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# MALIC ENZYME ACTIVITY AND CYANIDE-INSENSITIVE ELECTRON TRANSPORT IN PLANT MITOCHONDRIA

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SUMMARY. In the presence of exogenous $NAD^+$, malate oxidation by cauliflower mitochondria takes place essentially via an electron transport pathway that is insensitive to rotenone, antimycin and cyanide but is strongly sensitive to salicyl hydroxamic acid. It bypasses all phosphorylation sites. $NAD^+$ is reduced by an enzyme identified as malic enzyme (L-malate:NAD oxidoreductase (decarboxylating), EC 1.1.1.39). The NADH produced is reoxidized by an internal rotenone-insensitive NADH dehydrogenase that yields electrons directly to the cyanide-insensitive pathway.

## INTRODUCTION

It is currently accepted that the oxidation of malate by plant mitochondria is carried out through the action of two different enzymes: malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) and malic enzyme (L-malate:NAD oxidoreductase (decarboxylating), EC 1.1.1.39) (1,2,3). In plant mitochondria, malic enzyme prefers $NAD^+$, rather than $NADP^+$, as an electron acceptor (4). Depending on the mitochondrial environment (pH, cofactors, etc...) malate oxidation occurs through one or both pathways (1,4,5). In particular, direct measurements of the reaction products have shown that in cauliflower mitochondria (1), as also in other plant mitochondria (2,3), the major product of malate oxidation in the presence of added $NAD^+$ is not oxaloacetate but, instead, pyruvate. Under these conditions pyruvate is formed through an $NAD^+$-dependent malic enzyme (1,2,3,4).

On the other hand, it is also well known that besides having a normal cytochrome pathway, most plant mitochondria possess a non-phosphorylating, cyanide-insensitive, alternate electron transport pathway that can be specifically inhibited by hydroxamic acid derivatives (6). This paper reports

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on the observation that malate oxidation through the action of malic enzyme is preferentially mediated by the cyanide-insensitive, alternate pathway.

## MATERIALS AND METHODS

Mitochondria from cauliflower buds (*Brassica oleracea* L.) were isolated and subsequently purified by density gradient centrifugation as already described (7,8). Oxygen uptake was measured at 25°C using a Clark oxygen electrode in a 4 ml-cell containing: 300 mM Mannitol, 5 mM $MgCl_2$, 10 mM KCl, 1 g/l bovine serum albumin, 10 mM phosphate buffer (pH 7.2) and approximately 1 mg/ml mitochondrial protein (7). Reduction of $NAD^+$ was followed at 340 nm using the same medium as above in the presence of 600 µM KCN and 600 µM SHAM ( salicyl hydroxamic acid) to prevent reoxidation by the cytochrome and the alternate pathways. Mitochondrial proteins were determined by mineralization and nesslerization.

## RESULTS

In the absence of any added cofactor purified cauliflower mitochondria readily oxidize malate (Fig. 1A). Electron transport is linked to the

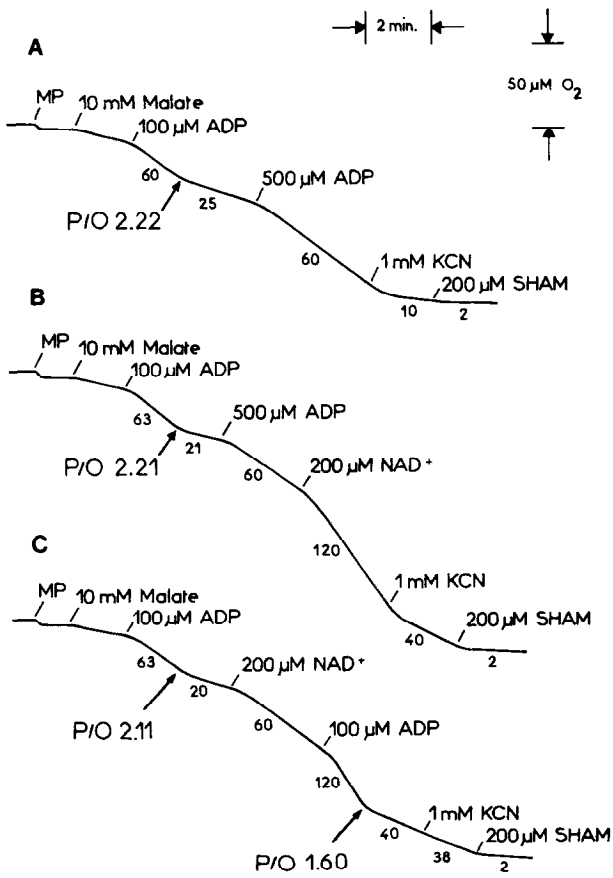


Fig. 1 Effect of exogenous $NAD^+$ on malate oxidation by purified cauliflower mitochondria. The numbers on the traces refer to nmoles $O_2$ consumed per min per mg protein.

synthesis of ATP. ADP/O ratios with this substrate are significantly higher than 2, indicating a satisfactory coupling between electron transport and phosphorylation. Addition of 1 mM cyanide to mitochondria oxidizing malate in state 3 strongly inhibits electron transport (83 %) and the subsequent addition of 200 μM SHAM brings the inhibition level to 97 %.

The addition of $NAD^+$ to mitochondria in state 3 markedly increases (+ 100 %) the rate of malate oxidation (Fig. 1B) and the rate of electron transport in the presence of cyanide is increased four times. As in Fig. 1A, the addition of SHAM almost completely inhibits all cyanide-insensitive electron transport. Addition of $NAD^+$ to mitochondria in state 4 brings about an increase of 200 % in the rate of oxidation (Fig. 1C). Then, the addition of a limiting amount of ADP (100 μM) gives a state 3/state 4 transition with an ADP/O ratio of 1.60. ADP/O ratios measured in the presence of $NAD^+$ are thus significantly lower than in its absence (Fig. 1C). The rate in the presence of cyanide is the same as in Fig. 2B.

From these results it is clear that the increment in malate oxidation brought about by the addition of exogenous $NAD^+$ is linked to the alternate pathway, as shown by the increase in cyanide resistance and the lowering of ADP/O ratios. The decrease of ADP/O ratios in the presence of exogenous $NAD^+$ has been explained by a switch of the electron path from an internal NADH dehydrogenase, linked to three phosphorylation sites, to an external NADH dehydrogenase linked to only two phosphorylation sites (2,9). Participation of the alternate cyanide-insensitive pathway seems to offer an equally good explanation, since it appears that the oxidation of malate via malate dehydrogenase is highly sensitive to cyanide (Fig. 1A) whereas the oxidation involving malic enzyme is strongly cyanide-resistant (Fig. 1B and C).

Experiments in the presence of various electron transport inhibitors point to the same conclusions. While rotenone has a strong effect on the oxidation of malate through malate dehydrogenase, it has only slight effect in the

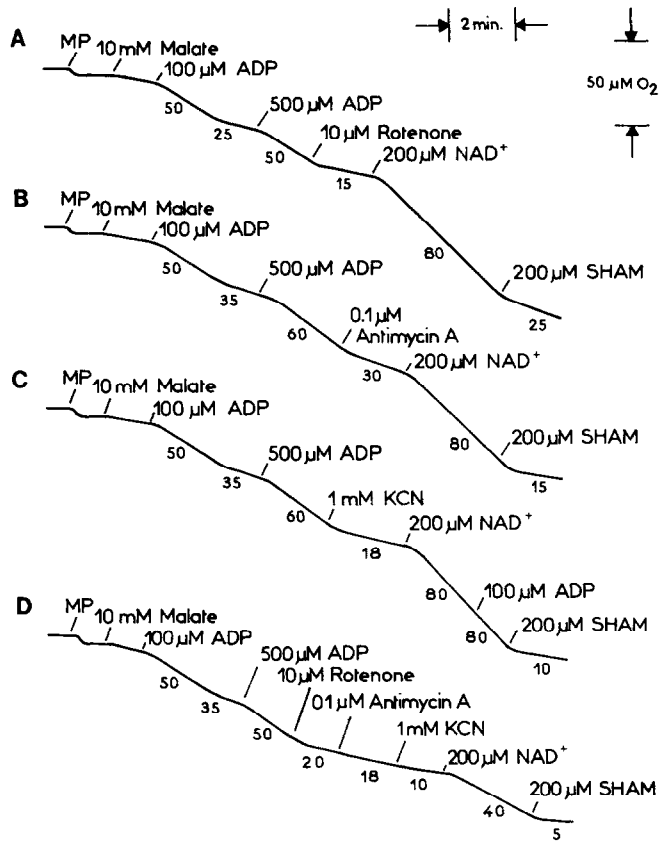


Fig. 2 Effect of exogenous $NAD^+$ on malate oxidation by purified cauliflower mitochondria in the presence of respiratory chain inhibitors. The numbers on the traces refer to nmoles $O_2$ per min per mg protein.

presence of added $NAD^+$, when oxidation takes place through malic enzyme (Fig. 2A). As a matter of fact, the rate in the presence of $NAD^+$ and rotenone is well above the state 3 rate and it amounts to 66 % of the rate in the absence of rotenone (comparison of Fig. 2A and 1B).

The same is true if electron transport is inhibited by antimycin (Fig. 2B) or cyanide (Fig. 2C). Furthermore, the oxidation of malate does not respond to addition of ADP in the presence of cyanide and added $NAD^+$, whereas it is sensitive prior to the addition of the latter compounds (Fig. 2C). Finally, provided $NAD^+$ has been added, oxidation of malate takes place even in the presence of inhibitors of complexes I, II and III (rotenone, antimycin and cyanide, respectively) which are known to abolish all electron transport from

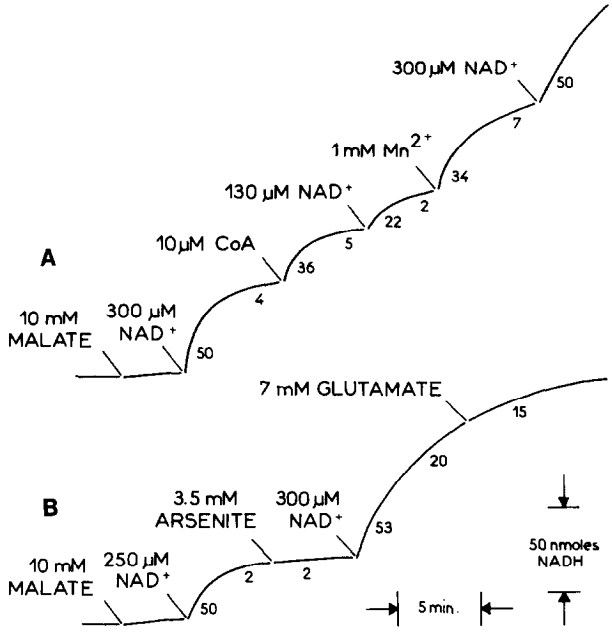


Fig. 3 Effects of malic enzyme cofactors (A) and malate dehydrogenase effectors (B) on the reduction of $NAD^+$ by purified cauliflower mitochondria oxidizing malate. The numbers on the traces refer to nmoles $NAD^+$ reduced per min per mg protein. The reaction mixtures and conditions are described in the text.

malate to oxygen through malate dehydrogenase, NADH dehydrogenase and the cytochrome system. Moreover, under these conditions, the rate of oxidation is still about 75 to 80 % of the state 3 rate in the absence of any inhibitor (Fig. 2D). Finally, this $NAD^+$-dependent oxidation of malate is strongly inhibited by SHAM.

A further evidence that malate is oxidized by malic enzyme in the presence of added $NAD^+$ is given in Fig.3. If, under appropriate conditions, one follows the reduction of $NAD^+$, one can see that cofactors which are known to stimulate malic enzyme, such as $Mn^{2+}$ ions and CoA (1,10) strongly increase the rate of reduction of $NAD^+$ (Fig. 3A). On the other hand, effectors that would directly (glutamate) or indirectly (arsenite) modulate the activity of malate dehydrogenase by acting on the level of oxaloacetate (5,11) have no action at all (Fig. 3B).

## DISCUSSION

Addition of $NAD^+$ to plant mitochondria oxidizing malate readily switches the path of malate oxidation from malate dehydrogenase to malic enzyme, as can be seen from direct measurement of the products of malate oxidation (2,4,12). All the above results point to the fact that the electron transport linked to the activity of the malic enzyme is essentially mediated by the cyanide-insensitive pathway and is inhibited by SHAM. Conversely, electron transport linked to the activity of malate dehydrogenase occurs via the cytochrome pathway and is inhibited by rotenone, antimycin and cyanide.

The main features of malate oxidation by malic enzyme are its insensitivity to all electron transfer inhibitors, except SHAM, and the absence of connection with any phosphorylation site (at least in the presence of inhibitors). This suggests that the NADH produced by malic enzyme is reoxidized by an electron carrier (presumably a flavoprotein NADH dehydrogenase) which bypasses phosphorylation site I. The nature of this NADH dehydrogenase is not clear at the moment. However, if one admits that malic enzyme is located in the mitochondrial matrix (13) and that $NAD^+$ slowly permeates the inner membrane (14), it may be surmised that such a rotenone-insensitive NADH dehydrogenase is located on the inner face of the inner membrane, as some other results also tend to suggest (9,15).

As a consequence, the above results indicate that the rotenone-sensitive NADH dehydrogenase of the inner mitochondrial membrane (12) does not participate in the electron transfer linked to the oxidation of malate via malic enzyme. The same is true of the rotenone-insensitive NADH dehydrogenases located respectively on the inner (16) and on the outer (8) membranes. Their participation would therefore lead, directly or indirectly, to a cyanide-sensitive electron transport (17), which, however, is not the case.

Finally, these results also suggest that slight changes in metabolic conditions (*e.g.* presence of $NAD^+$) can unveil a latent cyanide-insensitive electron

transport system. Similar events (malate and $NAD^+$ accumulation) accompanying changes in respiration are also observed during storage or aging of some plants tissues (18,19).

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