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Evidence for the presence of two rotenone-insensitive NAD(P)H dehydrogenases on the inner surface of the inner membrane of potato tuber mitochondria

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Abstract

Submitochondrial particles were isolated from potato (*Solanum tuberosum* L.) tubers. The latency of cytochrome-*c* oxidase and succinate dehydrogenase indicated that they were 90% inside-out. The inability of the submitochondrial particles to form a membrane potential inside negative as monitored by safranine absorbance changes and their ability to form a large membrane potential inside positive as monitored by oxonol VI absorbance changes confirmed this sidedness. Through the use of rotenone to inhibit Complex I, and diphenyleneiodonium to inhibit both Complex I (by binding to the FMN in the active site) as well as rotenone-insensitive NADPH oxidation, it was possible to distinguish three different NAD(P)H dehydrogenases on the inner surface of the inner mitochondrial membrane: (1) a rotenone-sensitive, diphenyleneiodonium-sensitive, Ca^{2+}-independent enzyme which prefers NADH as the substrate, i.e., Complex I; (2) a rotenone-insensitive, diphenyleneiodonium-sensitive, Ca^{2+}-dependent NAD(P)H dehydrogenase; (3) a rotenone-insensitive, diphenyleneiodonium-insensitive, Ca^{2+}-independent NADH dehydrogenase. All three enzymes were linked to the electron transport chain before Complex III as shown by antimycin A sensitivity and to proton pumping as shown by the generation of a membrane potential. The possible significance of these three enzymes for the function of the mitochondrion in the plant cell is discussed.

Keywords: Mitochondrion (plant); NAD(P)H dehydrogenase; Potato; Respiratory chain; Rotenone-insensitive; (*Solanum tuberosum*)

1. Introduction

The electron transport chain of all mitochondria except those from baker's yeast contains Complex I, the rotenone-sensitive NADH dehydrogenase. This is a multisubunit complex encoded by both nuclear and mitochondrial genes [1]. It is the only NAD(P)H dehydrogenase in mammalian mitochondria in which, as a consequence, the oxidation of NAD^+-linked substrates is completely inhibited at very low concentrations of rotenone. In contrast, inhibition of the oxidation of NAD^+-linked substrates by plant mitochondria requires much higher concentrations of rotenone and there is a considerable proportion of rotenone-insensitive oxidation ([2] and references therein). In the presence of rotenone, the oxidation of NAD^+-linked substrates has an ADP/O ratio only 2/3 that of uninhibited electron transport, indicating that a site of proton pumping is bypassed, i.e., that in Complex I [3–6].

Plant and fungal mitochondria have the ability to oxidize exogenous NADH and NADPH [7,8]. This oxidation is dependent on Ca^{2+} at neutral pH [9–11]. Circumstantial evidence has indicated that this oxidation is caused by two separate enzymes [7,12,13], a model recently confirmed in a study that showed that diphenyleneiodonium completely inhibits external NADPH oxidation but has a much smaller effect on NADH oxidation [14].

Møller and Palmer [15] detected two NADH dehydrogenase activities on the inner surface of the inner membrane of Jerusalem artichoke mitochondria. One had a low K_m for NADH and was sensitive to rotenone and was concluded to be due to Complex I. The other activity had a 10-fold higher K_m for NADH and was rotenone-insensi-

Abbreviations: DPI, diphenyleneiodonium; SMP, submitochondrial particles.

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tive. It was suggested to be due to a separate dehydrogenase, although a pathway within Complex I bypassing the sites of rotenone inhibition and proton pumping could not be excluded [15]. The discovery of the presence of two internal NAD(P)H dehydrogenases with different kinetic properties made it possible to rationalize many puzzling observations on substrate oxidation in plant mitochondria [6,7].

Deamino-NADH and deamino-NADPH oxidation by inside-out SMP from potato tubers is completely rotenone-sensitive, showing that it is only oxidized by Complex I. In contrast, the oxidation of NADH and NADPH is partly rotenone-insensitive, indicating that it is indeed partly caused by at least one separate dehydrogenase [16]. These results were confirmed by Menz et al. [17]. The rotenone-insensitive dehydrogenase activity can use both NADH and NADPH as substrates [16] and the NADPH oxidation is Ca^{2+}-dependent [18].

Previous studies have thus identified a total of four NAD(P)H dehydrogenases in the electron transport chain of plant mitochondria, two on the outer surface of the inner membrane and two on the inner surface, one of which is Complex I. In the present study we provide evidence for the presence of two rotenone-insensitive NAD(P)H dehydrogenases on the inner surface of the inner membrane of plant mitochondria, one Ca^{2+}-dependent that prefers NADPH as substrate, the other Ca^{2+}-independent and specific for NADH. This brings the total number of NAD(P)H dehydrogenases associated with the electron transport chain in plant mitochondria up to five and we speculate on the possible roles of these enzymes in plant metabolism.

2. Materials and methods

2.1. Preparation of mitochondria and inside-out submitochondrial particles

Potato (*Solanum tuberosum* L.) tubers were purchased at the local market. Mitochondria and inside-out submitochondrial particles (SMP) were isolated as described by Struglics et al. [19] and Rasmussen and Møller [16], respectively. When not used immediately, the SMP were frozen in liquid nitrogen with 5% (v/v) dimethylsulfoxide and stored at −80°C.

2.2. Monitoring the membrane potential

The transmembrane potential of inside-out submitochondrial particles was monitored with an Aminco DW2 spectrophotometer essentially as described by Bashford and Thayer [20] and Moore and Bonner [21] using oxonol VI (Molecular Probes, Inc., Eugene, OR, USA) and safranine (Sigma S-2255), respectively.

The reactions were run at room temperature (22–24°C) in 0.3 M sucrose, 5 mM morpholinopropanesulfonic acid, 5 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 0.02% (w/v) BSA (pH 7.2). The concentration of respiratory substrate was 1 mM deamino-NADH, 1 mM NADH, 1 mM NADPH or 10 mM succinate.

2.3. Enzyme assays

Cytochrome-*c* oxidase (CCO) (EC 1.9.3.1) activity was determined in the presence or absence of 0.025% (w/v) Triton X-100 [22].

NAD^+-linked malate dehydrogenase (MDH) (EC 1.1.1.37) activity was measured as in Ref. [23].

Succinate dehydrogenase (SDH) (EC 1.3.99.1) activity was measured at room temperature in 0.3 M sucrose, 5 mM morpholinopropanesulfonic acid, 5 mM KH_2PO_4, 2.5 mM $MgCl_2$ (pH 7.2), 10 mM succinate, 0.2 μg/ml antimycin A, 1 mM potassium ferricyanide plus or minus 0.025% (w/v) Triton X-100. Samples were preincubated with succinate for 5 min to activate SDH before adding antimycin and ferricyanide. The reaction was followed at 420 nm.

The percentage of latent activity of these enzymes was calculated as $100 \times \{[(\text{rate} + \text{Triton})-(\text{rate-Triton})]/(\text{rate} + \text{Triton})\}$.

Electron transport from several substrates to oxygen was measured in an oxygen electrode (Rank Brothers, Cambridge, UK) at 25°C in 0.3 M sucrose, 5 mM morpholinopropanesulfonic acid, 5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.4 μM FCCP and 0.02% (w/v) BSA (pH 7.2). The concentration of respiratory substrate was 1 mM deamino-NADH, 1 mM NADH, 1 mM NADPH or 10 mM succinate.

2.4. Protein

Protein was determined as described by Lowry et al. [24] using bovine serum albumin as standard.

2.5. Statistics

The results presented are from seven different preparations of SMP. Each experiment was done on at least three different preparations (fresh and/or frozen/thawed) and similar results were obtained.

3. Results

3.1. The experimental material

The SMP isolated as described in Section 2 were used either fresh or thawed after having been frozen in liquid nitrogen and stored at −80°C in the wash medium containing 5% (v/v) dimethylsulfoxide. No differences could be detected between freshly isolated and frozen/thawed SMP with respect to enzyme activities, enzyme latencies

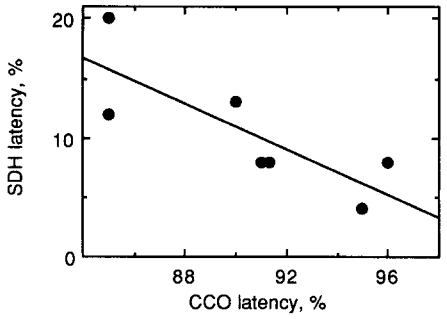

Fig. 1. Correlation between the latency of CCO and SDH measured on potato tuber SMP. Each point represents an independent preparation of SMP.

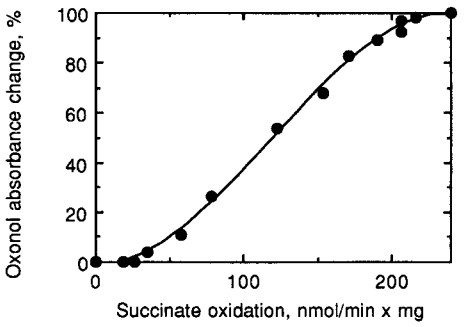

Fig. 2. The relationship between the oxonol absorbance change as a measure of the membrane potential and the rate of succinate oxidation by SMP. The rate of succinate oxidation was measured in the oxygen electrode with 10 mM succinate and increasing amounts of malonate (0.02–7.4 mM) added to inhibit the succinate oxidation. The oxonol absorbance change was measured in a spectrophotometer under similar conditions. Frozen/thawed SMP, 0.2 mg protein/ml.

or the ability to generate a membrane potential as monitored by oxonol absorbance changes (results not shown). However, every experiment was performed at least once on freshly isolated SMP to ensure that the results were not artifacts caused by the freezing and thawing procedure.

Seven independent preparations of SMP had an average latency value for CCO activity of $90 \pm 2\%$ (mean $\pm$ S.E.) and $10 \pm 2\%$ for SDH activity. The high latency of CCO activity indicates that the binding site for cytochrome *c* on CCO is behind a permeability barrier, i.e., inside a sealed vesicle. Since the active site for cytochrome *c* on CCO is on the outer surface of the inner mitochondrial membrane this means that the SMP were inside-out. The active site for SDH is on the opposite side of the inner membrane, i.e., the matrix side, and the low latency is therefore consistent with the presence of predominantly inside-out vesicles. Since there is a linear correlation between the two latencies (Fig. 1) and the latencies add up to 100%, it is likely that neither CCO nor SDH are activated or inactivated by the detergent [25].

The SMP could not generate a membrane potential inside negative as detected by safranine absorbance changes (not shown). This is consistent with the absence of right-side-out SMP in the preparations.

The SMP generated a membrane potential positive inside, as monitored by oxonol VI absorbance changes, with several substrates. Succinate, deamino-NADH and NADH gave potentials of similar size, whereas the membrane potential generated with NADPH as the substrate was usually smaller (not shown). This is probably due to the relatively slow rate of NADPH oxidation, insufficient to maintain the maximal potential in the face of proton leakage from the vesicles. The relationship between the rate of electron transport with succinate as the substrate and the oxonol absorbance change is shown in Fig. 2. An oxidation rate of about 200 nmol oxygen $min^{-1} mg^{-1}$ was required to give the maximum absorbance change, a rate that exceeds that observed with NADPH (see below). Note that NADPH oxidation, in the absence of rotenone, is catalyzed both by complex I and by a rotenone-insensitive NADPH dehydrogenase [16].

There is no simple relationship that permits the calculation of the size of the membrane potential from the magnitude of the oxonol response in SMP [20], so in the remainder of this paper we will treat the oxonol signal as being a semiquantitative measure of the membrane potential.

3.2. The effect of rotenone and diphenyleneiodonium on the oxidation of deamino-NADH and NAD(P)H

The oxidation of deamino-NADH by inside-out SMP was completely inhibited by rotenone (Table 1), confirming previous reports that deamino-NADH is only oxidized by Complex I [16,17]. The membrane potential generated with deamino-NADH was also completely collapsed by the addition of rotenone (Fig. 3).

Table 1
The effect of rotenone, diphenyleneiodonium and antimycin A on the oxidation of deamino-NADH, NADH and NADPH by inside-out SMP

Additions	Oxygen consumption, nmol/min × mg
Deamino-NADH	445
+ DPI	14
Deamino-NADH	576
+ rotenone	0
+ NADPH	71
+ antimycin A	0
Deamino-NADH	576
+ rotenone	0
+ NADPH	71
+ DPI	16
+ NADH	348
+ antimycin A	0

The three separate experiments were done in the presence of 1 mM $CaCl_2$. Final concentrations were 1 mM deamino-NADH, 1 mM NADH, 1 mM NADPH, 0.4 μM antimycin A, 20 μM rotenone and 6 μM diphenyleneiodonium (DPI). The plus signs indicate consecutive additions.

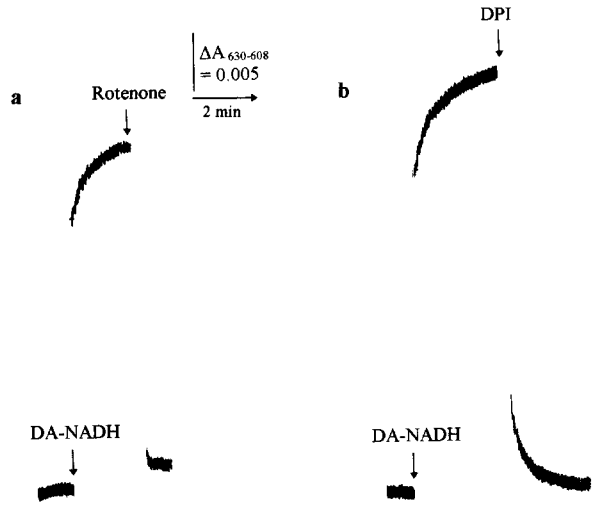

Fig. 3. Effect of rotenone (a) and DPI (b) on the membrane potential generated by the oxidation of deamino-NADH. Inside-out SMP at 0.2 mg protein/ml and Ca^{2+} at 1 mM were present. Where indicated deamino-NADH at 1 mM, rotenone at 20 μM and DPI at 6 μM were added.

Diphenyleneiodonium gave the same results as rotenone (Table 1, Fig. 3), consistent with the conclusion by Majander et al. [26] that diphenyleneiodonium inhibits Complex I by binding to the FMN. The concentration of diphenyleneiodonium chosen (6 μM) is well in excess of that required to inhibit Complex I [26]. At the same time, it has little or no effect on external NADH oxidation by intact potato tuber mitochondria or on the membrane potential this oxidation creates (i.e., no uncoupling effect), while giving a complete inhibition of external NADPH oxidation [14]. The advantage with an inhibitor like diphenyleneiodonium that binds directly at the active site of Complex I is that its presence precludes the possibility of rotenone-insensitive NAD(P)H oxidation by Complex I. Such an activity has been proposed to be induced by the addition of UQ1 to SMP [17] and could be caused by a bypass of the rotenone binding site. This binding site is near the quinone binding site at the opposite end of the electron transport chain within the complex [1]. The rationale of the following experiments is therefore that any NAD(P)H oxidation by the inside-out SMP observed in the presence of diphenyleneiodonium will be catalyzed by an enzyme or enzymes distinct from Complex I.

When NADPH was added after deamino-NADH oxidation had been inhibited by rotenone, oxygen consumption resumed due to electron transport through complex III as shown by the antimycin sensitivity of the NADPH oxidation (Table 1). At the same time a small membrane potential was generated that could be collapsed by antimycin (Fig. 4a). The rotenone-insensitive oxidation of NADPH and the resulting generation of a membrane potential were both inhibited strongly by diphenyleneiodonium (Table 1, Fig. 4b), indicating that the Ca^{2+}-dependent NAD(P)H dehydrogenase described by Rasmusson and Møller [16,18] is diphenyleneiodonium-sensitive.

In the presence of both rotenone and diphenyleneiodonium, i.e., under conditions where Complex I is inhibited at the flavin and the rotenone-insensitive, Ca^{2+}-dependent NAD(P)H dehydrogenase is also inhibited, the addition of NADH gave rise to a rapid rate of oxygen consumption and the generation of a membrane potential which were inhibited by antimycin A and collapsed by antimycin A, respectively (Table 2, Fig. 4b). This clearly indicates that there is a third dehydrogenase on the inner surface of the inner membrane of potato tuber mitochondria also linked to the electron transport chain via complexes III and IV and proton pumping by those two complexes. The signal

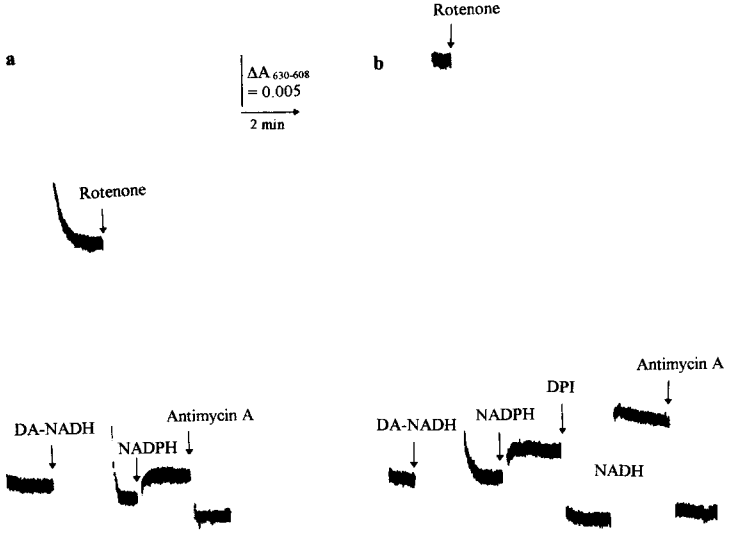

Fig. 4. Generation of a membrane potential by different NAD(P)H dehydrogenase activities on inside-out SMP. Inside-out SMP at 0.2 mg protein/ml and Ca^{2+} at 1 mM were present. Other additions were 1 mM deamino-NADH, 1 mM NADPH, 1 mM NADH, 20 μM rotenone, 0.4 μg/ml antimycin A and 6 μM DPI.

Table 2
The oxidation of deamino-NADH, NADH and NADPH by inside-out SMP in the presence and absence of Ca^{2+}

Substrate	Oxygen consumption, nmol/min × mg	
	EGTA	$CaCl_2$
Deamino-NADH	522	518
NADH	536	533
NADPH	70	130

The SMP were preincubated for 1 min in the presence of either EGTA (1 mM) or $CaCl_2$ (1 mM) and the reaction started by the addition of substrate. Final concentrations were 1 mM deamino-NADH, 1 mM NADH and 1 mM NADPH.

with NADH is relatively small in the experiment shown in Fig. 4b, in spite of the rapid rate of NADH oxidation observed (Table 4).

A membrane potential generated by NADH oxidation can be consecutively reduced by addition of rotenone, DPI and finally antimycin A (Fig. 5) consistent with the experiments shown in Fig. 4.

3.3. The requirement for calcium ions

All of the previous experiments on NAD(P)H oxidation were performed in the presence of Ca^{2+}. In the presence of EGTA to remove all Ca^{2+} there was no effect on the oxidation of NADH and deamino-NADH (Table 2). Only the oxidation of NADPH was partly Ca^{2+}-dependent (Table 2). NADPH oxidation is catalyzed both by complex I, which is independent of Ca^{2+}, as well as by a rotenone-insensitive, Ca^{2+}-dependent NAD(P)H dehydrogenase ([18]; Table 2).

In the presence of rotenone to inhibit Complex I, the oxidation of NADPH was completely Ca^{2+}-dependent (Table 3), consistent with the results of [18]. When Complex I as well as the Ca^{2+}-dependent NAD(P)H dehydrogenase were inhibited by diphenyleneiodonium, the residual oxidation of NADH was almost completely independent of Ca^{2+} (Table 3). We conclude that Complex I and the rotenone-insensitive NADH dehydrogenase do not require Ca^{2+}, whereas the rotenone-insensitive NADPH dehydrogenase requires Ca^{2+} for activity.

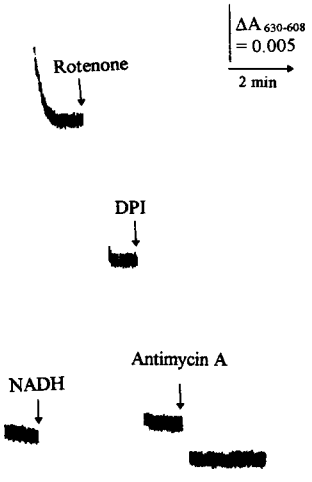

Fig. 5. Generation of a membrane potential by three different NADH-oxidizing enzymes on inside-out SMP. Additions were (final concentrations): SMP, 0.2 mg protein/ml; Ca^{2+}, 1 mM; NADH, 1 mM; rotenone, 20 μM; diphenyleneiodonium (DPI), 6 μM; antimycin A, 0.4 μg/ml.

Table 3
The Ca^{2+}-dependence of rotenone-insensitive NAD(P)H oxidation by inside-out SMP

Additions	Oxygen consumption (nmol/min × mg)
EGTA + rotenone	
+ NADPH	16
+ $CaCl_2$	147
+ antimycin A	0
EGTA + DPI	
+ NADH	207
+ $CaCl_2$	258
+ antimycin A	0

The SMP were preincubated for 1 min in the presence of either 1 mM EGTA + 20 μM rotenone (top) or 1 mM EGTA + 6 μM diphenyleneiodonium (DPI) (bottom) and the reaction started by the addition of substrate. Final concentrations were 1 mM NADPH, 1 mM NADH, 2 mM $CaCl_2$ and 0.4 μM antimycin A. The plus signs indicate consecutive additions.

4. Discussion

Based on the above results we conclude that there are three distinct NAD(P)H dehydrogenases on the inner surface of the inner membrane of potato tuber mitochondria. One is a rotenone-sensitive, diphenyleneiodonium-sensitive, Ca^{2+}-independent enzyme that prefers NADH as the substrate, i.e., Complex I. The second is a rotenone-insensitive, diphenyleneiodonium-sensitive and Ca^{2+}-dependent NAD(P)H dehydrogenase. The third is a rotenone-insensitive, diphenyleneiodonium-insensitive and Ca^{2+}-independent NADH dehydrogenase. All three enzymes are linked to the electron transport chain before Complex III as shown by antimycin A sensitivity and to proton pumping as shown by the generation of a membrane potential. Their properties are summarized in Table 4 and compared to those of the external NAD(P)H dehydrogenases.

Using SMP that were about 50% inside-out and for which external NAD(P)H oxidation by the contaminating right-side-out SMP was inhibited by EGTA, Møller and Palmer [15] detected two NADH dehydrogenases on the inner surface of the inner membrane of Jerusalem artichoke mitochondria. One had a low K_m for NADH and was sensitive to rotenone, while the other had a much higher K_m for NADH and was rotenone-insensitive. Since these measurements were carried out in the presence of EGTA, both of those NADH dehydrogenase activities

Table 4
Summary of the properties of respiratory NAD(P)H dehydrogenases on the inner surface of the inner membrane of potato tuber mitochondria

Parameter	Complex I	Rotenone-insensitive dehydrogenases			
		Internal		External	
		NADPH	NADH	NADPH	NADH
Substrate specificity					
DA-NADH	+	–	–	–	–
NADH	+	(+)	+	–	+
NADPH	(+)	+	–	+	(+)
Inhibitor sensitivity					
Rotenone	+	–	–	–	–
DPI	+	+	–	+	–
Ca^{2+}-dependence	–	+	–	+	+

(+) indicates a weaker reaction. The data on the internal dehydrogenases is based on the present manuscript and on [16–18], whereas the data on the external dehydrogenases is based on [7,8,14] and refs. therein.

were clearly not Ca^{2+}-dependent. These results were later confirmed and extended using > 90% inside-out SMP from potato tubers: the rotenone-insensitive dehydrogenase oxidized both NADH and NADPH [16] and the NADPH oxidation was Ca^{2+}-dependent [18]. Thus, if previous results obtained on mitochondria from two different species are combined, they do point to the presence of two rotenone-insensitive NAD(P)H dehydrogenases on the inner surface of the inner membrane of plant mitochondria, one Ca^{2+}-dependent and oxidizing NADPH, the other Ca^{2+}-independent and oxidizing NADH, precisely the conclusion reached in the present study based on more complete experiments with SMP from one species.

There are a number of similarities between the rotenone-insensitive NAD(P)H dehydrogenases on the outer and the inner surface of plant mitochondria (a) They are both easily released from the membrane [27,28]; (b) The NAD(P)H-specific enzyme is DPI-sensitive, whereas the NADH-specific enzyme is not (this paper; [14]); (c) NADPH oxidation by both mitochondria and inside-out SMP requires Ca^{2+} for activity [10,18]; (d) they are both inhibited by sulfhydryl group reagents [9,11,18] and dicumarol [18,29], and (e) the addition of UQ-1 decreases the K_m(NADH) for rotenone-insensitive NADH oxidation in both intact mitochondria [30] and inside-out SMP [31]. However, while both of the two external NAD(P)H dehydrogenases in potato tuber mitochondria are completely Ca^{2+}-dependent [18], the rotenone-insensitive NADH dehydrogenase on the inner surface is not Ca^{2+}-dependent (Table 4). This further supports the view that the SMP used in the present study do not expose the outer surface of the inner membrane to the medium. In other words, the SMP are not only completely inside-out, but all of the inside-out vesicles are also sealed and do not permit the diffusion of NAD(P)H into the vesicles to be oxidized there.

Both NADH and NADPH in the matrix can be oxidized via two routes. For each substrate, one route passes three sites of proton pumping (through Complex I) and the other only two sites. The affinity of Complex I for NADPH is very low [16] which raises the question of whether NADPH oxidation by Complex I has any physiological significance. In mammalian mitochondria the NADP(H) content is 0.5–2.0 nmol/mg protein [32] or about 0.5–2.0 mM assuming a matrix volume of 1 μl/mg mitochondrial protein. The matrix NADP(H) in mammals is kept much more reduced than the NAD(H) by the activity of a transhydrogenase linked to the electrochemical proton gradient [33] with an $NADPH/NADP^+$ ratio of more than ten [32]. The concentration of NADPH is therefore in the 0.5–2.0 mM range in the matrix of mammalian mitochondria or sufficient to engage Complex I. The conditions in the matrix of plant mitochondria are much less well-described: the presence of transhydrogenase activity and an NADP(H) content of 0.2–1.0 nmol/mg protein has been reported for potato tuber mitochondria [34–36], indicating conditions similar to those in mammalian mitochondria. An $NADPH/NADP^+$ ratio of 0.15, only marginally higher than the $NADH/NAD^+$ ratio of 0.05, has been reported for mitochondria isolated from potato tuber tissue before and after wounding [34]. However, the latter mitochondria were not energized and the transhydrogenase, if present, was therefore not able to maintain a disequilibrium between the two nucleotides. The NADPH content of these unenergized mitochondria was 0.02–0.15 mM [34] or not enough to engage complex I significantly [16].

The oxidation of NADPH by the rotenone-insensitive NADPH dehydrogenase in plant mitochondria, which has a low K_m for NADPH (25 μM, [16]), could have important functions if, and when, Complex I cannot contribute to the oxidation. Under conditions in the matrix where the Ca^{2+} concentration is high enough to engage this enzyme (> 1 μM, [18]) it can reoxidize NADPH produced by the action of the $NADP^+$-dependent isocitrate dehydrogenase present in the matrix of plant mitochondria [22,37–39].

The physiological role of the rotenone-insensitive NADH dehydrogenase may be to act as an overflow mechanism for the oxidation of matrix NADH under conditions where the reduction level of NAD(H) is high but the cell has little need for ATP, i.e., under conditions approaching state 4.

Since matrix NADH and NADPH are oxidized by separate enzymes, the turnover of these two coenzymes can be regulated not only at the enzymatic level, e.g., via the concentration of NAD(P)H and/or Ca^{2+} in the matrix, but also at the level of transcription and translation. Thus, it is possible that the relative amounts of these three enzymes vary with species, with tissue and even with environmental conditions. An indication that this is the case comes from the observation that rotenone-insensitive oxidation of NAD^+-linked substrates is induced in parallel with the induction of alternative oxidase activity in aged potato tuber slices ([40] and refs. therein).

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