washed free of bovine serum albumin by centrifugation and resuspension in extraction medium. Analysis was by a semimicro Kjeldahl technique followed by nesslerization (14).

Respirometry. O_2 uptake, in short-term experiments, was measured polarographically using a Clark electrode and potentiometric recorder. For longer term experiments, standard manometric techniques were employed. In both cases the reactions were carried out at 30° in 2.0 ml of the standard reaction medium containing 0.4 M sucrose, 0.5 mM EDTA, 5 mM $MgCl_2$, 10 mM phosphate, and 10 mM Tris, pH 7.2. CO_2 evolution was measured by the Warburg direct method.

Measurement of Oxidative Phosphorylation. ATP formation was measured by including glucose and hexokinase in the reaction medium. After an incubation period of 30 minutes, an aliquot of the reaction mixture was added to a centrifuge tube containing 0.2 ml cold perchloric acid. The tubes were chilled, the precipitated protein spun down, and aliquots of the supernatant fluid were neutralized and analyzed for glucose-6-P by reduction of TPN in the presence of glucose-6-P dehydrogenase.

Radioisotope Techniques. $C^{14}O_2$ was collected by trapping in 1 M NaOH in the center well of the Warburg vessel. Upon completion of the incubation, the filter paper wick and washings from the center well were transferred to a 50 ml centrifuge tube containing 20 ml of distilled water. The tube was capped, shaken vigorously to disentegrate the paper,

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and allowed to stand overnight. Centrifugation removed the debris. After washing the residue the radioactive carbonate was precipitated as $BaCO_3$ by addition of $BaCl_2$. All $BaCO_3$ samples were plated on Celite-coated sintered steel planchets with the aid of a chimney funnel. Counting was done with an end-window Geiger-Müller tube.

Results

Preparation. The use of isolation media buffered with 10 mM phosphate, pH 7.2, resulted in preparations which showed high rates of substrate oxidation when DPN and cytochrome c were added, but exhibited no increase in rate upon addition of ADP. Mitochondria prepared by the standard procedure, as described, showed good respiratory control and were not dependent upon added cofactors. Most satisfactory results were obtained by omitting Tris and EDTA from all but the initial grinding medium. In order to obtain actively phosphorylating mitochondria, it was necessary to include bovine serum albumin in either the reaction mixture or the isolation medium.

Oxidation and Phosphorylation with Different Substrates. The highest respiratory rate was obtained with succinate (table I). High values for QO_2 were obtained for oxidation of L-glutamate and α-ketoglutarate with lower values for other substrates. Pyruvate gave low rates of oxidation which increased upon addition of tricarboxylic acid cycle intermediates. L-Alanine provided some enhancement of O_2 uptake while oxaloacetate, L-aspartate, and γ-aminobutyrate provided none.

Table I. *P/O Ratios and Oxidation of Several Substrates by Pea Root Mitochondria*

The reaction mixture contained 2.0 ml of the standard reaction medium and 1 mM DPN, 1 mM ADP, 0.05 mM cytochrome c, and 3 mg/ml bovine serum albumin. The substrate concentrations were 20 mM. The QO_2 was measured as μl O_2/mg N per hour.

Substrate	QO_2	P/O
Alpha-ketoglutarate	390	1.54
Citrate	240	1.46
Succinate	540	0.81
Fumarate	250	...
Pyruvate	150	...
Pyruvate + L-Malate	309	...
L-Glutamate	350	1.61
L-Glutamine	225	...
L-Alanine	165	...
Endogenous	53	...

The highest P/O ratios were obtained for the pyridine nucleotide-linked substrate oxidations. The inclusion of 10 mM NaF, as an adenosine triphosphatase inhibitor, had little effect upon the P/O ratios and was inhibitory to O_2 uptake.

DNP enhances O_2 uptake, presumably by a removal of the regulatory influence of phosphate and phosphate acceptor upon the rate of oxidation. Phosphorylation is inhibited resulting in a low P/O ratio (table II).

Respiratory Control. The oxidation of L-glutamate as well as of succinate was increased at least 2-fold upon addition of ADP (figure 1). Respiration continued at this higher rate until the added ADP became phosphorylated then the rate of oxidation decreased. Further additions of ADP resulted in similar cycles of rapid and slow respiration. The low respiratory control ratios and the rather high state 4 respiratory rate (6) indicate that oxidation is rather loosely coupled to phosphorylation in these mitochondria. Calculation of the ratio of added ADP to the increment of O_2 uptake results in values of ADP/O near 2 for glutamate and above 1 for succinate, values in agreement with the ratios of P/O. It was found that 40 % of the O_2 uptake during succinate oxidation was not inhibited by 1.0 mM KCN. This cyanide resistant respiration may explain the high state 4 rate as well as the below theoretical values obtained for P/O and ADP/O.

Addition of ATP had no effect upon the state 4 rate of succinate oxidation suggesting that the respiratory rate was actually controlled by the rate-limiting ADP concentration rather than by oxaloacetate inhibition. Such inhibition has been observed by Wiskich and Bonner (15) in plant mitochondrial preparations.

Cofactor Requirements. Unlike many plant mitochondrial preparations, no respiratory stimulation could be obtained by addition of pyridine nucleotides, cytochrome c, or several other cofactors in short-term incubations. In experiments of 30 minutes duration or longer, however, addition of DPN and cytochrome c was necessary to maintain maximal rates of

Table II. *Effect of DNP Upon Oxidation and Phosphorylation by Pea Root Mitochondria*

The reaction mixture contained the same reactants as listed in table I. L-Glutamate concentration was 20 mM.

DNP Conc (mM)	O_2 uptake (μ atoms/mg N per hr)	Glucose-6-P (μmoles/mg N per hr)	P/O
None	43.2	69.5	1.61
0.05	80.0	61.5	0.77
0.10	79.0	47.4	0.60
0.50	49.5	13.8	0.28

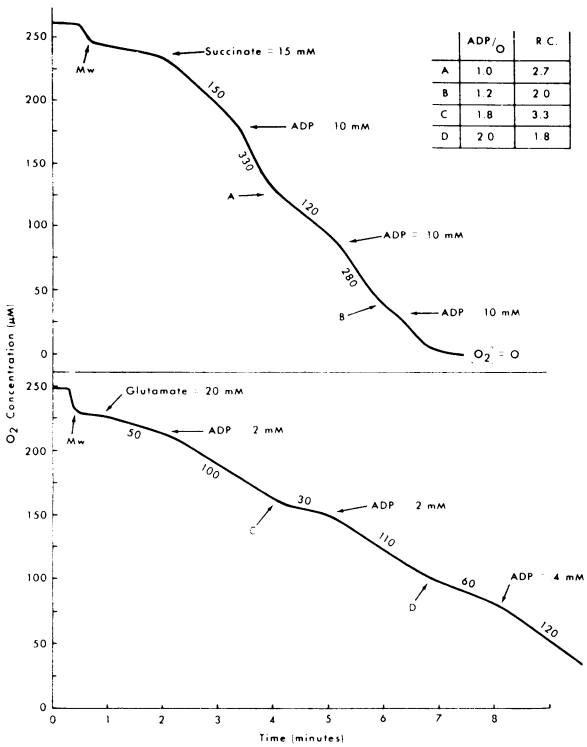

FIG. 1. Respiratory control during oxidation of succinate and L-glutamate by pea root mitochondria. Assay in 2.0 ml of the standard reaction medium at 30°. Rates expressed as mμmoles O_2/minute. Additions are shown as final concentrations. Mw indicates addition of mitochondria.

α-ketoglutarate or L-glutamate oxidation. Mitochondria which had been preincubated in the standard reaction medium without substrate for 30 minutes at 30°, exhibited a 50 % and 30 % increase in the rate of glutamate oxidation upon addition of DPN and cytochrome c respectively.

Magnesium stimulated the oxidation of L-glutamate having an optimum concentration of 5 mM. In view of the presence of zinc in beef liver glutamic dehydrogenase (1) and reports that manganese could serve as a cofactor for glutamate oxidation in rat liver mitochondria (10), the effects of these ions were also investigated. Manganese, at 1 mM, was inhibitory in the absence of magnesium, but had little effect in the presence of magnesium. Zinc, at 1 mM, was inhibitory in both the presence and absence of magnesium.

Oxidation of Glutamate. As noted in table I, L-glutamate was readily oxidized resulting in a high QO_2. This rate of oxidation, higher than that previously reported for pea root mitochondria (13), is near that of α-ketoglutarate and higher than those of the other amino acids investigated. The oxidation of L-glutamate followed Michaelis-Menten kinetics

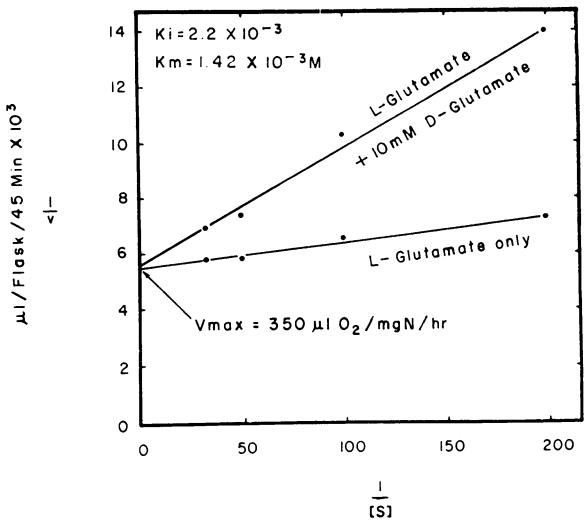

FIG. 2. Competitive inhibition of L-glutamate oxidation by D-glutamate. The reaction mixture contained the same reactants as listed in table I. L-Glutamate concentration was 20 mM.

(fig 2) with a Km of 1.42×10^{-3} M. A L-glutamate concentration of 30 mM resulted in a QO_2 approaching V_{max}. The reaction was stereo-specific as D-glutamate acted as a competitive inhibitor of L-glutamate oxidation, with a K_i of 2.2×10^{-3} (fig 2). Competitive inhibition by D-glutamate has been reported for carrot root discs (9) as well as plant mitochondria (8).

Respiratory Quotients. The values for RQ obtained for α-ketoglutarate and L-glutamate oxidation varied somewhat, but the value for glutamate was consistently lower than that obtained for α-ketoglutarate (table III). These figures indicate that

Table III. *Respiratory Quotients for Mitochondrial Oxidation*

The reaction mixture contained the same reactants as listed in table I. The substrate concentrations were 20 mM.

Substrate	Respiratory quotient*	
	Theoretical	Observed
L-Glutamate	0.5**	0.35
a-ketoglutarate	0.67	0.75
Succinate	0.0	0.0

* Theoretical values are based upon oxidation of the substrate to the oxaloacetate stage. Observed values were calculated by subtraction of endogenous values.

** This value is for the glutamic dehydrogenase pathway. If oxidation proceeds via transamination, the RQ would be the same as that for *a*-ketoglutarate.

for L-glutamate oxidation more O_2 is utilized per mole of substrate oxidized. This additional utilization may result from the oxidative deamination of glutamate to α-ketoglutarate.

It is likely that under the conditions of the present experiments, the mitochondria did not oxidize substrates beyond the oxaloacetate stage. This is suggested by the absence of CO_2 evolution during succinate oxidation, producing a RQ of O, as well as by the failure of oxaloacetate to stimulate respiration.

Pathway of L-Glutamate Oxidation. Arsenite is known to inhibit oxidation of keto acids by binding lipoic acid (2). At an arsenite concentration of 1.0 mM, α-ketoglutarate oxidation is completely inhibited. Glutamate oxidation is inhibited only 70 % whereas the $C^{14}O_2$ released from oxidation of glutamate-1-C^{14} is inhibited 95 %. This suggestion that O_2 uptake results from the conversion of glutamate to α-ketoglutarate is confirmed by the respiratory stimulation observed upon addition of glutamate to an arsenite-inhibited system (fig 3).

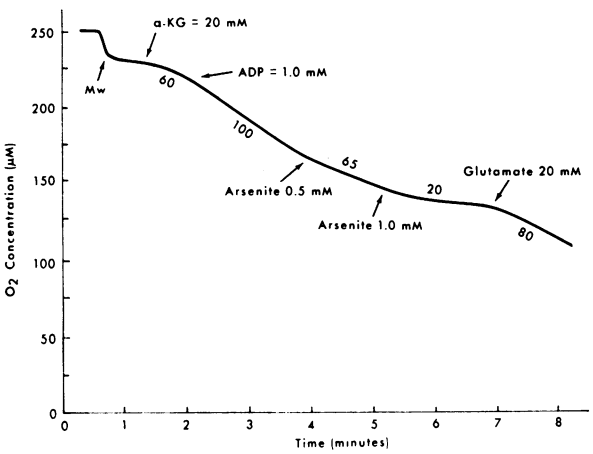

FIG. 3. Stimulation of O_2 uptake upon addition of L-glutamate to an arsenite-inhibited system. Assayed in 2.0 ml of the standard reaction medium at 30°. Rates expressed as mμmoles O_2/minute. Additions are shown as final concentrations. Mw indicates addition of mitochondria. α-KG designates α-ketoglutarate.

Discussion

The inclusion of phosphate in the homogenization medium seemed to prevent the preparation of coupled mitochondria showing respiratory control, even though high rates of substrate oxidation could be obtained with added cofactors. Since phosphate can cause swelling and uncoupling in mitochondria under certain conditions (11), it seems likely that its use in the medium caused damage to the phosphorylation mechanism. These results suggest that in earlier reports, the respiratory stimulation observed, by the use of phosphate in the grinding medium (3, 12), may have been obtained at the expense of phosphorylation. This view would be supported by the low P/O ratios reported in these instances (3, 4).

It is well known that the phosphate acceptor system can regulate the rate of mitochondrial respiration. In the present experiments, O_2 uptake increased 2-fold upon addition of ADP to a system containing oxidizable substrate and Pi. Respiration continued at this increased rate until the added ADP had been phosphorylated then the rate decreased. The ADP/O and respiratory control ratios obtained indicate rather loose coupling of phosphorylation to oxidation and suggest that this mechanism may play a role in the regulation of root respiration.

The initial reaction in the oxidation of glutamate is conversion to α-ketoglutarate, which may occur via either transamination or oxidative deamination. In certain animal mitochondria, transamination is the principal pathway of conversion to α-ketoglutarate (5). In contrast, Das and Roy (7) have shown that glutamic dehydrogenase is the predominant pathway of oxidation in mitochondria isolated from cow peas. In the present experiments, arsenite was shown to inhibit respiration with glutamate to a lesser extent than with α-ketoglutarate, whereas the amount of $C^{14}O_2$ obtained from glutamate-1-C^{14} was inhibited almost completely. The latter result would be expected in the presence of arsenite since the first carbon atom of glutamate is released as CO_2 during the oxidative decarboxylation of α-ketoglutarate. The lower value for RQ obtained with glutamate as compared to α-ketoglutarate as well as the stimulation of O_2 uptake upon addition of glutamate to an arsenite inhibited system, further indicates that O_2 is utilized in the conversion of glutamate to α-ketoglutarate. This utilization probably results from reoxidation of DPNH produced in the glutamic dehydrogenase pathway.

Summary

Oxidative activity and oxidative phosphorylation were studied in mitochondria isolated from root tissue of pea seedlings (*Pisum sativum* L.). The highest respiratory rates were obtained with succinate, α-ketoglutarate, and L-glutamate.

Addition of adenosine diphosphate increased rates of oxidation 2-fold. Phosphorylation of the added adenosine diphosphate produced a decrease in rates characteristic of loose coupling of oxidation to phosphorylation. ADP/O ratios of 1 and 2 were obtained for succinate and glutamate. Values for P/O were also obtained. Dinitrophenol uncoupled phosphorylation with stimulation of oxygen uptake.

The oxidation of L-glutamate by root mitochondria was stereo-specific and followed Michaelis-Menten kinetics. Studies with arsenite and values obtained for RQ indicate that conversion to α-ketoglutarate via glutamic dehydrogenase is the initial reaction in the oxidation of glutamate.

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