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Regulation of malate oxidation in plant mitochondria

Response to rotenone and exogenous NAD^+

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Exogenous NAD^+ stimulated the rotenone-resistant oxidation of all the NAD^+-linked tricarboxylic acid-cycle substrates in mitochondria from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers. The stimulation was not removed by the addition of EGTA, which is known to inhibit the oxidation of exogenous NADH. It is therefore concluded that added NAD^+ gains access to the matrix space and stimulates oxidation by the rotenone-resistant NADH dehydrogenase located on the matrix surface of the inner membrane. Added NAD^+ stimulated the activity of malic enzyme and displaced the equilibrium of malate dehydrogenase; both observations are consistent with entry of NAD^+ into the matrix space. Analysis of products of malate oxidation showed that rotenone-resistant oxygen uptake only occurred when the concentration of oxaloacetate was low and that of NADH was high. Thus it is proposed that the concentration of NADH regulates the activity of the two internal NADH dehydrogenases. Evidence is presented to suggest that the rotenone-resistant NADH dehydrogenase is engaged under conditions of high phosphorylation potential, which restricts electron flux through the rotenone-sensitive dehydrogenase (coupled to ATP synthesis).

Plant mitochondria differ from mammalian mitochondria in several respects. One important difference is the way in which they oxidize malate as a sole respiratory substrate without the necessity of removing oxaloacetate and the way in which the rate of malate oxidation responds to the addition of rotenone and exogenous NAD^+ (Palmer, 1976).

Product removal in the malate dehydrogenase reaction is a major problem, because of the low equilibrium constant (Stern *et al.*, 1952). If only the NADH is removed by oxidation via the internal NADH dehydrogenase, then the concentration of oxaloacetate will rise, thereby decreasing the equilibrium concentration of NADH and preventing respiration. The ability of plant mitochondria to oxidize malate is due to the presence of an NAD^+-linked malic enzyme (EC 1.1.1.39; Macrae, 1971). This enzyme circumvents the inhibition of malate oxidation imposed by high concentrations of oxaloacetate in two ways. Firstly, it will be able to provide a constant source of NADH even when malate dehydrogenase is in equilibrium. Secondly, pyruvate, the product of the malic enzyme, can be converted into acetyl-CoA by pyruvate dehydrogenase, thus enabling citrate synthase (EC 4.1.3.7) to remove oxaloacetate by condensing it with acetyl-CoA to form citrate.

Isolated plant mitochondria are more permeable to oxaloacetate than are mammalian mitochondria, and addition of oxaloacetate to plant mitochondria oxidizing malate results in a strong inhibition of oxygen consumption. The oxygen consumption eventually re-appears when the oxaloacetate has disappeared (Douce & Bonner, 1972; Brunton & Palmer, 1973). This has been explained by assuming that malate dehydrogenase, the equilibrium of which is displaced by the addition of oxaloacetate, uses the NADH produced by the malic enzyme to reduce oxaloacetate to malate. This process only stops when the concentration of oxaloacetate is sufficiently low to permit the concentration of NADH, in equilibrium with it, to act as a substrate for the NADH dehydrogenase.

The interaction of oxaloacetate with the two malate-oxidizing enzymes and the respiratory chain results in very complex patterns of respiration. The

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rate of malate oxidation progressively declines under State-3 (Chance & Williams, 1955) conditions owing to accumulation of oxaloacetate (Lance *et al.*, 1965, 1967; Palmer *et al.*, 1978; Tobin *et al.*, 1980), the rate of decrease being dependent on pH (Palmer *et al.*, 1978). It has also been observed that when rotenone was added to plant mitochondria oxidizing malate the initial inhibition was strong, but decreased after a few minutes (Brunton & Palmer, 1973). The initial strong inhibition caused by rotenone disappeared in the presence of an oxaloacetate-removing system (Palmer & Arron, 1976).

The rate of malate oxidation can frequently be stimulated by the addition of NAD^+, a coenzyme which has not been thought to be able to traverse the inner membrane of the mitochondrion. It is generally reported that exogenous NAD^+ selectively stimulates the rotenone-resistant oxidation of malate (Coleman & Palmer, 1972; Day & Wiskich, 1974*a*,*b*; Marx & Brinkmann, 1978). This phenomenon has been explained in three different ways.

(1) Palmer and co-workers have suggested that the stimulation of malate oxidation by NAD^+ could be due to the stimulation of malic enzyme located in the intermembrane space (Coleman & Palmer, 1972) and the stimulation of malate dehydrogenase from a small percentage of 'broken' mitochondria (Palmer, 1980). Both would have the effect of producing NADH in the intermembrane space which is oxidized by the external rotenone-resistant NADH dehydrogenase. However, a stimulation of piericidin-resistant and antimycin-sensitive electron transport from malate to ferricyanide by NAD^+ was also observed (Brunton & Palmer, 1973; Palmer & Arron, 1976). This indicates that an internal piericidin-resistant pathway was affected by exogenous NAD^+.

(2) Day & Wiskich (1974*a*,*b*) argued that malate uptake was a pre-requisite for the stimulation by NAD^+ and suggested that NAD^+ was acting as an external substrate for a transmembrane transhydrogenase which received its reducing equivalents from internal NADH. This model did not require a rotenone-resistant dehydrogenase present on the inside of the inner membrane.

(3) Finally, Neuburger & Douce (1978) and Tobin *et al.* (1980) have reported that NAD^+ can be taken up into intact plant mitochondria. They proposed that the concentration of endogenous NAD^+ is too low to saturate the malic enzyme and added NAD^+ enters the matrix space and stimulates the activity of the malic enzyme (Neuburger & Douce, 1978). This proposal provides no explanation concerning the mechanism by which the stimulated component of respiration became resistant to rotenone, the fact that favoured the external location of NADH oxidation adopted in the alternative proposals. There is a rotenone-resistant NADH dehydrogenase associated with the inner face of the inner membrane (Brunton & Palmer, 1973; Marx & Brinkmann, 1978). It is therefore possible that added NAD^+ could be accumulated in the matrix space and could give rise to NADH which could be oxidized directly from that compartment in a rotenone-resistant manner.

In the present study the interaction between the effect of added NAD^+ on the respiration of NAD^+-linked substrates with the respiratory state, rotenone-sensitivity and oxaloacetate concentrations is described in detail for Jerusalem-artichoke mitochondria. The results, which are most simply explained by assuming that NAD^+ does indeed penetrate the inner membrane of plant mitochondria (Neuburger & Douce, 1978; Tobin *et al.*, 1980), make it possible to predict the presence of two internal NADH dehydrogenases with different kinetic properties. One is the 'classical' rotenone-sensitive dehydrogenase linked to Site I of phosphorylation and having high affinity for NADH. The other is rotenone-resistant, bypasses Site I and has a much lower affinity for NADH. Their relative activity is controlled by the concentration of NADH in the matrix.

Direct kinetic analysis, using submitochondrial particles, shows that the rotenone-resistant dehydrogenase has a K_m of 80 μM for NADH, whereas the rotenone-sensitive component has a K_m of 10 μM (Møller & Palmer, 1982), which is consistent with the above proposals.

Materials and methods

Isolation of mitochondria

Mitochondria were isolated from Jerusalem-artichoke (*Helianthus tuberosus* L.) tubers essentially as described by Palmer & Kirk (1974).

Measurement of the respiratory activity

The mitochondrial respiration was measured polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, U.K.). Each assay was performed at 25°C with approx. 1 mg of mitochondrial protein in a final volume of 1.0 ml. The medium contained 0.3 M-sucrose, 10 mM-potassium phosphate buffer (pH 7.0), 10 mM-KCl, 5 mM-$MgCl_2$ and 0.1% (w/v) bovine serum albumin (Schwitzguébel *et al.*, 1981). Measurements were performed with or without 1 mM-EGTA as specified.

Citrate (BDH), *cis*-aconitate (Calbiochem), 2-oxoglutarate (Sigma), L-malate (Sigma), all at 25 mM, 50 mM-DL-isocitrate (Sigma) and 0.5 mM-NADH (Boehringer) were used as substrates. All were dissolved in water and neutralized. The oxidation of 2-oxoglutarate was measured in the presence of 1 mM-malate and 0.2 mM-thiamin pyro-

phosphate. When required, 0.1–0.5 mM-ADP or 0.5 mM-NAD^+ (both Boehringer) was added to the assay mixture. Rotenone (final concn. 20 μM) was used as an inhibitor of the NADH–ubiquinone oxidoreductase. It was dissolved in dimethyl sulphoxide at a concentration of 10 mM on the day of the experiment.

Analysis of product formation during malate oxidation

Mitochondria were incubated in a 40 ml Clark-type oxygen electrode fitted with a floating lid containing a sampling hole. The medium used was the respiratory medium described above plus 1 mM-EGTA. Final volume was 30 ml and the temperature 23–25°C. Additions were made as indicated in the legends to the Figures. Small-scale experiments (1 ml total volume) were always run in parallel as a control, and these gave similar oxygen-consumption curves.

At the times indicated a 1.0 ml sample was taken out and the reaction stopped by mixing with 125 μl of ice-cold 5.0 M-$HClO_4$. This mixture was left for 10 min and centrifuged at 12000 *g* for 5 min. Then 1 ml of the supernatant was neutralized by mixing with 175 μl of 0.83 M-triethanolamine/HCl + 2.5 M-K_2CO_3. This was left for 10 min and centrifuged at 12000 *g* for 5 min.

The concentrations of oxaloacetate and pyruvate in the supernatant of the neutralized sample were measured as follows. In a 3.0 ml cuvette were mixed 600 μl of 0.5 M-triethanolamine (pH 7.0), 1500 μl of water, 600 μl of neutralized sample and 30 μl of 14 mM-NADH in 5% (w/v) K_2CO_3. The A_{340} was read and checked after 5 min. The stability of the absorbance showed that no residual malate dehydrogenase activity was present. This contrasts with what has been reported by Wedding *et al.* (1976) and Tobin *et al.* (1980), and indicates that the above procedure for killing and neutralizing the sample was efficient in removing interfering enzymes.

Malate dehydrogenase (10 μl, containing 60 units; Boehringer no. 127 248) was added and A_{340} read after 10 min and again after 15 min as a control. The absorbance change was used to calculate the amount of oxaloacetate ($\varepsilon = 6.2\,mM^{-1} \cdot cm^{-1}$). Lactate dehydrogenase (10 μl, containing 11 units; Boehringer no. 127 221) was then added and again A_{340} was read after 10 and 15 min. From these absorbance changes the amount of pyruvate was calculated. Samples of the reaction mixture before and immediately after the addition of mitochondria were treated precisely as described above to determine the amounts of metabolites present at the start of the experiment.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951) after solubilizing the mitochondria with 0.5% (w/v) deoxycholate. Bovine serum albumin was used as the standard.

ADP determination

The concentration of ADP in the stock solution was determined by the use of a Boehringer Test Kit (no. 15 980).

Calculation of apparent ADP/O ratio for the NAD^+-induced component of mitochondrial respiration

The rate of oxidation with citrate, aconitate, isocitrate, malate and 2-oxoglutarate was measured in States 3 and 4 in the absence or presence of 0.5 mM-NAD^+ (1 mM-EGTA was always presesnt to inhibit exogenous NADH oxidation). ADP/O ratios were calculated for the two assay conditions. It was assumed that the flow of electrons in State 3 in the presence of NAD^+ was composed of two components: (a) an unaffected State-3 rate as measured in the absence of NAD^+ with the given ADP/O ratio and (b) an NAD^+-induced component, the ADP/O ratio of which was to be determined. That could be done by applying the following formula:

$$(r_+ - r_-) \times (ADP/O)_{NAD} + r_- \times (ADP/O)_- = r_+ \times (ADP/O)_+$$

where $(ADP/O)_{NAD}$, $(ADP/O)_-$ and $(ADP/O)_+$ are the ADP/O ratios of the NAD^+-induced component and of the overall rate in the absence and presence of NAD^+ respectively, and r_+ and r_- are the State-3 rates of oxidation in the presence and absence of NAD^+ respectively.

Results

Effect of NAD^+ and rotenone on the oxidation of NAD^+-linked substrates

State 3. The effect of NAD^+ on malate oxidation has been the subject of much debate, and has given rise to three different models to explain the phenomenon as outlined in the introduction. It is a well-established fact that exogenous NADH oxidation is inhibited strongly by EGTA (Coleman & Palmer, 1971; Earnshaw, 1975; Cowley & Palmer, 1978; Møller *et al.*, 1980, 1981), and this provides us with a tool for removing possible interference from this external rotenone-resistant component of NADH oxidation. A >90% inhibition of NADH oxidation by Jerusalem-artichoke mitochondria is observed with EGTA both in the absence and in the presence of rotenone (Table 1). Under these conditions (0.5 mM-NADH) the maximal interference from external NADH oxidation will thus be 21 or 14 nmol of O_2/min per mg of protein in the absence or presence of rotenone respectively. It should be emphasized that the oxidation of neither malate nor

Table 1. *Effect of EGTA on the oxidation of malate, citrate and NADH in the presence of rotenone and NAD$^+$*
The rate of oxidation in the presence of ADP is expressed as nmol of O_2/min per mg of protein. Results are means ± S.E.M. for the numbers of preparations of mitochondria shown in parentheses. Assay conditions are described in Materials and methods section. Abbreviation: NT, not tried.

Conditions	EGTA (1 mM)	Substrate		
		Malate	Citrate	NADH
Control	−	53 ± 5 (3)	42 ± 2 (4)	226 ± 17 (3)
	+	53 ± 7 (3)	40 ± 2 (4)	21 ± 3 (5)
+NAD$^+$	−	121 ± 10 (2)	53 ± 5 (3)	NT
	+	86 ± 11 (3)	47 ± 5 (4)	NT
+rotenone	−	25 ± 2 (3)	19 ± 3 (4)	185 ± 14 (4)
	+	18 ± 2 (4)	20 ± 1 (5)	14 ± 3 (4)
+NAD$^+$ + rotenone	−	54 ± 6 (2)	47 ± 3 (4)	NT
	+	39 ± 2 (3)	49 ± 2 (6)	NT

Table 2. *Effect of NAD$^+$ and rotenone on the oxidation rates of NAD$^+$-linked substrates*
Rates of oxidation are expressed as nmol of O_2 min per mg. Results are means ± S.E.M. for the numbers of preparations of mitochondria shown in parentheses. All measurements were made in the presence of 1 mM-EGTA. Conditions were otherwise as described in the Materials and methods section. In (*b*) the fast State 4 is reported for malate oxidation (see Figs. 2 and 3).

	Substrate	Control	+NAD$^+$	+Rotenone	+NAD$^+$ + rotenone
(*a*) State 3	Citrate	40 ± 2 (4)	47 ± 5 (4)	20 ± 1 (5)	49 ± 2 (6)
	Aconitate	74 ± 9 (3)	81 ± 5 (4)	22 ± 2 (5)	64 ± 2 (4)
	Isocitrate	40 ± 5 (4)	50 ± 5 (3)	17 ± 2 (4)	46 ± 3 (2)
	2-Oxoglutarate	69 ± 7 (4)	73 ± 5 (4)	33 ± 4 (3)	74 ± 3 (3)
	Malate	53 ± 7 (3)	86 ± 11 (3)	18 ± 2 (3)	39 ± 2 (3)
(*b*) State 4	Citrate	12 ± 1 (4)	17 ± 2 (4)	10 (1)	21 ± 2 (2)
	Aconitate	14 ± 1 (3)	20 ± 2 (4)	13 ± 1 (3)	21 ± 4 (4)
	Isocitrate	13 ± 2 (3)	19 ± 3 (3)	10 ± 1 (2)	18 ± 3 (2)
	2-Oxoglutarate	18 ± 2 (3)	20 ± 2 (3)	16 ± 2 (2)	21 ± 2 (2)
	Malate	16 ± 2 (3)	30 ± 4 (3)	18 (1)	23 ± 1 (2)

citrate is affected by EGTA in the absence of NAD$^+$ (Table 1).

Data presented in Table 1 show that the addition of NAD$^+$ caused only a slight stimulation of citrate oxidation in the absence of rotenone and a much more marked stimulation if added in the presence of rotenone. This stimulation was unaffected by adding EGTA and is therefore assumed to be mediated by the rotenone-resistant dehydrogenase associated with the matrix face of the inner membrane. Malate oxidation is stimulated by exogenous NAD$^+$ in both the absence and the presence of rotenone; in both cases the rate of oxidation after adding NAD$^+$ was partially sensitive to inhibition by EGTA. This oxidation of external NADH, not obvious with citrate, is attributed to the presence of soluble malate dehydrogenase released from the 2–5% of broken mitochondria present in the preparation (Palmer, 1980). However, the addition of NAD$^+$ to the control treatment, oxidizing malate in the presence of EGTA, resulted in a stimulation of 33 nmol of O_2 consumed/min per mg, which is significantly higher than the 21 nmol/min per mg found with exogenous NADH. Since only 0.5 mM-NAD$^+$ was added and spectroscopic experiments show only a small proportion of this to be reduced during malate oxidation (Tobin *et al.*, 1980), the concentration of external NADH would be very much less than 0.5 mM. Since the K_m of the external NADH oxidation is 50 μM (Møller & Palmer, 1981), it seems likely that it is not fully saturated under the conditions of assay used in Table 1, and any interference from the external enzyme would be less that the 21 nmol/min per mg reported in Table 1. Thus it is safe to conclude that the addition of NAD$^+$ stimulated the oxidation of malate by both the rotenone-resistant NADH dehydrogenases associated with the cytosolic and matrix face of the inner membrane.

The data presented in Table 2(*a*) show that, under State-3 conditions and in the presence of EGTA and absence of rotenone, NAD$^+$ stimulated only the oxidation of malate. Rotenone inhibited the oxidation of all the substrates tested, and under these conditions added NAD$^+$ restored the rate to the

original State-3 rate, except with malate, where there was only a partial recovery. In the presence of rotenone, NAD^+ clearly stimulates respiration even in the presence of EGTA, and this stimulation is therefore not due to the external NADH dehydrogenase. This constitutes conclusive evidence against the involvement of a putative transmembrane transhydrogenase (Day & Wiskich, 1974*a*,*b*) in the stimulation by NAD^+.

State 4. In Table 2(*b*) is shown the effect of NAD^+ and rotenone on the State-4 respiration rate with the same substrates as in Table 2(*a*). NAD^+ has a slight stimulatory effect on the rate of oxidation of all substrates in both the presence and the absence of rotenone. The most important difference from State 3 is that rotenone does not inhibit State-4 respiration with any of the substrates in the absence or the presence of NAD^+.

Effect of NAD^+ on product formation during the oxidation of malate

The influence of added NAD^+ on the products of malate oxidation is shown in Fig. 1. The experiment was performed in the presence of ADP and EGTA. Oxaloacetate accumulated and reached a plateau, whereas pyruvate production was linear. NAD^+ was added after 5½min. Oxaloacetate production was resumed, but appeared to approach a new constant concentration. Both the production of pyruvate and the consumption of oxygen were stimulated by the addition of NAD^+. It is clear that exogenous NAD^+ stimulated the activity of the malic enzyme as well as shifting the equilibrium of the malate dehydrogenase. It is noteworthy that oxygen uptake always takes at least 30s to respond fully to added NAD^+, and in Fig. 1 it is clear that oxaloacetate did not start to increase until after 30s. This could represent the time taken to accumulate NAD^+ in the matrix space.

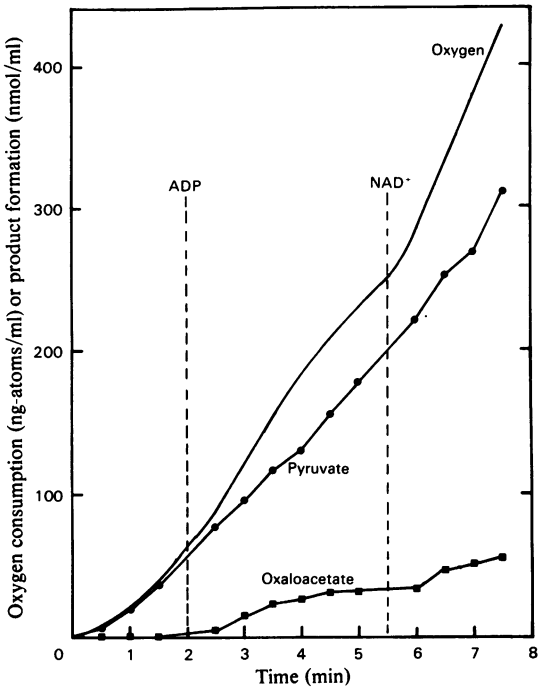

Fig. 1. *Product analysis during NAD^+ stimulation of malate oxidation*

The measurement of respiration and the analysis of samples taken at the times indicated were performed as described in the Materials and methods section, with 0.6mg of mitochondrial protein/ml. Oxygen consumption (continuous line) is given as ng-atoms/ml and the production of pyruvate (●) and oxaloacetate (■) as nmol/ml. ADP (600μM) and NAD^+ (0.5mM) were added where indicated.

Effect of NAD^+ on the ADP/O ratio

It has previously been observed that the ADP/O ratio with NAD^+-linked substrates was decreased by one-third in the presence of rotenone (Brunton & Palmer, 1973; Day & Wiskich, 1974*a*,*b*; Marx & Brinkmann, 1978), indicating that the rotenone-resistant pathway is non-phosphorylating. The results obtained with all substrates used in the present study (in the presence of EGTA) confirm this observation (results not shown). It has also been reported that the ADP/O ratio with malate seemed to decrease in the presence of added NAD^+ (Day & Wiskich, 1974*a*,*b*). This was taken to be evidence for the operation of the external NADH dehydrogenase (coupled with the postulated transmembrane transhydrogenase), which by-passes Site I (Palmer & Passam, 1971). However, this decrease in ADP/O ratio can be observed with all NAD^+-linked substrates in the presence of EGTA to inhibit the external NADH dehydrogenase (Table 3). When the apparent ADP/O ratio is calculated for the NAD^+-induced component of State 3 with the various substrates as explained in the Materials and methods section, one arrives at an ADP/O ratio of 0.9 ± 0.1. Compared with the ADP/O ratio of 1.8–2.0 observed when the electrons pass all three sites of phosphorylation (Table 3), this indicates that the NAD^+-induced component by-passed (at least) one site of phosphorylation.

Under no conditions tested could we detect the induction by NAD^+ of an alternative, cyanide-resistant, salicylhydroxamic acid-sensitive oxidation of malate in Jerusalem-artichoke mitochondria (results not shown). Such an induction has been reported for mitochondria from potatoes (Rustin & Moreau, 1979; Rustin *et al.*, 1980).

Non-linear rates of malate oxidation

NAD^+ was shown to affect product formation during malate oxidation (Fig. 1). NAD^+ also

Table 3. *Effect of NAD$^+$ on the ADP/O ratios*

The ADP/O ratio for the NAD$^+$-induced component was calculated (see the Materials and methods section) by using concomitant values from the original oxygen electrode traces to obtain maximal precision. Thus it is not possible to use the respiratory rates from Table 2(*a*) and the ADP/O ratios in this Table to arrive at the value given. Results are means ± S.E.M. for the numbers of preparations shown in parentheses.

	ADP/O ratio		
Substrate	No NAD$^+$	+NAD$^+$	NAD$^+$-induced component
Citrate	1.95 ± 0.03 (4)	1.70 ± 0.09 (4)	
Aconitate	1.89 ± 0.00 (2)	1.65 ± 0.02 (2)	
Isocitrate	1.77 ± 0.04 (2)	1.63 ± 0.02 (2)	0.89 ± 0.11 (12)
2-Oxoglutarate	2.32 ± 0.18 (2)	2.17 ± 0.14 (2)	
Malate	1.82 ± 0.07 (3)	1.60 ± 0.04 (3)	

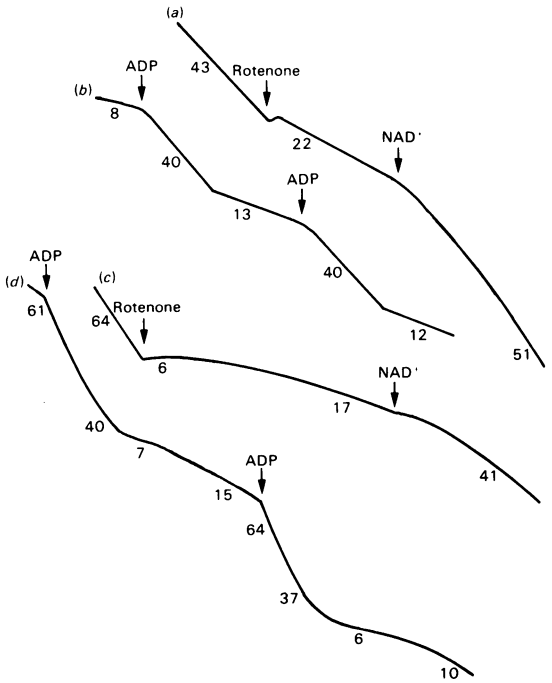

Fig. 2. *Oxidation of malate and citrate: effect of State 3, State 4, rotenone and NAD$^+$*

The oxidation of citrate (traces *a* and *b*) and malate (traces *c* and *d*) was measured as described in the Materials and methods section by using 0.7–1.2 mg of mitochondrial protein/ml. In (*a*) and (*c*) 1.0 mM-ADP was present from the start. Other additions were 20 μM-rotenone and 0.5 mM-NAD$^+$ (*a* and *c*) and 0.2 mM-ADP (*b* and *d*). Numbers on the traces are rates of oxidation in nmol of O_2/min per mg.

affected rotenone-sensitivity, which, in turn, was affected by the respiratory state of the mitochondria (Tables 1 and 2). It was therefore decided to characterize further the connection between rotenone-sensitivity, respiratory state and product formation during malate oxidation by Jerusalem-artichoke mitochondria.

The oxygen-electrode traces shown in Fig. 2 illustrate some of the complexities of malate oxidation as compared with oxidation of citrate (used as in Table 1 as a typical representative of other NAD$^+$-linked substrates) by Jerusalem-artichoke mitochondria. The following differences are apparent. (1) The rate of oxidation in neither State 3 nor 4 is linear when malate is the substrate. State 3 shows a slow decrease and State 4 is initially very slow, but increases rapidly to reach a linear rate (Fig. 2*d*). Such biphasic State-4 curves have been described previously (Lance *et al.*, 1965, 1967). With citrate, linear oxidation rates are always observed (Fig. 2*b*). (2) When rotenone is added to mitochondria oxidizing malate, a very strong initial inhibition is observed, but this is partly relieved with time (Fig. 2*c*). With citrate the inhibition is smaller and the resistant rate is constant (Fig. 2*a*). (3) When NAD$^+$ is added to mitochondria inhibited by rotenone, a stimulation is observed such that when citrate is used as the substrate the initial State-3 rate is fully recovered; when malate is used, NAD$^+$ causes only a partial recovery of the rotenone-inhibited rate (Figs. 2*a* and 2*c*).

Product formation during malate oxidation in States 3 and 4

It has previously been shown that oxaloacetate accumulates during State 3 when mitochondria oxidize malate at neutral pH, and because the malate dehydrogenase is in equilibrium the concentration of NADH must progressively decrease; this is the reason for the steadily decreasing rate of respiration (Lance *et al.*, 1965, 1967; Douce & Bonner, 1972; Palmer *et al.*, 1978; Tobin *et al.*, 1980). Fig. 3 shows the results of an experiment in which samples were analysed for pyruvate and oxaloacetate at intervals

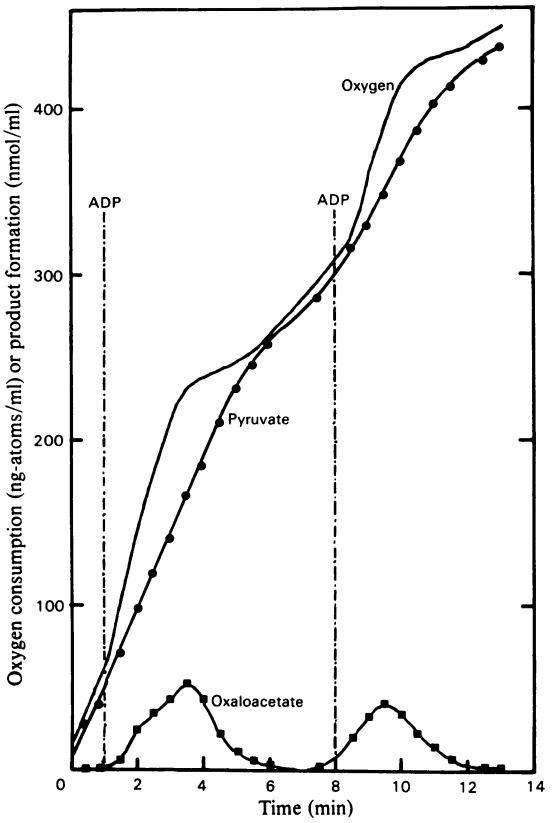

Fig. 3. *Product analysis during State-3/State-4 cycles of malate oxidation*

Methods and symbols are as for Fig. 1, except that 0.5 mg of mitochondrial protein/ml was used. ADP was added at 1 min (0.35 mM) and at 8 min (0.25 mM).

during two State-3/State-4 cycles of malate oxidation. The results confirm that oxaloacetate accumulates during State 3, but what is most striking is that oxaloacetate disappears again during the initial slow part of State 4. This is clearly observed in both ADP cycles (Fig. 3). Only when oxaloacetate has disappeared completely (at 6 min and at 12 min) is the faster State-4 rate seen. Under the conditions used, pyruvate accumulation only varies slightly between States 3 and 4. Thus there is a clear correlation between the rate of malate oxidation and the accumulation of oxaloacetate during State 4 as well as during State 3.

Product formation during rotenone-inhibition of malate oxidation

In Fig. 2(*c*) rotenone gave a strong initial inhibition of malate oxidation, and the rate increased after a few minutes. Products were determined

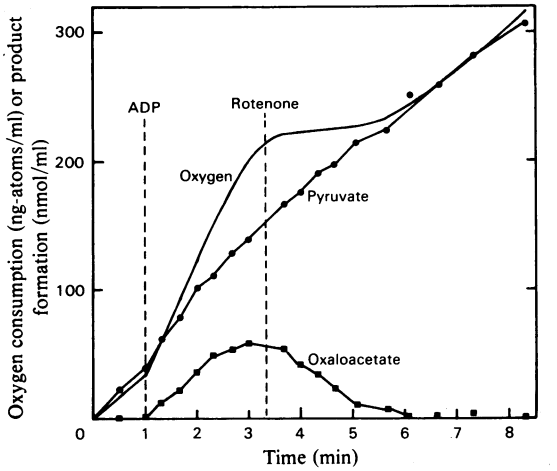

Fig. 4. *Product analysis during rotenone inhibition of malate oxidation*

Methods and symbols are as for Fig. 1, except that 0.7 mg of mitochondrial protein/ml was used. ADP (1 mM) and rotenone (20 μM) were added where indicated.

during a scaled-up version of this experiment, and the results are shown in Fig. 4. Oxaloacetate accumulates and causes a slight inhibition of O_2 uptake; at this point (after 3 min) rotenone was added. This resulted in a strong inhibition of oxygen uptake, which coincided with the removal of oxaloacetate. After 6 min oxaloacetate had completely disappeared and oxygen consumption reached a new linear rate, slower than before the addition of rotenone. Throughout this experiment pyruvate production was almost constant.

It should be noticed that in both Figs. 2 and 3 the sum of pyruvate and oxaloacetate produced matched the consumption of oxygen atoms consumed very closely, indicating that little or no pyruvate and oxaloacetate were removed in these mitochondria by pyruvate dehydrogenase and citrate synthase respectively. Tobin *et al.* (1980) included arsenite in the assays to inhibit pyruvate dehydrogenase activity. This was clearly unnecessary with Jerusalem-artichoke mitochondria, and it seems to be due to a lack of thiamin pyrophosphate in these mitochondria such that pyruvate dehydrogenase is inactive (Al-Sané, 1981; D. A. Moss & J. M. Palmer, unpublished work).

Discussion

NAD^+ stimulation of mitochondrial respiration

The results in Tables 1 and 2 clearly demonstrate that NAD^+ stimulates the rotenone-resistant oxidation of NAD^+-linked substrates by Jerusalem-

artichoke mitochondria in the presence of EGTA, which severely inhibits the activity of the external NADH dehydrogenase. The results are most easily explained by assuming that the added NAD^+ brought about the stimulation by entering the matrix space (Neuburger & Douce, 1978; Tobin *et al.*, 1980), where it was reduced by the NAD^+-linked dehydrogenases and re-oxidized by a rotenone-resistant NADH dehydrogenase located on the inner face of the inner membrane (Brunton & Palmer, 1973; Palmer & Arron, 1976; Marx & Brinkmann, 1978). This rotenone-resistant NADH dehydrogenase is only coupled to two sites of ATP synthesis (Table 3). The results are not in agreement with the involvement of a transmembrane transhydrogenase as suggested by Day (Day & Wiskich, 1974*a*,*b*; Hanson & Day, 1980). Neither are the results consistent with the effect being due to a stimulation of malic enzyme (Coleman & Palmer, 1972; Brunton & Palmer, 1973; Palmer & Arron, 1976) or malate dehydrogenase (Palmer, 1980) outside the inner membrane.

Jerusalem artichoke mitochondria contain a relatively low concentration of NAD^+ (1nmol/mg = 1 mM, assuming a matrix volume of 1 μl/mg; Al-Sané, 1981), and the accumulation of NAD^+ would alter the equilibrium of malate dehydrogenase towards the production of more NADH and oxaloacetate. This increase in oxaloacetate was detected in the results presented in Fig. 1. However, although the addition of NAD^+ would result in an increase in the concentration of NADH, the reduction level of the nicotinamide nucleotide pool would become more positive if the malate dehydrogenase reaction is maintained in equilibrium. It seems therefore that an increase in the concentration of NADH in the matrix space is responsible for the induction of the rotenone-resistant NADH oxidation. This would be possible if the rotenone-resistant NADH dehydrogenase has a higher K_m for NADH. In the following sections we shall see that the other results presented in this paper are also consistent with this suggestion.

The only assumption made in the analysis is that malate dehydrogenase is operating at or close to equilibrium. In Jerusalem-artichoke mitochondria malate dehydrogenase is present in great excess capable of reducing NAD^+ at the rate of 2000nmol/min per mg, compared with 300nmol/min per mg for isocitrate dehydrogenase (Palmer, 1980; Møller, 1981), and there is therefore good reason to believe that its equilibrium can respond rapidly to changes in the concentration of any one of the substrates. Results with mung-bean mitochondria show a similar relative excess of malate dehydrogenase activity over that of other enzymes (Bowman *et al.*, 1976), indicating that it may well be a general phenomenon in plant mitochondria.

Inhibition of mitochondrial respiration by rotenone

Rotenone inhibits the oxidation of all NAD^+-linked substrates strongly under State-3 conditions (Table 2*a*, Figs. 2 and 4) and very weakly or not at all under State-4 conditions (Table 2*b*). In the presence of NAD^+ only the oxidation of malate in State 3 is rotenone-sensitive (Table 2*a*). Thus the sensitivity to rotenone may be dependent on the concentration of NADH, which would be higher under State-4 conditions than under State-3 conditions. The degree of rotenone inhibition is also linked to the concentration of oxaloacetate. The data presented in Fig. 4 shows that rotenone inhibited strongly until all the oxaloacetate had disappeared. At this point it is assumed that the concentration of NADH in equilibrium with the oxaloacetate, via the malate dehydrogenase, would have risen sufficiently to engage the rotenone-resistant low-affinity NADH dehydrogenase postulated above.

Variation in the concentration of oxaloacetate during the oxidation of malate under State-3 and -4 conditions

The non-linear rates of oxidation of malate during State-3 or State-4 conditions (traces *c* and *d*, Fig. 2) were shown to be connected with changes in the concentration of oxaloacetate present in the assay system (Fig. 3). During State 3 oxaloacetate accumulated, and this caused a suppression of the oxidation rate, presumably by a lowering of the concentration of NADH in equilibrium with it through the malate dehydrogenase. When ADP was exhausted a very slow State 4 ensued, during which oxaloacetate disappeared, whereas pyruvate continued to be produced at the rate observed in State 3 (Fig. 3). Clearly the reducing equivalents produced by malic enzyme were being used to force malate dehydrogenase in the reverse direction and thereby remove oxaloacetate. Only when oxaloacetate had disappeared was the NADH concentration allowed to rise sufficiently to be oxidized via the NADH dehydrogenase by-passing the first site of phosphorylation. The rate of pyruvate production slowed down when oxaloacetate had disappeared (Fig. 3), presumably because the electron-transport chain was slower than malic enzyme under State-4 conditions.

The addition of rotenone to mitochondria oxidizing malate in State 3 caused a similar sequence of events (Fig. 4). The only difference was that pyruvate production was constant even after the resumption of oxygen consumption (Figs. 3 and 4), which indicates that malic enzyme and not electron transport was rate-limiting.

Conclusions

The results demonstrate the presence of a second internal NADH dehydrogenase in plant mitochon-

dria. It is only active under conditions where the matrix concentration of NADH is high, it is rotenone-resistant and the electrons from it bypass the first site of phosphorylation. The 'classical', rotenone-sensitive NADH dehydrogenase, on the other hand, can operate under conditions of low NADH concentration. The presence of the two internal dehydrogenases is in agreement with the results from a kinetic study of NADH oxidation by inside-out submitochondrial particles from plant mitochondria (Møller & Palmer, 1982).

The rate of oxidation of NAD^+-linked substrates by plant mitochondria is regulated by the presence of the two NADH dehydrogenases with their different kinetic properties and by the equilibrium of malate dehydrogenase.

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