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The internal rotenone-insensitive NADPH dehydrogenase contributes to malate oxidation by potato tuber and pea leaf mitochondria

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Inside-out submitochondrial particles from both potato (*Solanum tuberosum* L. cv. Bintje) tubers and pea (*Pisum sativum* L. cv. Oregon) leaves possess three distinct dehydrogenase activities: Complex I catalyzes the rotenone-sensitive oxidation of deamino-NADH, ND_{in}(NADPH) catalyzes the rotenone-insensitive and Ca^{2+}-dependent oxidation of NADPH and ND_{in}(NADH) catalyzes the rotenone-insensitive and Ca^{2+}-independent oxidation of NADH. Diphenylene iodonium (DPI) inhibits complex I, ND_{in}(NADPH) and ND_{in}(NADH) activity with a K_i of 3.7, 0.17 and 63 μM, respectively, and the 400-fold difference in K_i between the two ND_{in} made possible the use of DPI inhibition to estimate ND_{in}(NADPH) contribution to malate oxidation by intact mitochondria. The oxidation of malate in the presence of rotenone by intact mitochondria from both species was inhibited by 5 μM DPI. The maximum decrease in rate was 10–20 nmol O_2 mg^{-1} min^{-1}. The reduction level of NAD(P) was manipulated by measuring malate oxidation in state 3 at pH 7.2 and 6.8 and in the presence and absence of an oxaloacetate-removing system. The inhibition by DPI was largest under conditions of high NAD(P) reduction. Control experiments showed that 125 μM DPI had no effect on the activities of malate dehydrogenase (with NADH or NADPH) or malic enzyme (with NAD^+ or $NADP^+$) in a matrix extract from either species. Malate dehydrogenase was unable to use $NADP^+$ in the forward reaction. DPI at 125 μM did not have any effect on succinate oxidation by intact mitochondria of either species. We conclude that the inhibition caused by DPI in the presence of rotenone in plant mitochondria oxidizing malate is due to inhibition of ND_{in}(NADPH) oxidizing NADPH. Thus, NADP turnover contributes to malate oxidation by plant mitochondria.

Abbreviations – MDH, malate dehydrogense; ME, malic enzyme; ND_{in}(NADH), internal rotenone- and DPI-insensitive NADH dehydrogenase; ND_{in}(NADPH), internal rotenone-insensitive, DPI-sensitive NADPH dehydrogenase; OAA, oxaloacetate; SMP, submitochondrial particle(s); TX, Triton X-100.

Introduction

Malate, an intermediate of the Krebs cycle and a key compound involved in the dynamic regulation of many physiological processes in plants (e.g., stomate opening/closing, C_4 photosynthesis, CAM metabolism), can be oxidized by two distinct enzymes in the matrix of plant mitochondria. The Krebs cycle enzyme NAD^+-malate dehydrogenase converts malate + $NAD(P)^+$ into oxaloacetate (OAA) and NAD(P)H, whereas malic enzyme converts malate + $NAD(P)^+$ into pyruvate and NAD(P)H. The NAD(P)H can subsequently be reoxidized by the respiratory chain NAD(P)H dehydrogenases on the inner surface of the inner mitochondrial membrane to give rise to ATP synthesis.

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The pattern of malate oxidation by isolated plant mitochondria, and presumably also in the intact cell, is very complex due not only to the presence of two oxidizing enzymes, but also to the presence of several NAD(P)H dehydrogenases (Møller and Lin 1986 and references therein). There is general agreement that two dehydrogenases, one rotenone-sensitive with a low K_m(NADH) (complex I), the other rotenone-insensitive with a high K_m(NADH), are involved in the oxidation of matrix NADH (Møller and Palmer 1982, Palmer et al. 1982, Møller and Lin 1986). Until 1991 it was assumed that only NADH was involved, but following the discovery of the NADPH dehydrogenase on the inner, matrix surface of the inner membrane (Rasmusson and Møller 1991a,b, Melo et al. 1996) the question arose as to whether this enzyme also contributes to malate oxidation.

Diphenylene iodonium (DPI) is an inhibitor of a number of flavoproteins, amongst those complex I of the respiratory chain (Majander et al. 1994, Melo et al. 1996). DPI inhibits external NADPH oxidation by plant mitochondria at submicromolar concentrations (Roberts et al. 1995). It was recently found that in potato tuber submitochondrial particles (SMP), 5 μM DPI inhibits the internal NADPH dehydrogenase completely, apparently without affecting the rotenone-insensitive NADH dehydrogenase (Melo et al. 1996) and since DPI has an electron structure that permits a delocalized charge, we hypothesized that it might be able to cross the inner membrane of plant mitochondria despite its positive charge. In the present study we have used DPI (in addition to rotenone to inhibit complex I) to estimate the contribution of the DPI-sensitive NADPH dehydrogenase and therefore of NADP to mitochondrial malate oxidation. Throughout this study we compare the responses of mitochondria from potato tubers, a relatively inactive tissue, with mitochondria from pea leaves, a photosynthesizing tissue with quite different metabolic demands on its mitochondria (Krömer 1996).

Throughout the remainder of this paper we will use the nomenclature of Møller and Rasmusson (1998) and refer to the internal rotenone- and DPI-insensitive Ca^{2+}-independent NADH dehydrogenase (Møller and Palmer 1982) as ND_{in}(NADH) and to the internal rotenone-insensitive, DPI-sensitive, Ca^{2+}-dependent NADPH dehydrogenase (Rasmusson and Møller 1991a,b, Melo et al. 1996) as ND_{in}(NADPH).

Materials and methods

Preparation of mitochondria and inside-out submitochondrial particles

Tubers of potato (*Solanum tuberosum* L. cv. Bintje) were purchased from local markets. Mitochondria and inside-out submitochondrial particles (SMP) were isolated as described by Struglics et al. (1993) and Rasmusson and Møller (1991a), respectively.

Pea (*Pisum sativum* L. cv. Oregon sugar pod II) plants were grown in trays with soil in a controlled environment growth chamber for 14 days under a 12-h photoperiod at 25°C and 60% relative humidity. PAR was 400 $\mu mol\ m^{-2}\ s^{-1}$ provided by metal arc lamps (fluorescent tubes type F72T 12/CW/VHO, Osram Sylvania Inc., Danvers, MA, USA). Mitochondria and inside-out SMP were isolated from green leaves as described by Day et al. (1985) and Møller et al. (1987), respectively.

NAD^+ loading of potato tuber mitochondria

The mitochondria were suspended at a protein concentration of 2.8 $mg\ ml^{-1}$ in incubation buffer containing 0.3 *M* sucrose, 5 m*M* MOPS, 5 m*M* KH_2PO_4, 2.5 m*M* $MgCl_2$, 25 m*M* malate, 25 m*M* glutamate, 0.1 m*M* NAD^+, 2 m*M* ADP and 0.01% (w/v) BSA (pH 7.2). Following a 5-min incubation at room temperature (22–24°C), the mitochondrial suspension was diluted with wash medium (0.3 *M* mannitol, 10 m*M* TES [pH 7.2] and 0.1% [w/v] BSA) and centrifuged at 39 000 *g* for 5 min. This wash procedure was repeated to remove all external NAD^+.

Measurement of oxygen consumption

Respiration was measured polarographically as oxygen consumption using a Rank Brothers oxygen electrode (Rank Brothers, Cambridge, UK) at 25°C. For potato mitochondria and SMP the reaction medium contained 0.3 *M* sucrose, 5 m*M* MOPS, 5 m*M* KH_2PO_4, 2.5 m*M* $MgCl_2$, and 0.01% (w/v) BSA (pH 7.2). EGTA at 1 m*M* [to inhibit ND_{ex} activity (Palmer et al. 1982)] and ADP at 2 m*M* (to ensure state 3 throughout the experiment) were added when conducting experiments on mitochondria. FCCP was added at 0.4 μM when conducting experiments on SMP.

For pea leaf mitochondria and SMP, the reaction medium contained 0.3 *M* mannitol, 10 m*M* MOPS, 10 m*M* KH_2PO_4, 10 m*M* KCl, 5 m*M* $MgCl_2$, 0.1% (w/v) BSA (pH 7.2 or 6.8 as indicated in the legend to the tables). One m*M* EGTA was always added to mitochondria to inhibit ND_{ex} activity (Palmer et al. 1982).

The concentrations of respiratory substrates were 10 m*M* (pea) or 25 m*M* (potato) malate, 10 m*M* (pea) or 25 m*M* (potato) glutamate, 10 m*M* succinate, 1 m*M* deamino (DA)-NADH, 1 m*M* NADH, 1 m*M* NADPH.

Enzyme assays

Cytochrome *c* oxidase (EC 1.9.3.1) activity was determined in the presence or absence of 0.025% (w/v) Triton X-100 (TX). The percentage latency of this enzyme was calculated as $100 \times [(\text{rate} + \text{TX}) - (\text{rate} - \text{TX})/(\text{rate} + \text{TX})]$ (Møller et al. 1987, Rasmusson and Møller 1991a).

In potato tuber mitochondria the activity of NAD^+-malate dehydrogenase (EC 1.1.1.37) was assayed in the reverse direction [oxidation of NAD(P)H] according to Møller et al. (1987) with either 0.2 m*M* NADH or 0.2 m*M* NADPH and in the forward direction [reduction of $NAD(P)^+$] as described by Yoshida (1969).

In potato tuber mitochondria NAD^+-malic enzyme (EC 1.1.1.39) activity was assayed in the forward direction (decarboxylation) essentially as described in Day et al. (1984), however, with slight modifications in the assay medium which contained 50 m*M* MOPS, pH 6.8, 2 m*M* $MnCl_2$, 4 m*M* dithiothreitol (DTT), 1 m*M* NAD^+ or $NADP^+$, 5 μ*M* antimycin A, 10 m*M* malate, 14.5 μ*M* coenzyme A (CoA) and 25 μ*M* NADH.

In pea leaf mitochondria the activity of NAD^+-malate dehydrogenase (EC 1.1.1.37) was assayed in the reverse direction according to Cooper and Beevers (1969) at pH 7.5 in 50 m*M* MOPS-KOH buffer containing 2.3 m*M* oxaloacetate and 0.25 m*M* NADH or 0.25 m*M* NADPH. In the forward direction the reaction was determined essentially as described by Yoshida (1969).

In pea leaf mitochondria NAD^+-malic enzyme (EC 1.1.1.39) was assayed in the forward direction essentially according to Hatch et al. (1982) in 25 m*M* MOPS-KOH, pH 6.8, 5 m*M* malate, 0.1 m*M* CoA, 5 m*M* DTT, 0.2 m*M* EDTA, 4 m*M* $MnSO_4$, 2 m*M* NAD^+ or 2 m*M* $NADP^+$.

In all assays of malate dehydrogenase and malic enzyme activity 0.025–0.040% (w/v) Triton X-100 was added to disrupt the membrane.

Source of chemicals

DPI-chloride was from Molecular Probes, Inc. (Eugene, OR, USA) or from Sigma (D-2926) and $NADP^+$ was from Fluka (product number 93200, $<0.05\%$ NAD^+).

Protein estimation

Mitochondrial protein was measured according to Lowry et al. (1951), with BSA as standard. In the case of mitochondria isolated from pea leaves this was corrected for the contribution by broken thylakoids by assuming a thylakoid protein to chlorophyll ratio of 7 (Nash and Wiskich 1983). Chlorophyll was determined by the method of Arnon (1949). The contamination by chloroplast proteins never exceeded 5% of the amount of mitochondrial protein.

Results and discussion

The inhibition of malate oxidation by DPI (in the presence of rotenone to inhibit complex I) was used as a measure of the involvement of ND_{in}(NADPH) in mitochondria from potato tubers and pea leaves. Five conditions, however, have to be met to allow such an interpretation: (1) ND_{in}(NADPH) is present in both potato tuber and pea leaf mitochondria. (2) ND_{in}(NADPH) is at least one order of magnitude more sensitive to DPI than ND_{in}(NADH). (3) No other component in the electron transport chain is sensitive to DPI. (4) The soluble enzymes, malate dehydrogenase (MDH) and malic enzyme (ME), responsible for the oxidation of malate in the mitochondrial matrix are not inhibited by DPI. (5) DPI is able to penetrate both the outer and inner membrane of intact mitochondria and gain access to the NAD(P)H dehydrogenases on the matrix surface of the inner mitochondrial membrane.

In the following experiments we have tested the above conditions.

ND_{in}(NADPH) is also present in pea leaf mitochondria

Using inside-out SMP from potato tuber it has previously been shown that 5 μ*M* DPI inhibits complex I activity (measured as DA-NADH oxidation) and NADPH oxidation completely, but not rotenone-insensitive NADH oxidation. This was interpreted to mean that there are three NAD(P)H dehydrogenases on the inner, matrix surface of the inner membrane all linked to the respiratory chain (Melo et al. 1996). When experiments were performed on inside-out SMP from pea leaf very similar results were obtained (Table 1). Both NADH and NADPH gave increased oxidation rates when added on top of DA-NADH indicating that additional dehydrogenase(s) are engaged. DA-NADH oxidation was completely inhibited by rotenone and DPI (complex I activity; Rasmusson et al. 1991a). NADPH was oxidized in a Ca^{2+}-dependent manner in the presence of rotenone and this oxidation was completely inhibited by 5 μ*M* DPI [ND_{in}(NADPH) activity; Rasmusson and Møller 1991b, Melo et al. 1996]. In the presence of DPI and independent of the presence of Ca^{2+}, the addition of NADH gave rise to a high rate of oxidation [ND_{in}(NADH) activity; Melo et al. 1996]. All of the above activities measured as oxygen consumption were inhibited by antimycin A indicating that electron flow via complex III and complex IV of the electron transport chain was involved (Table 1).

ND_{in}(NADPH) in potato tuber mitochondria is at least two orders of magnitude more sensitive to DPI than ND_{in}(NADH)

SMP isolated from potato tuber mitochondria were found to be 90% inside-out as determined by the latency of cytochrome *c* oxidase (Table 1) (Møller et al.

Table 1. The oxidation of deamino-NADH, NADH and NADPH by inside-out SMP from pea leaf mitochondria and the sensitivity of these activities to calcium and inhibitors. Oxygen consumption was measured as nmol O_2 (mg protein)$^{-1}$ min^{-1}. EGTA (1 m*M*), $CaCl_2$ (2 m*M*) and antimycin A (0.4 μ*M*) were added where indicated. The results presented are from two independent preparations of SMP which showed a cytochrome *c* oxidase activity of 4.6 μmol (mg protein)$^{-1}$ min^{-1} with a latency of 90%.

Exp.	Additions	Oxygen consumption
1	Deamino-NADH	337
	+ NADH	711
2	Deamino-NADH	319
	+ NADPH	404
3	Deamino-NADH	425
	+ DPI	0
4	Deamino-NADH	430
	+ 20 μ*M* rotenone	0
	+ NADPH	134
	+ 5 μ*M* DPI	0
	+ NADH	422
	+ antimycin A	0
5	NADPH + EGTA + 20 μ*M* rotenone	25
	+ $CaCl_2$	142
	+ antimycin A	0
6	NADH + EGTA + 5 μ*M* DPI	266
	+ $CaCl_2$	248
	+ antimycin A	0

1987, 1996). The oxidation of (1) DA-NADH (complex I), (2) NADPH in the presence of rotenone and Ca^{2+} [ND_{in}(NADPH)], and (3) NADH in the presence of rotenone (+EGTA to inhibit any ND_{ex} activity contributed by the small contamination of right-side-out SMP) [ND_{in}(NADH)], was measured in the presence of various concentrations of DPI (Table 2). The inhibition by DPI was relatively slow to develop, similar to what has been observed with other enzymes (Majander et al. 1994, Roberts et al. 1995). With 5 μ*M* DPI, NADPH (+rotenone) oxidation was completely inhibited after 2 min whereas deamino-NADH oxidation (complex I) took 5–6 min to be fully inhibited (results not shown). For this reason the data presented in Table 2 were all calculated as the slope of the trace between 6 and 7 min after DPI addition.

ND_{in}(NADPH) was fully inhibited by submicromolar concentrations of DPI whereas ND_{in}(NADH) was unaffected by 25 μ*M* DPI. When the K_i was calculated it was found to be 400 times higher for ND_{in}(NADH) than for ND_{in}(NADPH) (Table 2).

Succinate oxidation is completely unaffected by high concentrations of DPI

The oxidation of succinate by intact potato tuber and pea leaf mitochondria was unaffected by the addition of 125 μ*M* DPI (results not shown), indicating that no electron transport component after ubiquinone was inhibited by DPI. This conclusion, however, depends on the ability of DPI to cross the inner mitochondrial membrane (see below).

MDH and ME both take NADP as coenzyme and neither is affected by DPI

MDH from both potato tuber and pea leaf mitochondria showed activity with NADH and NADPH, but the activity with NADH was about 100 times higher than that with NADPH (Table 3). Similarly, ME from both types of mitochondria showed activity with NAD^+ and $NADP^+$. However, by comparison, the activity with NAD^+ was only about twice as high as that with $NADP^+$. The above results are consistent with previous reports on mitochondrial ME and MDH (Macrae 1971, Grover et al. 1981, Rasmusson and Møller 1990).

MDH did not form any NADPH when incubated with $NADP^+$ and malate in either of the two types of plant mitochondria (Table 3). We are not aware of any previous observations of this kind with plant MDH. If this behaviour also reflects the properties of MDH in the matrix of intact mitochondria, any ND_{in}(NADPH) activity observed during malate oxidation must be due to the activity of malic enzyme alone.

None of the enzyme activities tested in Table 3 was sensitive to DPI even at 125 μ*M*, which is 25 times higher than the concentration used in the experiments presented in Tables 4–6.

On the basis of all these control experiments we conclude that four of the five conditions listed above are met. It follows that, provided DPI crosses both membranes, any inhibition of malate oxidation by 5 μ*M* DPI (over and above that of rotenone) in either potato tuber or pea leaf mitochondria must be due to the engagement of ND_{in}(NADPH) that cannot be compensated for by an increased activity of ND_{in}(NADH). Such an inhibition must therefore indicate an involvement of NADP in malate oxidation.

Table 2. Sensitivity of NAD(P)H dehydrogenases to DPI measured on inside-out SMP from potato tuber mitochondria. Complex I activity was measured as deamino-NADH oxidation, ND_{in}(NADPH) activity as NADPH oxidation in the presence of 20 μ*M* rotenone + 1 m*M* $CaCl_2$, and ND_{in}(NADH) activity as NADH oxidation in the presence of 20 μ*M* rotenone + 1 m*M* EGTA. In all cases DPI was added as soon as the rate was linear and the slope measured after 7 min. Data given as means ± SD (n = 3 independent preparations).

Activity	Rate, nmol O_2 (mg protein)$^{-1}$ min^{-1}	K_i(DPI), μ*M*
Complex I	476 ± 64	3.7 ± 0.4
ND_{in}(NADPH)	94 ± 15	0.17 ± 0.02
ND_{in}(NADH)	73 ± 13	63 ± 2

Table 3. The specificity and DPI sensitivity of MDH and ME in potato tuber and pea leaf mitochondria. Controls included 0.6% DMSO and usually showed 1–5% inhibition of the activity for pea leaf mitochondria and 0–10% stimulation for potato tuber mitochondria compared to controls measured without DMSO. All results are from a representative experiment. Similar results were obtained on 2–3 other preparations of mitochondria. None of the % changes are significant. NM, not measured.

Enzyme		Activity, μmol (mg protein)$^{-1}$ min^{-1}	
		Control	+125 μ*M* DPI (% change)
Potato tuber mitochondria			
MDH	NADH (reverse)	22.0	23.3 (+6)
	NADPH (reverse)	0.32	0.32 (±0)
	NAD^+ (forward)	10.2	NM
	$NADP^+$ (forward)	<0.01	NM
ME	NAD^+	0.63	0.65 (+3)
	$NADP^+$	0.23	0.25 (+8)
Pea leaf mitochondria			
MDH	NADH (reverse)	25.7	25.5 (−1)
	NADPH (reverse)	0.26	0.27 (+4)
	NAD^+ (forward)	15.6	NM
	$NADP^+$ (forward)	<0.002	NM
ME	NAD^+	0.65	0.66 (+2)
	$NADP^+$	0.34	0.32 (−6)

Malate oxidation by intact potato tuber mitochondria is inhibited by DPI

In the absence of rotenone, 5 μ*M* DPI inhibited state 3 malate oxidation strongly. The inhibition took about 6 min to be complete (results not shown) which is similar to the time-course for DPI inhibition of deamino-NADH oxidation by SMP reported above. This indicates that DPI does cross the inner mitochondrial membrane and inhibits the matrix-facing NAD(P)H dehydrogenases. Thus, all five conditions are met.

Malate (+glutamate) oxidation in state 3 was only partially inhibited by rotenone, whether or not the mitochondria had been loaded with NAD^+, in agreement with the literature (e.g., Tobin et al. 1980, Palmer et al. 1982, Neuburger et al. 1984). The rate in the presence of rotenone was linear for at least 10 min. DPI at 5 μ*M*, added 2 min after the addition of rotenone, decreased the malate oxidation rate reproducibly by about 10 nmol (mg protein)$^{-1}$ min^{-1}, again independent of whether the mitochondria had been loaded with NAD^+ or not (Table 4).

The effect of loading the potato tuber mitochondria with NAD^+ was to increase the rotenone- and DPI-insensitive rate of oxidation catalyzed by ND_{in}(NADH). Neither complex I activity (the inhibition by rotenone) nor ND_{in}(NADPH) activity (the inhibition by DPI) was affected (Table 4). This is consistent with previous observations that ND_{in}(NADH) has a high K_m(NADH) (Møller and Palmer 1982, Palmer et al. 1982, Møller and Lin 1986, Rasmusson and Møller 1991a). Interestingly, NAD^+-loading had no effect on the DPI inhibition in the presence of rotenone. Perhaps the concentration and/or the reduction level of NADP(H) is not coupled to that of NAD(H). A coupling between the reduction levels would be expected in the presence of a transhydrogenase (Carlenor et al. 1988, Hoek and Rydström 1988). Alternatively, the capacity of ND_{in}(NADPH) is fully engaged in the absence of added NAD^+ so that an increase in NADPH concentration will not lead to an increase in the rate of oxygen consumption.

Malate oxidation was also carried out at pH 6.5 in an attempt to optimise conditions for ME. This would increase the reduction levels of pyridine nucleotides in the mitochondrial matrix and engage the ND_{in}(NADPH). However, as rates were curvelinear during the incubation with both rotenone and DPI, the inhibitory effects were difficult to evaluate and are therefore not reported.

Although the inhibition of complex I by rotenone has a K_i of less than 1 μ*M* (Rasmusson and Møller 1991a) we thought it possible that the inhibition by DPI could be due to an incomplete inhibition of complex I by rotenone whereby the addition of DPI would merely complete that inhibition. However, the same inhibitory effect of DPI was observed in the presence of 20 and 40 μ*M* rotenone (results not shown), ruling out this possibility.

DPI inhibition of malate oxidation depends on the NAD(P) reduction level in pea leaf mitochondria

Malate oxidation (in the presence of rotenone) by pea leaf mitochondria was inhibited by 5 μ*M* DPI at pH 7.2, but only when NAD^+ was added (Table 5). At pH 7.2 MDH is relatively active compared to ME and the equilibrium concentration of NAD(P)H is likely to be low (Palmer et al. 1982, Neuburger et al. 1984, Møller and Lin 1986). Therefore, the effect of DPI was tested under two different conditions where the reduction level of NAD(P) would be higher: malate oxidation at lower pH (pH 6.8) and in the presence of an OAA-removing system.

Table 4. Effect of DPI on malate (+glutamate) oxidation in state 3 by potato tuber mitochondria as affected by NAD^+ loading. Rotenone and DPI were added at 20 μ*M* and 5 μ*M*, respectively. Data are given as mean ± SD (n = number of independent preparations).

Conditions	Oxidation rate, nmol O_2 (mg protein)$^{-1}$ min^{-1}		
	Control mitochondria (n = 6)	NAD^+ loaded mitochondria (n = 3)	Effect of NAD^+
Mal/glu	75 ± 10	124 ± 20	+49
+Rotenone	44 ± 10	91 ± 14	+47
+DPI	33 ± 10	79 ± 13	+46
ΔRotenone	32 ± 10	34 ± 10	+2
ΔDPI	11 ± 3	11 ± 2	+0

At pH 6.8 ME is more active than MDH (Tobin et al. 1980) and the NAD pool is more reduced (Neuburger et al. 1984). Under these conditions DPI had a clear inhibitory effect both in the presence and absence of NAD^+ (Table 5). As for potato tuber mitochondria, NAD^+ loading of pea leaf mitochondria (done during the assay) strongly increased the activity of ND_{in}(NADH) (=rate in the presence of rotenone and DPI) at both pH 7.2 and 6.8 (Table 5).

Another way of increasing the reduction level of NAD(P) is by removing oxaloacetate, the product of MDH activity (Tobin et al. 1980, Palmer et al. 1982). This was done by adding glutamate to the reaction mixture in all the experiments shown for potato tuber mitochondria and in Table 6 for pea leaf mitochondria. Under these conditions DPI inhibited malate oxidation by pea leaf mitochondria even in the absence of external NAD^+, in fact the inhibition seemed smaller in the presence of NAD^+ (Table 6). Since MDH does not reduce $NADP^+$, at least not in vitro (Table 3), the NADPH oxidized by ND_{in}(NADPH) must derive from ME. The effect of glutamate is therefore to remove oxaloacetate and prevent the reverse reaction of MDH which can oxidize both NADH and NADPH.

Table 5. Effect of DPI on malate oxidation in state 3 by pea leaf mitochondria as affected by NAD^+ loading at pH 7.2 and 6.8. NAD^+ loading was done during the assay with 0.5 m*M* NAD^+. Malate, 20 μ*M* rotenone and 5 μ*M* DPI were added consecutively where indicated. Each one of the values is an average of 2–4 measurements on a representative preparation of mitochondra, but similar results were obtained on two other preparations.

Conditions	Oxidation rate, nmol O_2 (mg protein)$^{-1}$ min^{-1}		
	Control mitochondria	+NAD^+	Effect of NAD^+
pH 7.2			
Mal	102	159	+57
+Rotenone	12	73	+61
+DPI	9	57	+48
ΔRotenone	90	86	−4
ΔDPI	3	16	+13
pH 6.8			
Mal	77	104	+27
+Rotenone	39	68	+29
+DPI	25	53	+28
ΔRotenone	38	36	−2
ΔDPI	14	15	+1

The involvement of NADP in malate oxidation

The results presented in Tables 4–6 show that DPI inhibited malate oxidation in the presence of rotenone, both for potato tuber and pea leaf mitochondria. This indicates that ND_{in}(NADPH) was active and that it contributed to malate oxidation by 10–20 nmol O_2 (mg protein)$^{-1}$ min^{-1}. The size of the contribution, however, depended on the relative reduction level of pyridine nucleotides, which in turn varies depending on the assay conditions (Neuburger et al. 1984; S. C. Agius, A. G. Rasmusson, H.-E. Åkerlund and I. M. Møller, unpublished results). In mitochondria oxidizing malate under conditions where MDH is operative (e.g. at pH >7), OAA accumulation will maintain a relatively oxidized pool of NAD(P)H, thereby preventing the oxidation of NAD(P)H via the rotenone-insensitive pathway (Møller and Lin 1986). This would explain the absence of inhibition by DPI of malate oxidation by pea leaf mitochondria at pH 7.2 in the presence of rotenone and absence of external NAD^+ (Table 5). However, an alternative explanation is that there was no NADPH synthesis during malate oxidation unless malic enzyme was active because MDH is unable to use $NADP^+$ in the forward direction (Table 3). The properties and regulation of matrix NAD^+-MDH require much more attention.

By comparison, under conditions where malic enzyme is active due to lower pH in the matrix (due to lower pH in the medium) (Table 5) or due to NAD^+ loading of the mitochondria (Tables 4–6) (Macrae 1971, Tobin et al. 1980, Palmer et al. 1982) the pyridine nucleotides will be relatively more reduced and this in turn will engage the rotenone-insensitive dehydrogenases (Neuburger et al. 1984, Møller and Lin 1986). Therefore under these conditions the activity of ND_{in}(NADPH) was most apparent.

Table 6. Effect of DPI on malate (+glutamate) oxidation in state 3 at pH 7.2 by pea leaf mitochondria as affected by NAD^+ loading. The results are from one preparation of mitochondria, but similar results were obtained on three other preparations.

Conditions	Oxidation rate, nmol O_2 (mg protein)$^{-1}$ min^{-1}		
	Control mitochondria	+ NAD^+	Effect of NAD^+
Mal/glu	94	133	+39
+Rotenone	33	78	+45
+DPI	16	70	+54
ΔRotenone	61	55	−6
ΔDPI	17	8	−9

The question though arises as to what extent the reduction level of NADP follows that of NAD? In mammalian mitochondria NADP is kept in a much more reduced state than NAD through the action of the energy-linked transhydrogenase (Hoek and Rydström 1988), the presence of which has been reported (Carlenor et al. 1988) but not confirmed (Rasmusson 1994, Møller and Rasmusson 1998) for plant mitochondria as yet.

The concentration of NADP in the matrix of potato tuber mitochondria has been reported to be 0.3–0.7 nmol (mg protein)$^{-1}$ as measured by enzymatic analysis (Brinkman et al. 1973) or NMR analysis (Roberts et al. 1997). Our own preliminary data for potato tuber mitochondria indicate a concentration of 0.1–0.2 nmol NADP (mg protein)$^{-1}$ as measured by HPLC analysis (S. C. Agius, A. G. Rasmusson, H.-E. Åkerlund and I. M. Møller, unpublished results). There is therefore agreement that the concentration of NADP in the mitochondrial matrix is below that of NAD which is 1–5 nmol (mg protein)$^{-1}$ (Tobin et al. 1980). The origin of this matrix NADP is unknown at present. It might be taken up from the cytoplasm as suggested by Rasmusson and Møller (1990), or it might be synthesized in the matrix perhaps from NAD^+ by a matrix NAD^+ kinase. Although the concentration of NADP is low, it should be kept in mind that even 0.1 nmol (mg protein)$^{-1}$ NADP(H) is equivalent to about 0.1 m*M* in the matrix (based on the assumption of a matrix volume of 1 µl per mg protein). Assuming that the NADP is fully reduced, this concentration is sufficient to engage ND_{in}(NADPH), the K_m(NADPH) of which is 25 µ*M* (Rasmusson and Møller 1991a), to at least 80% of its capacity.

The V_{max} for ND_{in}(NADPH) in potato tuber SMP is 40–150 nmol O_2 mg^{-1} min^{-1} at pH 7.2, but only about half that at pH 7.7 (Table 3; Rasmusson and Møller 1991a,b, Melo et al. 1996). Actively respiring mitochondria maintain a pH gradient across the inner mitchondrial membrane such that the pH in the matrix is higher than the pH of the cytoplasm which is typically around 7.5 (Kurkdjian and Guern 1989). Since the inner membrane enrichment is about 2-fold in SMP over intact mitochondria (Rasmusson and Møller 1991a) this means that in intact potato tuber mitochondria one should not expect a V_{max} for ND_{in}(NADPH) of more than 10–40 nmol O_2 (mg protein)$^{-1}$ min^{-1} when measuring at pH 7.2. Thus, the observed contributions of ND_{in}(NADPH) to malate oxidation are of the right magnitude.

The physiological importance of ND_{in}(NADPH)

We do not know to what extent ND_{in}(NADPH) contributes to malate oxidation in the absence of DPI, nor do we know whether the enzyme contributes to the oxidation of other Krebs cycle substrates. Upon inhibition of ND_{in}(NADPH) by DPI, the reduction level of NADP must increase. If there is a coupling between the reduction levels of NADP and NAD (see above), then the matrix concentration of NADH might also increase and this would lead to an increased ND_{in}(NADH) activity. In both cases electron flux through ND_{in}(NADPH) are diverted by DPI and the true contribution by the enzyme would be underestimated. A similar problem was encountered with the alternative oxidase and it was only when the oxygen isotope discrimination method was developed, which avoids the use of inhibitors, that it was possible to estimate the true contribution by the alternative oxidase to substrate oxidation (Ribas-Carbo et al. 1995).

Since ND_{in}(NADPH) is not proton pumping it may function as an overflow mechanism under conditions where the cell needs to turn over large amounts of respiratory substrate(s) at a high ATP/ADP ratio, e.g., at elevated rates of photorespiration or glycolysis. The regulation of ND_{in}(NADPH) activity will have a direct effect on the reduction level of NADP in the matrix and thus on a number of biochemical processes where NADP is a coenzyme such as folate biosynthesis, thioredoxin and glutathione turnover and activation of the alternative oxidase (Vanlerberghe et al. 1995, Møller and Rasmusson 1998).

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