# OXALACETATE CONTROL OF KREBS CYCLE OXIDATIONS IN PURIFIED PLANT MITOCHONDRIA

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## SUMMARY

Plant mitochondria survive separation on sucrose gradients and subsequent dilution to iso-osmolar conditions. Oxalacetate penetrates these remarkably uniform and intact preparations, and inhibits all Krebs cycle oxidations. With the exception of succinate these inhibitions are caused by oxidation of a common pool of NADH, reduced by dehydrogenases, during conversion of added oxalacetate to malate.

## INTRODUCTION

Oxalacetate is one of a small group of metabolites which serve as branch points in the complex region of interactions between the pathways of carbohydrate, amino acid, fatty acid and oxidative metabolism (1). In animal tissues, the rate of entry of oxalacetate into mitochondria is restricted (2,3) and is in fact under strict control. No direct measurements of the passage of oxalacetate through the plant mitochondrial membrane have so far been reported. The present work examines the permeability of plant mitochondria for this dicarboxylic acid.

## METHODS

The plant material used in these experiments included potato tubers (Solanum tuberosum) and etiolated mung bean hypocotyls (Phaseolus aureus). Mitochondria were prepared by methods that have been described previously (4). The washed mitochondria preparations were applied to the tops of sucrose gradients containing 0.1% BSA and 10 mM phosphate buffer, pH 7.2 (0.6-1.8 M in 0.3 M steps). After centrifugation in a swinging bucket rotor for 45 min at 40,700 x g (Beckman model L preparative centrifuge, SW 25:1 rotor) the mitochondria were found at the boundary between 1.2 M and 1.5 M sucrose. They were diluted slowly at 0° C with 10 mM phosphate buffer containing 0.1% BSA until a sucrose concentration of 0.3 M was achieved. After centrifugation (9000 x g, 15 min) the purified mitochondria (Mp) were suspended in a small volume of 0.3 M sucrose containing 10 mM phosphate buffer, pH 7.2 (100 mg protein per ml).

Oxygen uptake was measured at 25° C in a 3 ml stirred cell using a Clark oxygen electrode (Yellow Springs Instrument Company) as described by Estabrook (5). The reaction medium (medium A) contained: 0.3 M mannitol or sucrose, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer (pH 7.2) and 0.5-4.0 mg of mitochondrial protein.

The redox level of the endogenous NAD (membrane bound) was monitored fluorometrically (6) with an Eppendorf fluorometer equipped with a 366 nm primary filter for excitation and a secondary filter transmitting between 400 and 3000 nm to pass the emitted light. The reaction medium was medium A (final volume 3 ml).

Succinate cytochrome c oxidoreductase (EC 1.3.99.1) was assayed in the following medium: 5 mM phosphate buffer (pH 7.2), 0.05 mM cytochrome c, 1 mM KCl and 0.1-1.0 mg of mitochondrial protein. The reaction was initiated with 10 mM succinate. The final volume was 3 ml. The cytochrome c reduction was measured in a Zeiss spectrophotometer using the millimolar absorbance coefficient at 550 nm of 21.0 $mM^{-1} \times cm^{-1}$.

Oxalacetate and L-malate were assayed by the method of Williamson and Corkey (7). Mitochondrial protein was measured by a modified Lowry method (8) with crystallized bovine serum albumin (Miles Laboratories, Inc.) as the standard.

## RESULTS AND DISCUSSION

Purification of the mitochondria results in improved respiratory control and ADP:O ratios as well as marked increase in state 3 oxidation rates and carrier concentrations. In potato, this increase amounts to 1.7 times and is 3.0 times in mung bean. In the purified mung bean we regularly find succinate state 3 oxidation rates between 460 and 490 nmoles/$O_2$/min/mg protein. One integrity assay is used to confirm the intactness of the purified mitochondria (Mp). This assay of succinate: cytochrome c reductase shows the appropriate activity only if the outer membrane is damaged and the added cytochrome c has access to the outer surface of the inner membrane (9); the amount of activity is related directly to the extent of outer membrane damage. This integrity assay showed negligible activities with intact purified mitochondria, bursting the mitochondria in zero osmolar medium released maximal activities (Fig. 1). The integrity of the purified preparations is confirmed by electron micrographs which show only intact mitochondria.

In contrast to animal systems, oxalacetate quickly penetrates into the plant mitochondria. This penetration is demonstrated in Fig. 2A where addition to state 4 mitochondria supplemented with substrate causes immediate oxidation of pyridine nucleotide. With time the pyridine nucleotide goes reduced. The addition of two times the initial amount of oxalacetate again

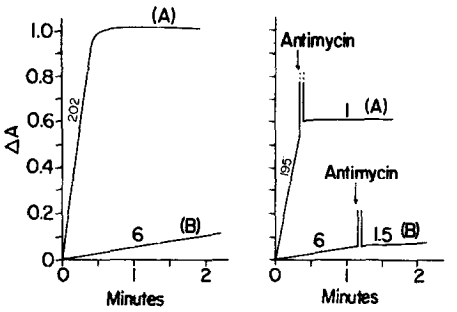


FIGURE 1. Succinate: cytochrome c oxidoreductase activity of purified potato mitochondria (Mp). (A) Burst mitochondria in 5 mM phosphate buffer. (B) Intact mitochondria in 5 mM phosphate buffer containing 0.3 M sucrose. Antimycin A (0.5 μg/ml) was added as indicated. The number on the traces refer to nmoles cytochrome c reduced per min per mg mitochondrial protein.

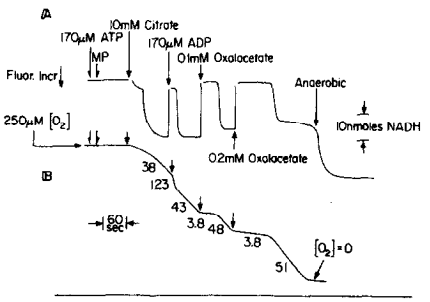


FIGURE 2. (A) Effect of oxalacetate on the oxidation state of endogenous NAD in purified mung bean mitochondria oxidizing citrate. (B) Effect of oxalacetate on citrate oxidation. The arrows correspond to the additions in (A), above. The numbers on the trace refer to nmoles $O_2$ consumed per min per mg mitochondrial protein.

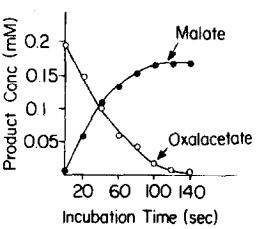


FIGURE 3. Accumulation of malate and disappearance of oxalacetate during citrate oxidation by purified mung bean mitochondria. The reaction mixtures and conditions were as in Fig. 2.

causes pyridine nucleotide oxidation, and the time period during which oxidation persists is two times the first one. The effect of oxalacetate on citrate oxidation is shown in Fig. 2B. This figure shows that upon addition of oxalacetate to mitochondria respiring in state 4 a clear inhibition of the respiration rate occurs which is gradually reversed. The inhibition is illustrated in state 4, but it occurs also in state 3 or in the presence of an uncoupler. Enzymatic analysis has shown that during the time of inhibition the oxalacetate is converted to malate (Fig. 3), a conversion that is dependent on the reduced pyridine nucleotide generated by the isocitric dehydrogenase. When the oxalacetate concentration becomes very low, the pyridine nucleotide becomes reduced and the inhibition is released. We have found that the same mechanism of oxalacetate inhibition pertains for the oxidation of pyruvate, α-ketoglutarate, glutamate and malate. In the case of the malate oxidation (Fig. 4) the reduced pyridine nucleotide is generated by the NAD-linked malic enzyme (10,11).

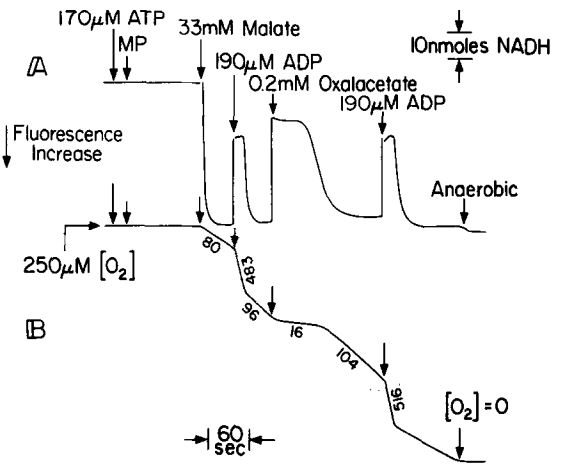


FIGURE 4. (A) Effect of oxalacetate on the oxidation state of endogenous NAD in purified mung bean mitochondria oxidizing malate. (B) Effect of oxalacetate on malate oxidation. The arrows correspond to the additions in (A), above. The numbers on the trace refer to nmoles $O_2$ consumed per min per mg mitochondrial protein.

During the course of the succinate oxidation by intact mitochondria the oxalacetate inhibition of succinic dehydrogenase is a competitive one; the inhibition is transient only in state 4 where the reduced NAD produced by reversed electron transport is used to remove the oxalacetate (Fig. 5).

These results show clearly that in plants the oxalacetate readily goes through the inner mitochondrial membrane. Anaerobiosis (Fig. 5) or the

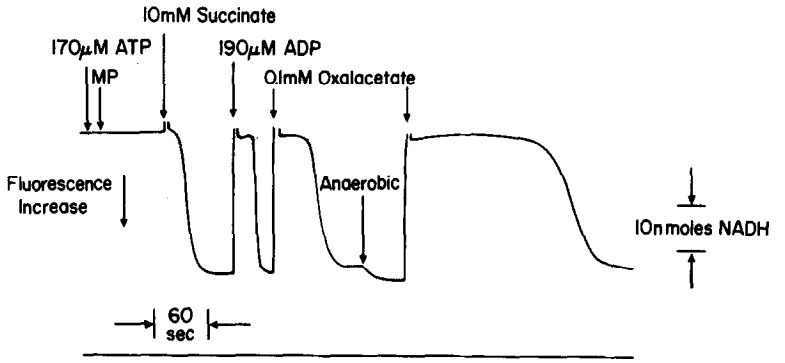


FIGURE 5. Effect of oxalacetate on the oxidation state of endogenous NAD in purified mung bean mitochondria oxidizing succinate.

presence of uncoupler in the incubation medium does not inhibit oxalacetate entry. This conclusion is in agreement with the fact that in plant mitochondria the space between the outer and the inner membrane is devoid of malate dehydrogenase activity, this enzyme being specifically localized inside the matrix (11).

Under the conditions described above we have shown that the addition of oxalacetate to intact animal mitochondria respiring in state 4 (rat and guinea pig liver, frog muscle and pigeon heart) is unable to cause immediate oxidation of pyridine nucleotide.

The oxalacetate inhibition of NAD-linked dehydrogenases is exerted through control of the oxidation state of the common NAD pool and undoubtedly represents a powerful mechanism for control of electron flow in the mitochondria of intact plant cells. The same oxalacetate control has been found in purified mitochondria prepared from cauliflower buds, jerusalem artichokes, skunk cabbage spadices and watermelon rinds.

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