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Malate Oxidation, Rotenone-Resistance, and Alternative Path Activity in Plant Mitochondria[1]

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ABSTRACT

The effect of cyanide and rotenone on malate (pH 6.8), malate plus glutamate (pH 7.8), citrate, α-ketoglutarate, and succinate oxidation by cauliflower (*Brassica oleracea* L.) bud, sweet potato (*Ipomoea batatis* L.) tuber, and spinach (*Spinacia oleracea* and *Kalanchoë daigremontiana* leaf mitochondria was investigated. Cyanide inhibited all substrates equally with the exception of malate plus glutamate; in this case, inhibition of O_2 uptake was more severe due to an effect of cyanide on aspartate aminotransferase. Azide and antimycin A gave similar inhibitions with all substrates. Subsequent addition of NAD had no effect with any substrate. Providing that oxalacetate accumulation was prevented, rotenone inhibited all NAD-linked substrates equally and caused ADP:O ratios to decrease by one-third. Addition of succinate to mitochondria oxidizing malate stimulated oxygen uptake, but adding citrate and α-ketoglutarate did not. These results indicate that there is no direct link between malic enzyme and the rotenone- and cyanide-resistant respiratory pathways, and that there is no need to postulate separate compartmentation of malic enzyme and the other NAD-linked enzymes in the matrix.

NADH oxidation by plant mitochondria is a complex process involving at least three, and possibly four, distinct dehydrogenases (14, 22). Extra mitochondrial NADH can be oxidized via two external dehydrogenases, one located on the outer membrane and the other on the outer surface of the inner membrane (13). The latter enzyme is linked to the respiratory chain and coupled to two phosphorylations. Endogenous NADH can also be oxidized via two pathways, one of these being coupled to three energy-transducing sites and sensitive to the electron transport inhibitors rotenone and piericidin A; this oxidation is accomplished by a dehydrogenase bound to the inner side of the inner membrane. The other pathway of internal NADH oxidation bypasses the rotenone-sensitive site and is coupled to only two phosphorylations (5, 18). This pathway probably involves another internally located NADH dehydrogenase. Although the internal bypass has properties similar to those of the external system, the two pathways are not identical (31).

Intramitochondrial NADH oxidation in many plant tissues can be linked to another 'bypass'—the well-known cyanide-insensitive, alternative pathway of electron transport (16). If the rotenone- and cyanide-insensitive pathways operate in series, then plant respiration can be completely nonphosphorylating. In some tissues, these two pathways develop together, although a functional link has yet to be established (7). On the other hand, in many tissues external NADH oxidation has only limited access to the alternative path (7).

Malate oxidation by plant mitochondria occurs via the action of two enzymes, MDH[2] and NAD-linked malic enzyme, both located in the matrix (29). In isolated mitochondria, these two enzymes may operate separately or together, depending on the conditions imposed (co-factors, pH, etc.).

It has been suggested that MDH and malic enzyme are compartmented within the matrix, being associated with different NAD-pools and NADH dehydrogenases, and having different access to the alternative path (22, 25, 26). The suggestion by Palmer and colleagues (5, 23) that malic enzyme is in a compartment which has access only to the rotenone-sensitive dehydrogenase, whereas MDH and the other Krebs cycle enzymes are associated with the rotenone-insensitive pathway, has been reinvestigated and refuted (30, 31). More recently, Rustin *et al.* (25, 26) have suggested that malic enzyme is functionally linked not only to the rotenone-insensitive NADH dehydrogenase, but also to the alternative path. This has important implications for plant respiration, considering the central role of malic enzyme (particularly in photosynthetic tissues), and therefore warrants close examination. We have thus reinvestigated the question of matrix compartmentation of NAD-linked enzymes and respiratory chain components in different plant tissues.

MATERIALS AND METHODS

Cauliflowers (*Brassica oleracea* L.) and sweet potatoes (*Ipomoea batatis* L.) were purchased from local markets. *Kalanchoë daigremontiana* plants were grown in soil in a naturally illuminated glasshouse. Reagents were purchased from Sigma and Calbiochem (Sydney, Australia).

Mitochondria were isolated from spinach leaves by disrupting leaves with a Polytron PTA-35 probe for 2 s at setting 7, in 200 ml cold medium containing 0.3 M sorbitol, 50 mM Tes buffer (pH 7.6), 10 mM KH_2PO_4, 1 mM EDTA, 10 mM isoascorbate, 0.2% (w/v) BSA, and 0.5% (w/v) PVP-40. The homogenate was filtered through two layers of Miracloth and centrifuged at 1,500*g* for 5 min in a Sorvall RC-2B centrifuge. The supernatant was centrifuged at 9,000*g* for 15 min and the pellets washed by resuspending in 60 ml 0.3 M sorbitol containing 20 mM Tes buffer (pH 7.2) and 0.1% BSA, and recentrifuging at 9,000*g* for 15 min. Final resuspension was in 3 to 4 ml of wash medium.

Mitchondria were isolated from the other tissues by published techniques as follows: cauliflower buds (9), sweet potato tubers (2), and *K. daigremontia* leaves (6). Mitochondria were purified,

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[2] Abbreviations: MDH, malate dehydrogenase; SHAM, salacylic hydroxamic acid; OAA, oxalacetate.

when indicated, by centrifuging on linear sucrose gradients essentially as described by Arron *et al.* (2). Protein was estimated by the method of Lowry *et al.* (17) and Chl according to Arnon (1). When necessary, mitochondrial protein was corrected for the contribution by broken thylakoids by assuming a thylakoid protein: Chl ratio of 7:1 (6).

O_2 consumption was measured at 25°C using either Rank (Rank Bros., Cambridge, U.K.) or Clark-type (Yellow-Springs, OH) O_2 electrodes. The standard reaction medium contained 0.25 M sucrose, 10 mM Tes buffer, 10 mM KH_2PO_4, 2 mM $MgCl_2$, and 0.1% BSA (pH 6.8–7.8, as indicated). O_2 in air-saturated medium was assumed to be 240 μM. Enzyme assays were performed spectrophotometrically at room temperature as described (11). Reduction of added NAD by detergent-disrupted cauliflower mitochondria was measured spectrophotometrically in 1 ml of standard reaction medium to which was added 50 μl mitochondrial suspension (0.5 mg protein), 1 mM NAD, 5 μM antimycin A, 0.04% deoxycholate, and 10 mM malate. Other additions are shown in Figure 1.

RESULTS AND DISCUSSION

The relative contribution of malic enzyme and MDH to malate oxidation by plant mitochondria can be varied according to the conditions chosen. Thus, at pH 6.8 to 7.2, O_2 consumption with malate as substrate is due to the combined activity of malic enzyme and MDH; at pH 7.4 to 7.8 in the presence of glutamate (to ensure transamination of oxalacetate formed) O_2 consumption is largely due to MDH activity inasmuch as malic enzyme is inhibited (6, 21). The effect of pH may be due to a greatly increased sensitivity of malic enzyme to inhibition by HCO_3^- at alkaline pH (21). We have taken advantage of these effects to study separately the effect of inhibitors on MDH- and malic enzyme-associated O_2 consumption by varying the pH of the medium. Hence, at pH 7.8, MDH predominates, whereas at pH 6.8 malic enzyme is active. In the experiments shown in Table I, malate alone was added to the mitochondria; the large decline in O_2 uptake rates at pH 7.8 reflect the accumulation of oxalacetate (which inhibits MDH) and the lack of malic enzyme activity. In subsequent experiments, glutamate and thiamine pyrophosphate were included in the reaction medium when malate was used, to secure maximum and consistent rates of O_2 uptake. Under these conditions other substrates may contribute to O_2 uptake since pyruvate, citrate, and α-ketoglutarate may be formed. Despite this, the greatest proportion of O_2 uptake is still likely to be due to malate oxidation (8), and the effects of the various inhibitors and NAD, shown in Tables II to IV, are the same as when malate alone is used (allowing for the faster O_2 uptake rates at high pH).

Table I. *Malate Oxidation by Cauliflower Bud Mitochondria*

Oxygen uptake was measured as described in "Materials and Methods," except that the temperature was 30°C. In experiment 1, 10 mM malate was used; in experiment 2, the malate concentration was 43 mM. Other additions as indicated were 1 μmol ADP, 1 mM KCN, and 1 mM NAD.

Sequential Additions to Vessel	Oxygen Consumption		
	pH 6.8	pH 7.2	pH 7.8
	nmol min^{-1} mg^{-1} protein		
Experiment 1			
Malate + ADP	245	102	34
KCN	22	16	3
NAD	22	14	3
Experiment 2			
Malate + ADP	228	141	50
KCN	22	19	3
NAD	20	16	3

Table II. *Effect of KCN and NAD on Cauliflower Mitochondria*

Oxygen consumption was measured as described in "Materials and Methods." Additions as indicated were 2 μmol ADP, 1.7 mM KCN, 1 mM NAD, 1 mM SHAM. All substrates were added to 10 mM; 0.25 mM thiamine pyrophosphate was included in the reaction medium when α-ketoglutarate was used. Except where indicated, the pH of the medium was 7.4. Glutamate (10 mM) was added with malate.

Sequential Additions	Oxygen Consumption				
	Malate, pH 6.8	Malate pH 7.8	α-Keto-glutarate	Citrate	Succinate
	nmol min^{-1} mg^{-1} protein				
ADP	172	132	94	42	103
KCN	12	0	13	10	10
NAD	11	0	13	9	
SHAM	0		0	0	0

Table III. *Effect of KCN and Azide on Malate Oxidation by Cauliflower Mitochondria*

Assay conditions as in Table II; 1.7 mM azide was used.

Sequential Additions	Oxygen Consumption	
	Malate, pH 6.8	Malate, pH 7.8
	nmol min^{-1} mg^{-1} protein	
Experiment 1		
ADP	110	83
KCN	9	0
NAD	10	0
SHAM	0	0
Experiment 2		
ADP	114	94
Azide	10	13
NAD	11	12
SHAM	1	2

Is There a Link between Malic Enzyme and the Alternative Path? The effect of KCN on cauliflower bud mitochondria is shown in Tables I, II and III. Rustin *et al.* (26) reported considerable cyanide-resistance in their cauliflower mitochondria, and found that with malate as substrate, adding NAD enhanced this resistance while simultaneously stimulating malic enzyme activity. In contrast to this, we find very little (<10%) cyanide-resistance in cauliflower mitochondria regardless of the choice of substrate, and adding NAD has virtually no effect (Tables I and II). Sweet potato mitochondria, on the other hand, show considerable cyanide-resistance with all substrates, but again addition of NAD is without effect (Table IV).

The only difference we observe between substrates in these mitochondria is the more severe inhibition of malate oxidation by KCN at pH 7.8 when glutamate is present (Tables II and III, experiment 1; Table IV). However, this difference is not observed when azide (Table III) or antimycin A (Table V) is used instead of KCN; again, NAD has no effect. At high (30–50 mM) malate concentrations, the effect of KCN at pH 7.8 is not as severe (data not shown). We attribute the effect of KCN on malate oxidation at the higher pH to an inhibition of aspartate aminotransferase (the operation of which is necessary for oxalacetate removal and continued MDH operation), and support for this idea is provided in Figure 1. In this experiment, NAD reduction by disrupted mitochondria in the presence of antimycin A (to prevent reoxidation of NADH by the respiratory chain), upon addition of malate, was used to measure MDH and malic enzyme activity. At both pH 7.8 and 6.8, an immediate burst of NAD reduction occurs

Table IV. *Effect of KCN on Sweet Potato Mitochondria*

Conditions were as described in Table II. Multiple substrates refer to the presentation of 10 mM succinate, malate, citrate, and α-ketoglutarate together. Mitochondria were purified as described in "Materials and Methods."

Sequential Additions to Vessel	Oxygen Consumption			
	Malate		Multiple substrates	
	pH 6.8	pH 7.4	pH 6.8	pH 7.4
	nmol min^{-1} mg^{-1} protein			
ADP	176	173	260	277
KCN	93	42	63	70
NAD	97	43	63	72
SHAM	8	10		

Table V. *Alternative Path Activity of Spinach Leaf Mitochondria*

Oxygen consumption was measured as described in "Materials and Methods." Antimycin A was presented at a final concentration of 5 μM; other details are given in Table II.

Substrate	Oxygen Consumption			
	State 3	+ Antimycin	+ SHAM	+ Antimycin and SHAM
	nmol min^{-1}			
Malate, pH 7.8	160	44	112	6
Malate, pH 6.8	110	46	70	6
Succinate	90	45		10

when malate is added, presumably due to MDH activity; at pH 6.8, this initial rapid reduction is followed by a linear, slower rate of NAD reduction (Fig. 1B) attributable to malic enzyme activity. This latter phase is unaffected by KCN or glutamate. At pH 7.8, malic enzyme is inactive and NAD reduction via MDH quickly ceases as OAA accumulates; subsequent addition of glutamate restores NAD reduction as the OAA is transaminated, but now KCN inhibits dramatically (Fig. 1A). This interpretation is reinforced by measurements of individual enzyme activities. Direct measurement of aminotransferase activity by the method of Sizer and Jenkins (27) confirmed that this enzyme is inhibited by KCN (1 mM KCN giving 50–60% inhibition). On the other hand, KCN has no effect on MDH itself (measured as NAD reduction at alkaline pH or as NADH oxidation in the presence of OAA).

Thus, results obtained with KCN when malate is substrate must be treated with caution; in particular, KCN inhibition may appear to be more pronounced when malic enzyme is inactive.

Table V shows results obtained with spinach leaf mitochondria. At first glance it appears that malate oxidation at pH 7.8 is less resistant to antimycin A (the per cent inhibition is greater); however, the absolute rate of O_2 uptake is the same at both pH values and similar to that with succinate. Moreover, SHAM reduced O_2 uptake by the same amount at high and low pH and in both instances this inhibition was very nearly equal to the cyanide-resistant rate (40–48 nmol min^{-1}). That is, the alternative path is engaged to the same extent (in fact it is fully engaged) regardless of whether MDH or malic enzyme predominates. We conclude that in the three species studied here, there is no special link between malic enzyme and the alternative path.

Effect of Rotenone and Oxalacetate. Compartmentation of NAD-linked enzymes and respiratory-linked NADH dehydrogenases was investigated by measuring the response of O_2 uptake to rotenone and oxalacetate. Results with *K. daigremontiana* mitochondria are shown because of their high resistance to rotenone (6), but similar results were obtained with cauliflower mitochondria.

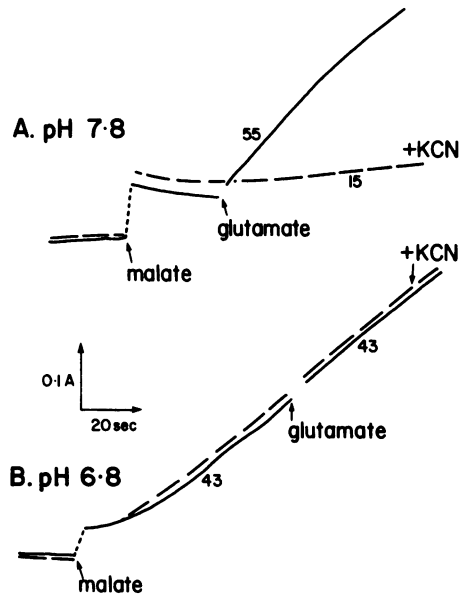

FIG. 1. NAD reduction by cauliflower bud mitchondria. Reduction of NAD was measured spectrophotometrically in disrupted mitochondria as described in "Materials and Methods." Where indicated, 10 mM malate and 10 mM glutamate were added. (——), control; (– – –), 1 mM KCN included in the reaction medium. Numbers on traces refer to nmol min^{-1}.

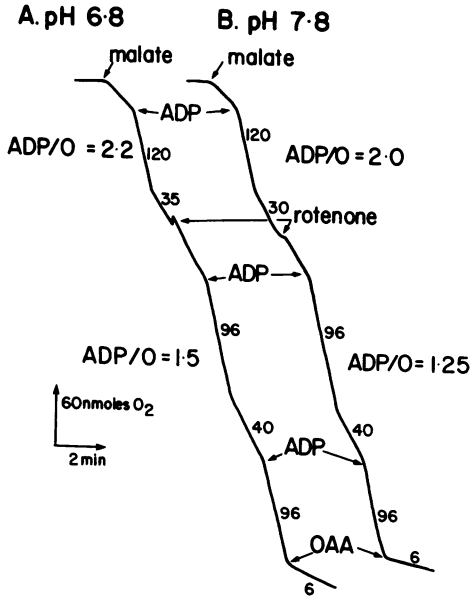

FIG. 2. Malate oxidation by *K. daigremontiana* leaf mitochondria. O_2 consumption was measured as described in "Materials and Methods." Additions as indicated were: 10 mM malate, 0.3 μmol (first and second additions), and 1 μmol (third addition) ADP, 20 μM rotenone and 1 mM oxalacetate. Numbers of traces refer to nmol min^{-1} mg^{-1} protein. At pH 6.8, 0.1 mM thiamine pyrophosphate, and at pH 7.8, 10 mM glutamate, were included in the reaction medium.

Figure 2 shows the effect of rotenone on malate-supported O_2 uptake at pH 6.8 and 7.8; the degree of inhibition in each case is virtually identical. Only when oxalacetate is allowed to accumulate

during malate oxidation does rotenone inhibition appear more severe (30). Similar degrees of inhibition of O_2 uptake by rotenone are found with citrate, α-ketoglutarate and glycine (6). Obviously, all NAD-linked substrates have equal access to the rotenone-bypass; inhibition of α-ketoglutarate oxidation may sometimes appear to be less sensitive to rotenone (23) but this is due to production and oxidation of succinate and is not seen when malonate is present (30).

In the presence of rotenone, ADP:O ratios decrease by almost exactly one-third (Fig. 2); this has been observed with other tissues and substrates (5, 18, 31), and indicates that one site of energy transduction has been bypassed. The fact that ADP:O values drop by the same proportion when rotenone is presented with different substrates (*e.g.* malate and α-ketoglutarate: 31), is further evidence that the different NAD-linked enzymes have equal access to the bypass. ADP:O values are consistently low in mitochondria isolated from the leaves of CAM plants (3, 6) and it was assumed that this reflected the high activity of the rotenone bypass. The effect of rotenone on ADP:O values in Figure 2, however, suggests that the bypass pathway is not engaged in the absence of inhibitors or restriction of the respiratory chain by lack of ADP. This is also true of other tissues (5, 18, 31).

Oxalacetate readily penetrates plant mitochondria, probably via a specific carrier (11), and rapidly oxidizes matrix NADH via MDH at the expense of the respiratory chain, thereby inhibiting O_2 uptake (12). If the various NAD-linked enzymes are in different compartments, then one might expect to see differential inhibition of oxalacetate. However, the data in Table VI indicate that O_2 uptake declines equally with all substrates when oxalacetate is added. That is, the NADH generated in the matrix upon oxidation of different NAD-linked substrates is equally accessible to MDH and oxalacetate, although some is still oxidized via the respiratory chain. The latter portion is less when rotenone is present (Fig. 2, compare to Table VI inhibition by oxalacetate is greater when rotenone is present), suggesting perhaps that the rotenone-insensitive bypass has a lower affinity for NADH than the rotenone-sensitive dehydrogenase.

Table VI. *Effect of Oxalacetate on the Respiration of Cauliflower and K. daigremontiana Mitochondria*

Assay conditions as in Table II; oxalacetate (OAA) was provided at 1 mM.

Substrate	State 3 O_2 Consumption		Inhibition
	Control	+ OAA	
	nmol min^{-1} mg^{-1} protein		%
K. daigremontiana			
Malate, pH 6.8	88	18	80
Malate, pH 7.8	92	22	77
Citrate	32	8	75
Cauliflower			
Malate, pH 6.8	138	15	91
Malate, pH 7.8	126	9	93

Table VII. *Concurrent Oxidation of More Than One Substrate*

The substrates shown were added sequentially to the vessel in the presence of ADP. For assay details see Table II.

Substrates	Oxygen Consumption			
	Sweet potato mitochondria		Cauliflower mitochondria	
	pH 6.8	pH 7.4	pH 6.8	pH 7.8
	nmol min^{-1} mg^{-1} protein			
Malate	138	156	102	103
Then α-ketoglutarate	177	180	80	98
Then citrate	156	186	88	106
Then succinate	249	294	121	145

Concurrent Oxidation of More Than One NAD-Linked Substrate. Previous experiments with cauliflower mitochondria (10) have shown that the Cyt chain is not saturated by oxidation of any one substrate; presentation of NADH or succinate together with malate produced O_2 uptake rates far in excess of those obtained with the three substrates separately (10). It was concluded that respiratory activity was limited by the respective respiratory linked dehydrogenases. In contrast to these results, addition of a second or third NAD-linked substrate in the presence of malate stimulates O_2 uptake very slightly, if at all (Table VII). This was observed with sweet potato and cauliflower mitochondria at both high and low pH. Further addition of succinate as a fourth substrate substantially stimulates O_2 uptake (Table VII). We interpret these results to indicate that the matrix NAD pool (or the internal NADH dehydrogenase complement) is saturated by malate (plus glutamate) alone at both pH values (*i.e.* by MDH and malic enzyme activity); hence, further addition of extra NAD-linked substrates does not stimulate O_2 uptake. In other words, the various NAD-linked enzymes compete with each other for matrix NAD and/or NADH dehydrogenase. This is unlikely to occur if the NAD-linked enzymes are in separate compartments or associated specifically with different respiratory-linked dehydrogenases.

GENERAL DISCUSSION

The results presented here argue strongly against a functional link between malic enzyme and the rotenone- and cyanide-resistant respiratory pathways in isolated plant mitochondria. Previous suggestions (5, 25, 26) to the contrary can be discounted by careful interpretation of the data published to date.

Rotenone Resistance. Plant mitochondria possess two routes for the oxidation of internal NADH, the rotenone-insensitive system apparently having a lower affinity for NADH (this is suggested by our results and those of ref. 18, and has been demonstrated more definitely in ref. 20). Hence, the resistant pathway does not operate unless the other path is restricted (*e.g.* by adding rotenone or during state four respiration, [5, 18, 31]). When malic enzyme or pyruvate dehydrogenase is inactive and glutamate absent during malate oxidation, matrix OAA levels increase and less NADH will be generated. That is, less NADH is available to the respiratory chain, and rotenone-resistance will appear to be less. The same argument applies when OAA is added to the mitochondria (Fig. 2). When steps are taken to ensure OAA removal, rotenone-insensitive O_2 uptake is the same whether MDH or malic enzyme is operative. Often, when rotenone or piericidin A is added to the mitochondria-oxidizing malate, the inhibition of O_2 uptake is initially severe but becomes less with time (23, 30). This phenomenon also reflects the sensitivity of MDH to OAA levels and the rate of turnover of the NAD(H) pool. Even when glutamate is present, small quantities of OAA may accumulate within the mitochondria, and subsequent addition of rotenone also raises matrix NADH levels. Thus, O_2 uptake suffers a transiently severe inhibition due to reversal of MDH; upon removal of OAA, O_2 consumption increases (30). Under the same conditions, oligomycin mimics rotenone (30), although it increases matrix NADH by a very different mechanism.

There is therefore no reason to postulate a direct link between malic enzyme and the rotenone-resistant pathway in the tissues we have examined. In a recent study (15), rotenone was found to be a direct inhibitor of isolated malic enzyme. However, rotenone did not seem to affect pyruvate production in intact mitochondria, suggesting that malic enzyme is not susceptible *in situ*. Our results also suggest this.

Cyanide Resistance. The same argument can explain why the alternative path appears to be more active when malic enzyme operates. Engagement of the alternative path requires a certain level of reduction of ubiquinone (4) and this occurs only when electron flow through the respiratory-linked dehydrogenases is rapid. The latter requirement is met when OAA is either not produced or is removed in some way. This explains why Miller and Obendorf (19) found only their 'fast-phase' state four O_2 uptake (after OAA had been reduced) to be SHAM-sensitive. The situation is further complicated by the cyanide-sensitivity of aspartate aminotransferase. Hence, malate, plus glutamate oxidation, in the absence of malic enzyme activity, appears to be inaccessible to the alternative path.

The situation is further complicated by the effect of exogenous NAD (9, 25, 26). The main difference between our results and those of Rustin *et al.* (26) is that adding NAD had no effect in the presence of cyanide in our mitochondria but stimulated alternative path activity in theirs. However, we consistently observe NAD stimulation of rotenone-resistant O_2 consumption (9). Adding NAD to plant mitochondria can have two effects; Douce and colleagues (21, 28) have presented evidence for active uptake of NAD leading to increased matrix NAD concentrations in mitochondria which have low endogenous NAD content. This, in turn, can stimulate substrate oxidation and electron transport (28). However, there is no doubt that under appropriate conditions reducing equivalents can be exported from the matrix in the presence of exogenous NAD (9). We have proposed the operation of a transmembrane transhydrogenase to explain this (9), but an exchange of added NAD for matrix NADH will also account for the results. Whatever the mechanism, NADH is generated in the intermembrane space and is reoxidized by the external dehydrogenase, thus relieving rotenone inhibition of O_2 uptake (9, 28). Inasmuch as external NADH oxidation in this tissue is largely cyanide-sensitive (7), it is unlikely that transhydrogenase activity will stimulate the alternative path. However, in NAD-depleted mitochondria, uptake of added NAD could stimulate alternative path activity (as well as malic enzyme). Obviously, the cauliflower mitochondria of Rustin *et al.* (25, 26) are physiologically different from ours; for example, they show substantial cyanide resistance, whereas ours do not. Other differences may also exist inasmuch as we find that the activity of cauliflower bud mitochondria varies significantly with the season, and with storage time and conditions. It is possible, therefore, that the mitochondria isolated by Rustin *et al.* (25, 26) are low in endogenous NAD, whereas ours are not, thus accounting for the different responses to added NAD.

In summary, we conclude that the NAD-linked enzymes of plant mitochondria have equal access to internal respiratory-linked NADH dehydrogenases, and to the alternative pathway. This fits well with the generally held concept of a concerted action of MDH and malic enzyme in the anaplerotic function of the citric acid cycle (14, 22, 29). That is, when carbon is exported from the mitochondria *in vivo* (*e.g.* as α-ketoglutarate for cytosolic nitrogen metabolism), carbon input to the tricarboxylic acid cycle must be in the form of an organic acid such as malate if the cycle is to continue to operate. Under these conditions, both malic enzyme and MDH function together to provide pyruvate and oxalacetate, respectively, and the action of the two enzymes must be coordinated to balance these metabolites. Such coordination would be difficult if the enzymes were associated with different respiratory chain components oxidizing NADH at different rates. Thus, if malic enzyme is specifically associated with nonphosphorylating electron transport pathways, it would operate faster than MDH under conditions of high phosphorylation potential, and the tricarboxylic acid cycle would cease to operate. Yet, it is under these very conditions that the cycle is likely to operate anaplerotically (14, 22). A possible exception to the above may occur in the leaves of C_4 and Crassulacean acid metabolism (CAM) plants (24), where malic enzyme operates independently of the tricarboxylic acid cycle to produce CO_2 and pyruvate (the latter leaving the mitochondria to be further metabolized by the chloroplasts). Under these conditions, it may be advantageous to have malic enzyme specifically linked to nonphosphorylating respiratory pathways. However, it should be noted that in CAM leaf mitochondria, the capacity of the alternative pathway is probably not great enough to support *in vivo* rates of malate decarboxylation, being no more than 20% of the capacity of the Cyt pathway (3, 6). It is also possible that malic enzyme may operate independently of the respiratory chain in these tissues, by means of substrate shuttles; in fact, in C_4 leaves this must be the case inasmuch as photosynthetic rates, and malic enzyme activity, are an order of magnitude faster than mitochondrial electron transport rates.

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