Plant Physiol. (1980) 66, 457–462
0032-0889/80/66/0457/06/$00.50/0

Malate Oxidation in Plant Mitochondria via Malic Enzyme and the Cyanide-insensitive Electron Transport Pathway[1]

Received for publication January 11, 1980 and in revised form April 14, 1980

PIERRE RUSTIN, FRANÇOIS MOREAU, AND CLAUDE LANCE
Université Pierre et Marie Curie, Laboratoire de Biologie Végétale IV, 12 rue Cuvier, 75005 Paris, France

ABSTRACT

Malate oxidation in plant mitochondria proceeds through the activities of two enzymes: a malate dehydrogenase and a NAD^+-dependent malic enzyme. In cauliflower, mitochondria malate oxidation via malate dehydrogenase is rotenone- and cyanide-sensitive. Addition of exogenous NAD^+ stimulates the oxidation of malate via malic enzyme and generates an electron flux that is both rotenone- and cyanide-insensitive. The same effects of exogenous NAD^+ are also observed with highly cyanide-sensitive mitochondria from white potato tubers or with mitochondria from spinach leaves. Both enzymes are located in the matrix, but some experimental data also suggest that part of malate dehydrogenase activity is also present outside the matrix compartment (adsorbed cytosolic malate dehydrogenase ?). It is concluded that malic enzyme and a specific pool of NAD^+/NADH are connected to the cyanide-insensitive alternative pathway by a specific rotenone-insensitive NADH dehydrogenase located on the inner face of the inner membrane. Similarly, malate dehydrogenase and another specific pool of NAD^+/NADH are connected to the cyanide- (and antimycin-) sensitive pathway by a rotenone-sensitive NADH dehydrogenase located on the inner face of the inner membrane. A general scheme of electron transport in plant mitochondria for the oxidation of malate and NADH can be given, assuming that different pools of ubiquinone act as a branch point between various dehydrogenases, the cyanide-sensitive cytochrome pathway and the cyanide-insensitive alternative pathway.

The mechanisms by which malate is oxidized are distinctive features of plant and animal mitochondria. In animal mitochondria, the oxidation of malate is extremely slow and requires the presence of other substrates, such as pyruvate or glutamate (12). On the other hand, plant mitochondria readily oxidize malate with no need for any additional factors (2, 12, 16). In recent years, it has become more evident that malate oxidation in plant mitochondria is a complex process. At least two mitochondrial enzymes, MDH[2] and NAD^+-dependent ME, contribute to the oxidation of this substrate (4, 6, 16). Recently, an emphasis was put on the participation of ME. The balance between the two enzyme systems competing for malate oxidation may be controlled by various internal or external factors, such as intramitochondrial pH or addition of cofactors (4, 15, 16). To make things still more intriguing, it was recently shown (22) that the oxidation of malate via malic enzyme essentially proceeds through the cyanide-insensitive alternative electron transport pathway, whereas the oxidation of malate via MDH appears strongly linked to the cyanide-sensitive Cyt pathway.

This paper reports on different aspects of malate oxidation in plant mitochondria. It also shows that different pools of NAD^+/NADH are present, one of them being closely linked to the cyanide-insensitive pathway through a specific NADH-dehydrogenase and a specific pool of ubiquinone.

MATERIALS AND METHODS

Preparation of Mitochondria. Mitochondria from cauliflower buds (*Brassica oleracea* L.) and potato tubers (*Solanum tuberosum* L.) were isolated according to Lance (10) with some minor modifications. Tissues (750 g) were homogenized for 7 s in a Waring Blendor in 1.5 liters ice-cold medium consisting of 0.3 M mannitol, 1 mM EDTA, 4 mM cysteine, 1 g/l BSA. The pH was adjusted at 7.5 with 100 mM NaOH. The crude extract was quickly filtered through a nylon net, the pH was brought to 7.3, and the filtrate was centrifuged at 650*g* during 15 min. The supernatant was further centrifuged at 9,000*g* for 20 min. The pellet of crude mitochondria was washed with the same medium minus cysteine and centrifuged again for 10 min, first at 270*g* and then at 9,000*g*. The washed mitochondria were subsequently purified on a discontinuous sucrose density gradient. The gradient was made of four layers of 0.9, 1.2, 1.5, and 1.8 M sucrose in 10 mM phosphate buffer (pH 7.2) and centrifuged at 35,000*g* for 1 h. Purified mitochondria were collected from the gradient (interface, 1.2:1.5 M), diluted to 0.3 M, and centrifuged again at 9,000*g* for 15 min.

Mitochondria from spinach leaves (*Spinacia oleracea* L.) were isolated using the medium described by Douce *et al.* (9) and made of 30 mM morpholinopropanesulfonic acid (pH 7.5), 0.3 M mannitol, 1 mM EDTA, 4 mM cysteine, 6% PVP, and 2 g/l BSA. Spinach leaves (500 g) were chopped with scissors and homogenized at low speed in a Waring Blendor in 1.5 liters of the medium described above. Spinach mitochondria were used without any further purification since such procedures are still not too satisfactory for mitochondria from green leaves.

Oxygen Uptake and Protein Determination. Oxygen uptake was measured at 25 C in a 4-ml cell using a Clark O_2 electrode in a medium consisting of 10 mM phosphate (pH 7.2), 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 1 mg/ml BSA, and approximately 1 mg/ml mitochondrial protein. Mitochondrial protein was determined by mineralization and nesslerization. The amount of protein for spinach leaves mitochondria was corrected for the contamination by broken thylakoids, assuming a protein to Chl ratio of 7 (13). Chl was determined according to McKinney (14).

Assays of Enzymes. NAD^+-dependent ME (EC 1.1.1.39) was measured spectrophotometrically by following the reduction of NAD^+ at 340 nm in the following medium: 100 mM phosphate (pH 6.8), 30 mM malate, 0.5 mM NAD^+, and 1 mM $MnCl_2$. The reaction was started by the addition of $MnCl_2$, an obligate cofactor for malic enzyme activity (4, 15). The amount of mitochondrial protein used was about 1 mg/ml. In addition, 0.5 mM KCN and

[1] This work was supported by a fellowship from the Délégation Générale à la Recherche Scientifique et Technique (to P. R.).

[2] Abbreviations: MDH, malate dehydrogenase; ME, malic enzyme; OAA, oxaloacetate; SHAM, salicylhydroxamic acid; states 3 and 4: rates of oxygen uptake in the presence or absence of ADP, respectively.

1 mM SHAM were also present to cut the access to O_2. MDH (EC 1.1.1.37) was measured by following the oxidation of NADH in a medium consisting of 100 mM phosphate (pH 7.5), 0.2 mM NADH, 0.5 mM OAA, and 1 mM KCN. The reaction was started by the addition of OAA. About 50 μg/ml mitochondrial protein was used for the assay of MDH.

Measurement of Products of Malate Oxidation. Mitochondria were incubated in the medium used for O_2 uptake measurements in the presence of 30 mM malate, 3.5 mM arsenite (to inhibit pyruvate oxidase), and eventually 200 μM NAD^+, 1 mM KCN, or 10 μM rotenone. The reaction was stopped by addition of an excess of $HClO_4$. After centrifugation at 3,000*g* for 15 min, the supernatant was neutralized by K_2CO_3, centrifuged at 3,000*g* for 10 min, and used for pyruvate and OAA assays. Pyruvate and OAA were measured with purified lactate dehydrogenase and MDH respectively, by following the oxidation of NADH at 340 nm in a medium consisting of 100 mM phosphate (pH 7.5), 120 μM NADH, and aliquots of supernatant. The reaction was started by addition of the enzyme.

RESULTS

Pattern of Malate Oxidation. Figure 1A shows an O_2 electrode trace for the oxidation of malate by cauliflower mitochondria. No cofactor is required. The oxidation rates, ADP/O, and RC values are typical of the oxidation of this substrate by plant mitochondria (6, 12). When 1 mM cyanide is added, the state 3 rate is only 65 to 70% inhibited, but a subsequent addition of 1 mM SHAM, a specific inhibitor of the alternative pathway, completely inhibits the residual cyanide-resistant electron transport. When compared with a similar trace for succinate oxidation (Fig. 1B), although the oxidation pattern is quite similar, the sensitivity of succinate oxidation to cyanide appears to be significantly higher (93%) than with malate. This indicates that the alternative pathway carries a larger share of the electron flux, both in relative and absolute amounts, when malate is the substrate being oxidized. If one assays for the products of malate oxidation (see Table I, malate alone), very little OAA (0.64 nmol) can be measured, whereas a large amount of pyruvate (116 nmol) is found. The formation of these products does not entirely account for the disappearance of malate during the same time (approximately 220 nmol, computed from the state 3 rate in Fig. 1A). The low level of OAA cannot be taken as a true measure of MDH activity since several mechanisms contribute to the removal of this product (4, 8). This accounts for a part of the discrepancy observed above.

Effect of NAD^+. When exogenous NAD^+ is added to mitochondria oxidizing malate in state 4 (ADP-exhausted), a sharp increase occurs in the rate of oxidation (Fig. 2A). Upon addition of ADP, RCs are observed but the measured ADP/O ratios are significantly lower than when measured before NAD^+ addition. The addition of cyanide shows that the electron transfer through the alternative pathway is increased by a factor of 2 (*cf.* Fig. 1A). The residual rate of oxidation is completely inhibited by SHAM.

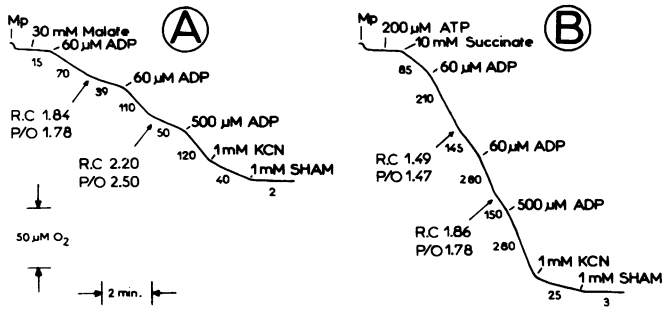

FIG. 1. Malate (A) and succinate (B) oxidation by cauliflower mitochondria. The numbers on the traces refer to nmol O_2 consumed/min·mg protein. Mp: purified mitochondria.

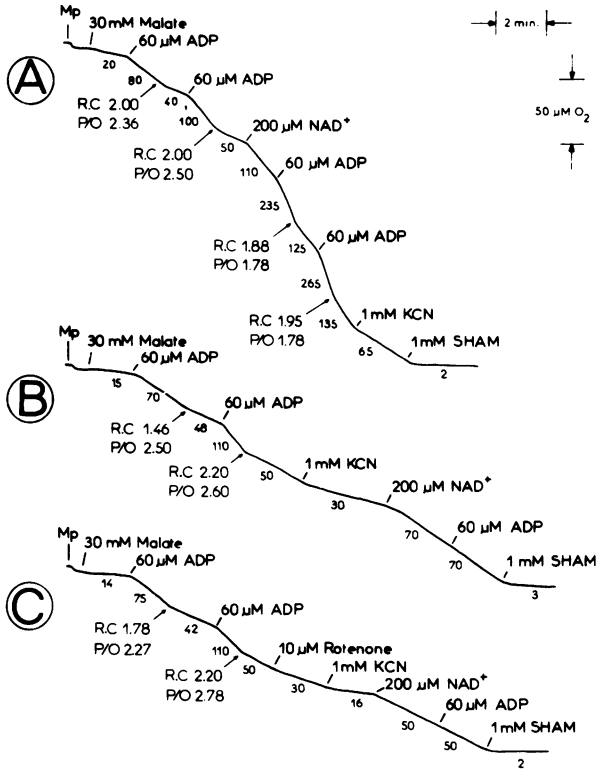

FIG. 2. Effect of exogenous NAD^+ on malate oxidation by cauliflower mitochondria. A, addition of NAD^+ during a state 4 rate; B, addition of NAD^+ in the presence of cyanide; C, addition of NAD^+ in the presence of rotenone and cyanide. The numbers on the traces refer to nmol O_2 consumed/min·mg protein. Mp: purified mitochondria.

Table I. *Products of Malate Oxidation in Cauliflower Mitochondria*

These determinations were made as described under "Materials and Methods," but in the presence of an excess of ADP.

Addition	Pyruvate	OAA
	nmol/min·mg protein	
Malate	116	0.64
Malate + NAD^+	193	0.32
Malate + KCN	57	0.05
Malate + KCN + NAD^+	103	0.30
Malate + rotenone	29	0.00
Malate + rotenone + NAD^+	64	0.00

Cyanide added before NAD^+ considerably reduces the rate of malate oxidation (rate equal to 60% of the state 4 rate and to 27% of the state 3 rate) (Fig. 2B). However, in that case, NAD^+ is still able to stimulate a cyanide-insensitive oxidation of malate. The rate is nearly the same as in the absence of cyanide. Then, ADP has no stimulating effect. From this, one concludes that part of the oxidation of malate is carried out through the cyanide-sensitive Cyt system, whereas NAD^+ essentially stimulates an oxidation which takes place through the cyanide-insensitive alternative pathway. Exactly the same conclusions are reached if rotenone is used as a specific inhibitor of the oxidation of malate via MDH and the Cyt sytem (Fig. 2C).

Further information can be gained by the examination of the products of malate oxidation under these different experimental conditions. Table I clearly show that the presence of cyanide or rotenone reduces both the rates of formation of pyruvate and OAA. In the presence of rotenone, no OAA can be measured.

However, the important fact is that, in all circumstances, the addition of NAD^+ specifically increases the rate of pyruvate production. Therefore, the presence of NAD^+ in the medium stimulates an oxidation of malate via ME and the cyanide-insensitive electron transport pathway.

Other Plant Materials. Such a situation is not limited to cauliflower mitochondria. It also occurs in a variety of other plant mitochondria. Mitochondria from white potato tubers are known as a typical example of highly cyanide-sensitive mitochondria (11). However, if malate is the substrate being oxidized and if the mitochondria are given NAD^+ in the presence of cyanide, then they show a typical cyanide-insensitive electron transport (Fig. 3A). In that case, one observes again that this phenomenon is correlated with the production of pyruvate (Table II). Mitochondria from spinach leaves also behave in a similar way (Fig. 3B; Table II). In this particular instance, however, rather large amounts of OAA are present. This can be partly due to the fact that contaminating broken plastids contribute to the production of OAA through a chloroplastic MDH. At any rate, the large amounts of OAA present in these mitochondria certainly explain the progressive inhibition of the rate of malate oxidation (compare the three successive state 3 rates in Fig. 3B).

From these observations, the conclusion can be drawn that the stimulation of malate oxidation via ME as a consequence of an addition of exogenous NAD^+ is a general phenomenon in plant mitochondria. Moreover, the resulting electron transport is cyanide-resistant.

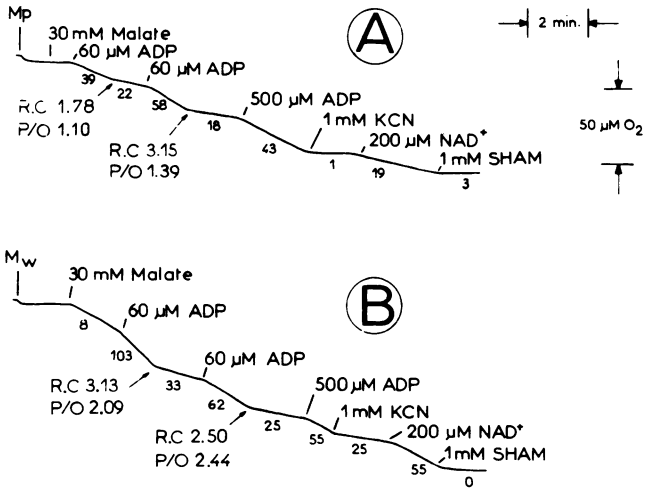

FIG. 3. Cyanide-insensitive oxidation of malate induced by exogenous NAD^+ in potato tuber (A) and spinach leaf (B) mitochondria. The numbers on the traces refer to nmol O_2 consumed/min·mg protein. Mp: purified mitochondria; Mw: washed mitochondria.

Table II. *Products of Malate Oxidation in Potato Tuber and Spinach Leaf Mitochondria*

Determinations were made as in Table I.

Addition	Pyruvate	OAA
	nmol/min·mg protein	
A. Potato tubers		
Malate	26.6	2.0
Malate + NAD^+	46.6	0.4
Malate + KCN	7.0	0.4
Malate + KCN + NAD^+	18.0	0.0
B. Spinach leaves		
Malate	58.6	42.6
Malate + NAD^+	80.0	48.0
Malate + KCN	20.0	16.0
Malate + KCN + NAD^+	34.6	18.0

Enzyme Location in Plant Mitochondria. Purified cauliflower mitochondria usually show a high degree of structural and biochemical integrity (6, 17). This can be assayed by the measurement of the succinate-Cyt *c* reductase activity, which gives information on the structural integrity of mitochondrial membranes (21). In cauliflower mitochondria, ME activity measured by the reduction of exogenous NAD^+, which freely permeates the outer membrane and slowly permeates the inner membrane (20), is very slow (Fig. 4A). After disruption of the inner membrane by sonication, this activity is increased by a factor of 10 at least. This result suggests that the NAD^+-dependent ME is located exclusively in the matrix compartment. In contrast, the behavior of MDH activity appears to be somewhat different (Fig. 4B). With intact mitochondria MDH activity is already important and sonication increases the activity only by a factor of 4. These results seem to indicate that the two enzymes are located within the matrix compartment. However, the difference in the effect of sonication on both enzyme activities suggests (as will be shown later) that some MDH could also be located outside the matrix.

We also observed that a factor of 10^3 exists between the activities of the two enzymes (Fig. 4; Table III). This is due to the fact that the enzyme activities are measured in different directions: towards the formation of malate for MDH ($\Delta G_0' = -6.7$ kcal/mol), towards the formation of pyruvate for ME ($\Delta G_0' = +0.36$ kcal/mol). Comparison of Tables I, II and III shows that there is very little relationship between the activity of MDH as measured *in vitro* and the actual amounts of OAA produced in the mitochondria. The formation of OAA is thermodynamically dependent on the removal of this product in order to displace the equilibrium in that direction. On the other hand, the correlation between ME activity and the amounts of pyruvate produced is quite satisfactory.

Reversal of Malate Dehydrogenase Activity. OAA, the product of MDH activity, is also a strong inhibitor of several Krebs cycle

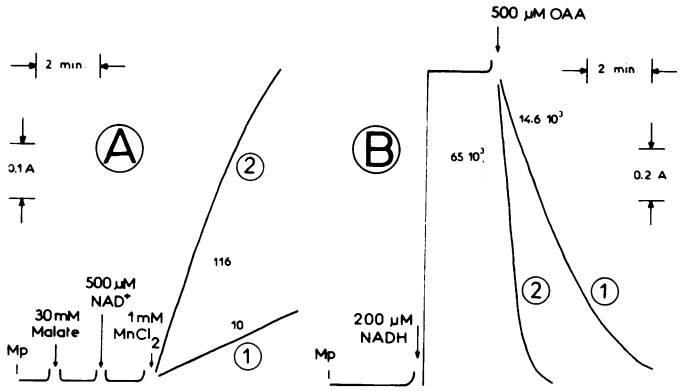

FIG. 4. Effect of sonication on ME (A) and MDH (B) activities in cauliflower mitochondria. 1, intact mitochondria; 2, sonicated mitochondria (6 × 15 s, 30 kiloHertz, Biosonik, Bronwill III). The numbers on the traces refer to nmol NADH produced (A) or consumed (B)/min·mg protein. Mp: purified mitochondria.

Table III. *Malic Enzyme and Malate Dehydrogenase Activities in Various Plant Mitochondria*

Measurements were made according to Figure 4.

Material	ME[a]	MDH[b]
	nmol/min·mg protein	
Cauliflower buds	116	65×10^3
Potato tubers	55	53×10^3
Spinach leaves	133	150×10^3

[a] Measured as NAD^+ reduced (= malate oxidized).

[b] Measured as NADH oxidized (= malate produced).

dehydrogenases (2, 8). For instance, 10 mM OAA completely inhibits succinate oxidation (Fig. 5A), thus indicating its ability to move readily across the inner mitochondrial membrane. Even in the presence of cyanide, OAA permeates mitochondrial membranes and is able to inhibit succinate dehydrogenase, as can be seen by the effect of OAA on succinate-Cyt *c* reductase activity in 10 mM phosphate and in the presence of 1 mM KCN (result not shown). Moreover, the addition of OAA can reverse the MDH reaction (1). This can be easily shown by supplying mitochondria with NADH and OAA (Fig. 5B). NADH is oxidized by the respiratory chain, as shown by the decrease of *A* at 340 nm (continuous trace). In the presence of cyanide, no oxidation took place (discontinuous trace). The addition of OAA brings about an extremely rapid oxidation of all the added NADH by reversal of the MDH reaction (1, 8). The rate of malate formation is 30 times higher than the rate of NADH oxidation (Fig. 5B). Such a fast kinetics does not seem to be compatible with the prerequisite of the access of OAA and more specifically of NADH, to a matrix-located MDH. It strongly suggests that the reaction takes place outside the inner membrane. This raises the important question of the existence of an external pool of MDH. This view is in agreement with the experiment reported in Figure 4.

In the same way, the addition of OAA to mitochondria oxidizing malate partially and transitorily inhibits the rate of electron transport (Fig. 5C). By direct inhibition of MDH by OAA, intramitochondrial NADH formation is reduced and so is the electron flux through the respiratory chain. In the presence of cyanide, the addition of NAD^+ stimulates the oxidation of malate via ME and the cyanide-insensitive pathway. OAA has no inhibitory effect under these circumstances. This shows that the NADH produced by ME has no access to MDH. If so, there would be a strong inhibition of the rate of oxidation in the presence of added OAA. Therefore, MDH and ME appear to be linked to two different pools of NADH.

Effect of Oxaloacetate on the Exogenous NADH Oxidation. As shown in Figure 6A, the addition of a limiting amount of NADH (100 μM) to mitochondria gives rise to a stochiometric uptake of O_2. If OAA is added and then NADH, O_2 consumption is reduced in direct proportion to the amounts of OAA introduced in the medium. By reversal of MDH, OAA is able to divert all external NADH that otherwise would be oxidized by the respiratory chain. This gives rise to the formation of malate which is oxidized more

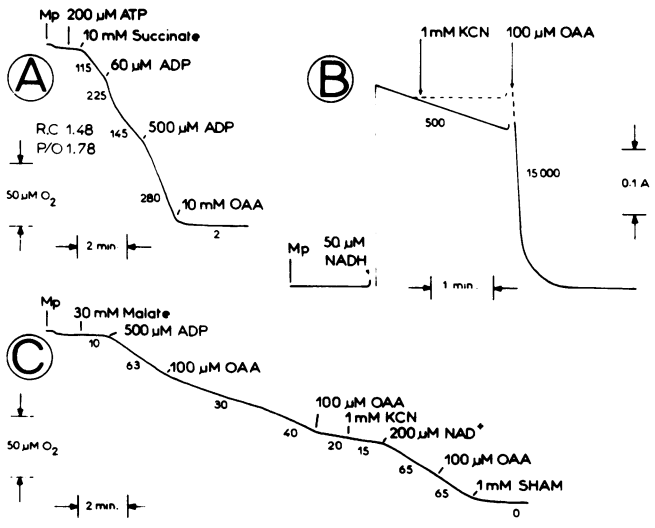

FIG. 5. Effect of addition of OAA on succinate (A), NADH (B), and malate (C) oxidation by cauliflower mitochondria. Numbers on the traces refer to nmol O_2/min·mg protein, except for trace B where they refer to nmol NADH oxidized. Mp, purified mitochondria.

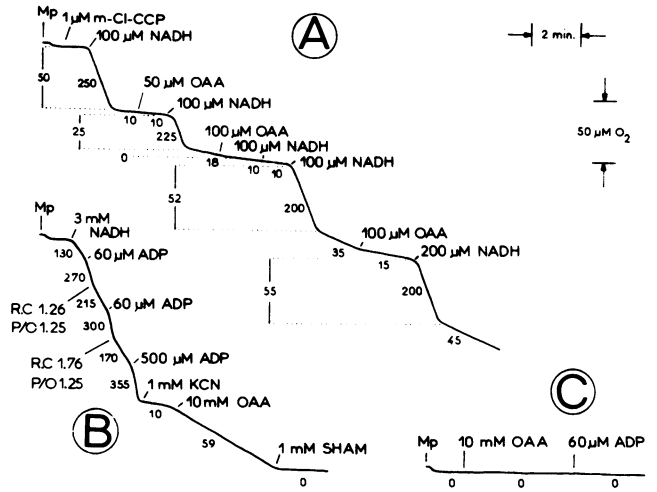

FIG. 6. Reversal of MDH in the presence of OA. A, stoichiometric dependence of NADH oxidation on the presence of OAA. Mitochondria were uncoupled by carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP); B, effect of OAA on NADH oxidation in the presence of cyanide; C, control trace for the oxidation of OAA alone. The numbers on the traces refer to nmol O_2/min·mg protein. Mp: purified mitochondria.

slowly than NADH, as it is shown by the increasing rates of oxidation, once all the added NADH has been oxidized.

On the other hand, in the presence of cyanide and an excess of NADH (3 mM) (Fig. 6B), the addition of a large amount of OAA (10 mM), which is not readily oxidized (Fig. 6C), generates a fair amount of cyanide-insensitive electron transport. This experiment can be explained in the following way. The oxidation of NADH by external NADH dehydrogenases generates exogenous NAD^+. In the presence of a MDH located outside the matrix, NADH and OAA generate exogenous NAD^+ and malate. Then, both exogenous malate and NAD^+ permeate the inner membrane and become the substrates of ME, giving rise to a cyanide-insensitive electron transport. This experiment also demonstrates that exogenous NADH has no access to the cyanide-insensitive pathway: its oxidation is strongly inhibited by cyanide (Fig. 5B), whereas NADH generated intramitochondrially by ME has access to this pathway.

Access of NADH to NADH Dehydrogenases. In plant mitochondria, exogenous NADH is normally oxidized with low ADP/O ratios by a pathway that is both rotenone-insensitive and cyanide-sensitive (Fig. 7A). The explanation is that the NADH dehydrogenase concerned is located on the outer face of the inner membrane and that the electron bypass phosphorylation site I (6, 21). In addition, another NADH dehydrogenase is also present on the outer membrane and could contribute to this type of NADH oxidation (17).

If mitochondria are sonicated (Fig. 7B), the rate of NADH oxidation is significantly reduced and becomes quite sensitive to rotenone (about 30 to 40%). It remains strongly sensitive to cyanide. Addition of exogenous Cyt *c* markedly increases the rate of oxidation but does not change the results. NADH then has become accessible to the rotenone-sensitive NADH dehydrogenase located on the inner face of the inner membrane and normally connected with the various NAD^+-dependent Krebs cycle dehydrogenases.

In sonicated mitochondria, NADH oxidation remains as strongly sensitive to cyanide (Fig. 7B) as it is in intact mitochondria (Fig. 7A). However, under these drastic conditions, an addition of malate and NAD^+ can still generate a cyanide-insensitive electron transport through the activity of ME. This requires that the NADH resulting from the activity of ME is reoxidized by a specific NADH dehydrogenase linked to the alternative pathway.

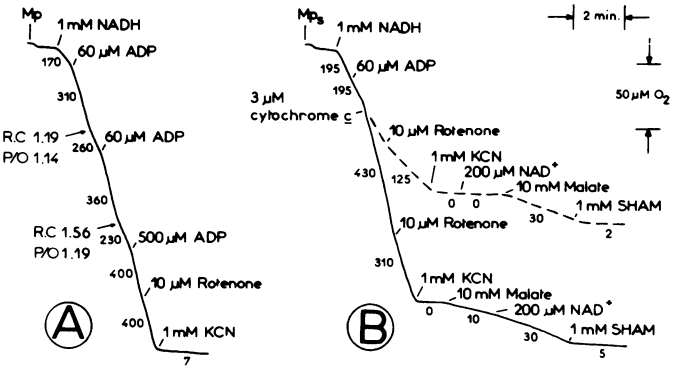

FIG. 7. Pathways of NADH oxidation in cauliflower mitochondria. A: effect of rotenone and cyanide on the oxidation of exogenous NADH; B: effect of rotenone and cyanide on the oxidation of exogenous NADH by sonicated mitochondria, in the presence of exogenous Cyt *c* (——) or in its absence (- - -). Sonication as described in Figure 4. The numbers on the traces refer to nmol O_2/min·mg protein. Mp: purified mitochondria; Mp_s: sonicated Mp.

An important point is that exogenous NADH present in the reaction medium has no access to this NADH dehydrogenase, which is closely connected with the NADH produced by ME. The same is true of NADH produced by the activity of MDH in the matrix (Fig. 2B). These experiments lead to the conclusion that two NADH dehydrogenases must be present on the inner face of the inner membrane: a rotenone-sensitive one connected with MDH and the cyanide-sensitive pathway and a rotenone-insensitive one connected with ME and the cyanide-insensitive pathway.

DISCUSSION

Important conclusions can be drawn from the results reported above. They deal with the peculiarities of malate oxidation but also with the general scheme of electron transport in plant mitochondria.

In these mitochondria, malate oxidation takes place through the action of two oxidizing enzymes: an NAD^+-dependent ME and MDH (16, 21). The results reported above indicate that ME is present in the matrix. It appears to be strongly adherent to the inner membrane as demonstrated by the action of detergents (7). MDH is also present in the matrix compartment (16, 21). Nevertheless, the results show that part of MDH activity can be found outside the matrix compartment, although one cannot assign a precise location (outer face of the inner membrane, intermembrane space, outer membrane).

The postulate of an externally located MDH activity is required to account for the fast kinetics of exogenous NADH oxidation in the presence of OAA (*cf.* Fig. 5B).The only alternative explanation would be to assume that NADH permeates the inner membrane, at the same rate as OAA does, to react at the level of the matrix-located MDH. Such an assumption cannot be held at the present time (2, 6). Other arguments are in favor of a peripheral MDH. Such an activity, originally considered as a contaminant, is present on isolated outer mitochondrial membranes devoid of contamination by inner membrane fragments (18). In mammalian mitochondria, the existence of different pools of MDH, free or more or less bound to the inner membrane, has also been demonstrated (5). Similarly, free MDH can bind to mitochondrial membranes with various degrees of specificity (25). This suggests that part of the cytosolic MDH could well be retained by nonspecific adsorption on mitochondria during the extraction and isolation process and, thus, be responsible for the external MDH activity that is observed.

Another part of this work indicates that the addition of NAD^+ to mitochondria oxidizing malate increases the rate of oxidation, with a concomitant decrease in ADP/O ratios. This has been known for some time (6), but the new element is that the increase in rate is cyanide-insensitive (22). In the presence of cyanide or rotenone, NAD^+ is still able to stimulate a malate oxidation that is inhibited by SHAM. This type of electron transport presents all the characteristics of an alternative pathway with respect to inhibitor action (23). The true participation of ME in this NAD^+-stimulated malate oxidation can be demonstrated by the formation of pyruvate. Furthermore, although the inner membrane has for a long time been considered as being rather impermeable to NAD^+ (2, 4, 21), the fact that NAD^+ is able to stimulate a matrix-located ME leads to the conclusion that, one way or another, NAD^+ must have some access to the matrix compartment or at least to the inside of the inner membrane (20).

Depending on the way malate is oxidized, by MDH and the Cyt pathway or by ME and the alternative pathway (in the presence of exogenous NAD^+), the same mitochondria can be considered as cyanide-sensitive or cyanide-insensitive (Fig. 3). This is probably one of the best demonstrations that all plant mitochondria should constitutively possess the two electron-transport pathways, with the alternative pathway being functional only under particular circumstances (11).

These results also suggest that strong compartmentations of enzymes and cofactors exist in plant mitochondria. For instance, the NADH produced by ME cannot be reoxidized by MDH in the presence of OAA (Fig. 5C). On the other hand, addition of OAA causes the oxidation of external NADH (Fig. 5B), as well as of intramitochondrial NADH which has been produced by MDH (Fig. 5C). This leads to the conclusion that at least two pools of intramitochondrial NADH are present.

With the exception of the NADH dehydrogenase associated with the outer membrane, it is generally assumed that the segments of the respiratory chain up to the flavoproteins and both terminal segments (Cyt and alternative) share a common intermediate: ubiquinone (23, 24). The organization of the branch point of the alternative and Cyt pathways in plant mitochondria is still not clear. However, many observations indicate that ubiquinone constitutes the branch point (23, 24). Taking this point for granted, if ubiquinone plays a central role in electron distribution between the two pathways, one nevertheless has to admit that different pools of ubiquinone must be present to explain various typical situations. In particular, if the electrons from different substrates show the same behavior with respect to cyanide, it is to be assumed that they share the same pool of ubiquinone. One the other hand, if their behaviors are different, they must be linked to different pools of ubiquinone.

Based on this simple idea, the results reported above, as well as some other well-established results (11), can be easily integrated, assuming that three different pools of ubiquinone are present in plant mitochondria (Fig. 8).

First, broadly speaking, malate oxidation and succinate oxidation always behave the same way with respect to the sensitivity to inhibitors (Fig. 1) (11). This indicates that their respective dehydrogenases (malate and rotenone-sensitive NADH dehydrogenases, succinate dehydrogenase) are connected to the same pool of ubiquinone before the electrons are fed into the Cyt or into the alternative pathway. However, if, as here, one discriminates according to the way malate is oxidized, one has to conclude that malate oxidation via MDH is essentially linked to the cyanide-sensitive electron transport pathway by a rotenone-sensitive NADH dehydrogenase. On the other hand, malate oxidation via ME is essentially linked to the cyanide-insensitive alternative pathway by a rotenone-insensitive NADH dehydrogenase and, therefore, is connected to another pool of ubiquinone. Also, in some particular instances (sweet potato, aged white potato slices) where one observes a fair degree of cyanide-resistance, exogenous NADH oxidation remains strongly cyanide-sensitive, whereas suc-

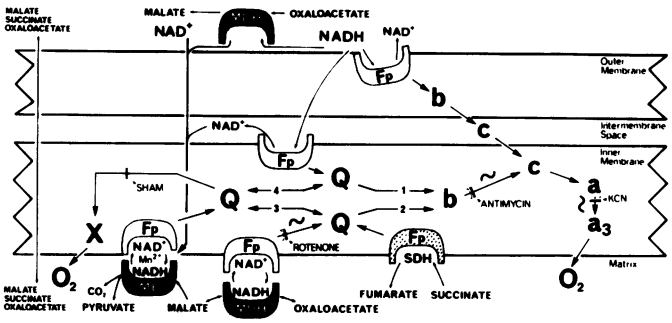

FIG. 8. Organization of electron transport in plant mitochondria. Fp, flavoproteins (various NADH dehydrogenases or succinate dehydrogenase); Q, various pools of ubiquinone; *a*, a_3, *b*, and *c*, cytochrome; X, supposed terminal oxidase of the alternative cyanide-insensitive pathway. SDH, succinate dehydrogenase.

cinate and malate oxidation are cyanide-resistant (11). This suggests that existence of a third independent pool of ubiquinone linked to the rotenone-insensitive NADH dehydrogenase of the outer face of the inner membrane.

All these facts are integrated in Figure 8, based on the above considerations. Three pools of ubiquinone are present and their various connections account for all the situations and changes observed in the organization of electron transport in plant mitochondria. Connections 1 and 2 are always present in plant mitochondria. They account for the cyanide- and antimycin-sensitive malate, NADH and succinate oxidations, but also for the rotenone-sensitive malate oxidation and the rotenone-insensitive NADH oxidation. Another pathway of NADH oxidation (through the center membrane) bypasses the antimycin-sensitive site. As a matter of fact, this is the general situation encountered in all cyanide-sensitive plant mitochondria. When, in addition, connections 3 and 4 are present, all substrate oxidations show various degrees of cyanide resistance since the electrons can reach the alternative pathway (3). The peculiarities of malate oxidation reported in this paper are quite well understood if connections 3 and 4 are missing. All substrate oxidations are cyanide-sensitive, except for the oxidation of malate through ME, its particular rotenone-insensitive NADH dehydrogenase, and its particular pool of ubiquinone, that is cyanide-resistant. Finally, in the case where succinate and malate oxidations are cyanide-resistant, whereas NADH oxidation remains strongly cyanide-sensitive (11), only connection 4 is missing.

The situation as depicted above is probably too schematic since it would forbid the oxidation of malate via ME in a 100% cyanide-sensitive plant mitochondria (connections 3 and 4 missing). So far, all plant mitochondria have been found to show a small percentage of cyanide resistance. Moreover, exogenous NAD^+ in the presence of malate can reveal a cyanide resistance that would have remained unnoticed with other substrates (Fig. 3). Finally, the results reported here clearly show that ME, its NADH dehydrogenase, and its pool of quinone behave as an autonomous cyanide-insensitive unit, whereas all other electron transports can be cyanide-sensitive. For reasons given above, the external MDH has also been represented in Figure 8. However, its nature and precise location are still uncertain.

Contrary to animal mitochondria, plant mitochondria show the unique feature of being able to oxidize malate and NADH in a number of ways that bypass definite numbers of phosphorylation sites (Fig. 8). Thus, they are able to oxidize malate and NADH in a way that does not increase the phosphate potential of the cell (21). This could be of some utility to such plants that have to eliminate an excess of reducing power (high rate of glycolysis) or to use large amounts of malate over a short periode of time (CAM plants) (19).

Acknowledgments—The authors are deeply indebted to Miss M. F. Alin and Mrs. M. Pouget for their expert technical assistance.

LITERATURE CITED

1. BERGMEYER HU, E BERNT 1963 Malic dehydrogenase. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, Ed 1. Verlag Chemie, Academic Press, New York, pp 757–760
2. BRUNTON CJ, JM PALMER 1973 Pathway for the oxidation of malate and reduced pyridine nucleotide by wheat mitochondria. Eur J Biochem 39: 283–291
3. CHAUVEAU M 1976 La chaine respiratoire des mitochondries d'Arum. II. Inhibition de la voie d'oxydation insensible au cyanure. Physiol Vég 14: 325–337
4. COLEMAN OD, JM PALMER 1972 The oxidation of malate by isolated plant mitochondria. Eur J Biochem 26: 499–509
5. COMTE J, DC GAUTHERON 1978 The markers of pig heart mitochondrial subfractions. II. On the association of malate dehydrogenase with inner membrane. Biochimie 60: 1299–1305
6. DAY DA, JT WISKICH 1974 The oxidation of malate and exogenous reduced nicotinamide adenine dinucleotide by isolated plant mitochondria. Plant Physiol 53: 104–109
7. DAY DA, GP ARRON, GG LATIES 1978 Malic enzyme localization in potato mitochondria. *In* G Ducet, C Lance, eds. Plant Mitochondria. Elsevier-North Holland, Amsterdam, pp 125–132
8. DOUCE R, WD BONNER 1972 Oxalacetate control of Krebs cycle oxidations in purified plant mitochondria. Biochem Biophys Res Commun 47: 619–624
9. DOUCE R, AL MOORE, N NEUBURGER 1977 Isolation and oxidative properties of intact mitochondria isolated from spinach leaves. Plant Physiol 60: 625–628
10. LANCE C 1971 Le mitochondries de l'inflorescence de Chou-fleur. I. Propriétés oxydatives. Physiol Vég 9: 259–279
11. LANCE C, P DIZENGREMEL 1978 Slicing-induced alterations in electron transport systems during aging of storage tissues. *In* G Kahl, ed, Biochemistry of Wounded Plant Tissues. Walter de Gruyer and Co, New York, pp 467–501
12. LANCE C, GE HOBSON, RE YOUNG, JB BIALE 1967 Metabolic processes in cytoplasmic particles of avocado fruit. IX. The oxidation of pyruvate and malate during the climacteric cycle. Plant Physiol 42: 471–478
13. LILLEY MCC, MD FITZGERALD, KG RIENITS, DA WALKER 1975 Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol 75: 1–10
14. MCKINNEY G 1941 Absorption of light by chlorophyll solutions. J Biol Chem 140: 315–322
15. MACRAE AR 1971 Isolation and properties of a "malic" enzyme from cauliflower bud mitochondria. Biochem J 122: 495–501
16. MACRAE AR, R MOORHOUSE 1970 The oxidation of malate by mitochondria isolated from cauliflower buds. Eur J Biochem 16: 96–102
17. MOREAU F 1976 Electron transfer between outer and inner membranes in plant mitochondria. Plant Sci Lett 6: 215–221
18. MOREAU F, C LANCE 1972 Isolement et propriétés des membranes externes et internes de mitochondries végétales. Biochimie 54: 1335–1348
19. MOREL C, O QUEIROZ 1978 Dawn signal as a rhythmical timer for the seasonal adaptative variation of CAM: a model. Plant Cell Environ 1: 141–149.
20. NEUBURGER M, R DOUCE 1978 Transport of NAD^+ through the inner membrane of plant mitochondria. *In* G Ducet, C Lance, eds. Plant Mitochondria. Elsevier-North Holland, Amsterdam, pp 109–116
21. PALMER JM 1976 The organization of electron transport in plant mitochondria. Annu Rev Plant Physiol 27: 133–157
22. RUSTIN P, F MOREAU 1979 Malic enzyme activity and cyanide-insensitive electron transport in plant mitochondria. Biochem Biophys Res Commun 88: 1125–1131
23. SOLOMOS T 1977 Cyanide-resistant respiration in higher plants. Annu Rev Plant Physiol 28: 279–297
24. STOREY BT 1976 The respiratory chain of plant mitochondria. XVIII. Point of interaction of the alternate oxidase with the respiratory chain. Plant Physiol 58: 521–525.
25. STRASBERG PM, KA WEBSTER, HU PATEL, KB FREEMAN 1979 Binding of mitochondrial malate dehydrogenase to mitoplasts. Can J Biochem 67: 662–665