Direct evidence for the presence of two external NAD(P)H dehydrogenases coupled to the electron transport chain in plant mitochondria

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Abstract **Exogenous NADPH oxidation by purified mitochondria from both potato tuber and *Arum maculatum* spadix was completely and irreversibly inhibited by sub-micromolar diphenyleneiodonium (DPI), while exogenous NADH oxidation was inhibited to only a small degree. Addition of DPI caused the collapse of the membrane potential generated by NADPH oxidation, while the potential generated by NADH was unaffected. We conclude that there are two distinct enzymes on the outer surface of the inner membrane of plant mitochondria, one specific for NADH, the other relatively specific for NADPH, with both enzymes linked to the electron transport chain.**

Key words: Diphenyleneiodonium; Electron transport chain; Mitochondria, plant; NAD(P)H dehydrogenase

1. Introduction

Unlike mammalian mitochondria, the electron transport chain of plant mitochondria contains at least two NAD(P)H dehydrogenases in addition to Complex I [1]. One of these rotenone-insensitive dehydrogenases is located on the outer surface of the inner mitochondrial membrane, the other on the inner, matrix surface, and in the following we will refer to them as ND_{ex} and ND_{in}, respectively.

Due to the presence of ND_{ex}, plant mitochondria oxidize exogenous NADH and NADPH. The resulting electrons enter the electron transport chain at ubiquinone bypassing Complex I, the site of rotenone inhibition [1–5]. Compared to NADH oxidation, NADPH oxidation has a lower pH optimum [6–8], is more sensitive to thiol reagents [7,9] and appears to require more Ca^{2+} for activity [9–12]. NADH oxidation has been reported to be induced in red beetroots without a concomitant induction of NADPH oxidation [13]. Likewise, mitochondria from suspension-cultured cells of sugar beet oxidize NADH, but exhibit very low rates of NADPH oxidation [14]. On the basis of this circumstantial evidence it has been concluded that it is most likely that two separate enzymes exist, one for each coenzyme [2,4,14]. However, no direct evidence has ever been presented to support this conclusion.

Diphenyleneiodonium (DPI) inhibits Complex I, probably by reacting irreversibly with FMN [15]. Here we test the effect of DPI on NADH and NADPH oxidation by intact mitochondria from potato tubers and *Arum maculatum* spadices. On the basis of these results we conclude that two distinct ND_{ex} enzymes are present.

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2. Materials and methods

Mitochondria were prepared from potato (*Solanum tuberosum* L.) tubers according to [16]. Crude mitochondria were isolated from *Arum maculatum* spadices according to [17] and purified according to [18].

Oxygen consumption was measured in a medium containing 0.3 M sucrose, 5 mM MOPS (pH 7.2), 5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.4 μM FCCP using an oxygen electrode (Rank Bros.). The membrane of the oxygen electrode was washed with 70% (v/v) ethanol between assays to avoid carry-over of DPI.

Membrane potential measurements using 16 μM safranine as the probe were made according to [19]. The medium used was the same as that above except that FCCP was omitted, $CaCl_2$ was 0.1 mM and 20 $\mu g \cdot mg^{-1}$ BSA was included.

NAD(P)H oxidation with oxygen as the electron acceptor was assayed spectrophotometrically at 340 nm in the same medium used for the oxygen electrode measurements. Activities were calculated using an extinction coefficient for NAD(P)H of 6.2 $mM^{-1} \cdot cm^{-1}$.

All concentrations given throughout the text are final concentrations.

3. Results and discussion

NADPH oxidation was completely inhibited by sub-micromolar DPI (Fig. 1), suggesting irreversible binding as found for Complex I from bacteria [15]. For mitochondria isolated from dormant potatoes NADH oxidation was slightly inhibited by concentrations of DPI that gave almost instant complete inhibition of NADPH oxidation (Fig. 1).

DPI had no effect on the membrane potential generated by NADH oxidation (Fig. 2A), consistent with the small effect on the rate of oxygen consumption (Fig. 1). In contrast, DPI completely collapsed the potential generated by NADPH oxidation – the subsequent addition of NADH regenerated the potential (Fig. 2B).

These results, obtained with five independent preparations of mitochondria isolated from dormant potato tubers in winter, show clearly that there are two separate enzymes on the outer surface of the inner membrane of potato tuber mitochondria catalyzing the oxidation of exogenous NAD(P)H.

The oxidation of NADH was more sensitive to DPI for mitochrondria isolated from non-dormant potato tubers (in summer) (Fig. 3) than for those isolated from dormant potato tubers (in winter) (Fig. 1). NADPH oxidation by the mitochondria isolated in summer was still inhibited completely by submicromolar DPI, but NADH oxidation was also substantially inhibited. At 0.6 μM DPI, which completely inhibited NADPH oxidation within 1 min (Fig. 3H), the inhibition of NADH oxidation was 44% (Fig. 3A,D). Very similar results to those obtained for the potato tuber mitochondria isolated in summer were obtained with purified *Arum maculatum* mitochondria (not shown).

The DPI inhibition of NADH oxidation by potato tuber mitochondria was not due to a binding site further down the

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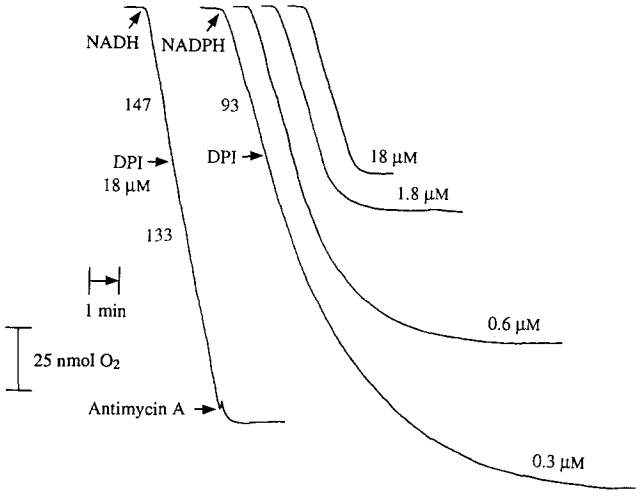

Fig. 1. Oxygen electrode traces showing the effect of DPI on the oxidation of exogenous NADH and NADPH by potato tuber mitochondria isolated in winter. Values (without units in the figure) indicate rates of oxygen consumption in nmol·(mg protein)$^{-1}$·min^{-1}. The concentrations of DPI used for each assay are given in the figure. The concentrations of NADH and NADPH were 1 mM; antimycin A, 0.4 μM; mitochondrial protein, 0.49 mg·ml^{-1}.

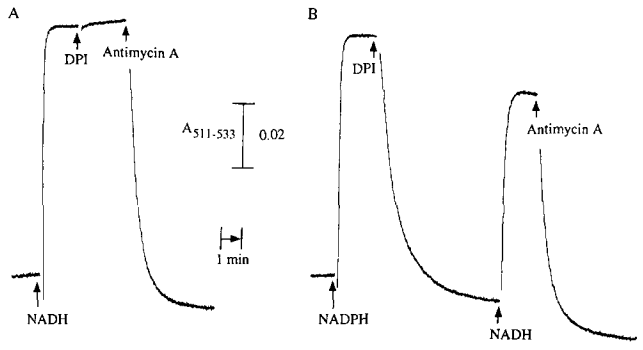

Fig. 2. The effect of DPI on membrane potentials (probed with safranine) generated by oxidation of exogenous NADH and NADPH by potato tuber mitochondria isolated in winter. The concentrations of NADH and NADPH were 1 mM; DPI, 6 μM; antimycin A, 0.4 μM; mitochondrial protein, 0.19 mg·ml^{-1}.

respiratory chain from the NADH dehydrogenases since succinate oxidation was unaffected by 6 μM DPI (not shown).

NADH added after DPI inhibition of NADPH oxidation by potato tuber mitochondria resulted in oxidation at low, but substantial, rates for some preparations (Fig. 3H,I) and at virtually the uninhibited rate for other preparations made in winter (not shown).

When potato tuber mitochondria were preincubated with NADPH and DPI and then assayed for NAD(P)H oxidation in the absence of DPI, NADPH oxidation was inhibited by 80% whereas NADH oxidation was inhibited by only 41% (Table 1). This very large inhibition of NADPH oxidation following the removal of free DPI supports the interpretation of irreversible binding. Since 0.18 μM DPI gave full inhibition of NADPH oxidation with 0.44 mg protein (Fig. 3G), inhibition required the binding of less than 1 nmol DPI/mg mitochondrial protein, and much less than the 20–30 nmol·(mg protein)$^{-1}$ required to inhibit Complex I from bovine heart mitochondria [20].

DPI had almost the same effect on NAD(P)H oxidation assayed spectrophotometrically with oxygen as electron acceptor as for the assays using the oxygen electrode, both for mitochondria from potato tubers and *Arum maculatum* spadices (Table 2).

4. Conclusions

With intact mitochondria from both potato tubers and *Arum maculatum* spadices, DPI inhibits NADPH oxidation, apparently irreversibly, with a high degree of specificity relative to NADH oxidation. We conclude that there are two rotenone-insensitive dehydrogenases on the outer surface of the inner mitochondrial membrane, both of which donate electrons to the electron transport chain. One enzyme oxidizes only NADH and the other oxidizes primarily NADPH. We do not have an explanation for the change in DPI sensitivity of NADH oxidation by potato tuber mitochondria with the change of season.

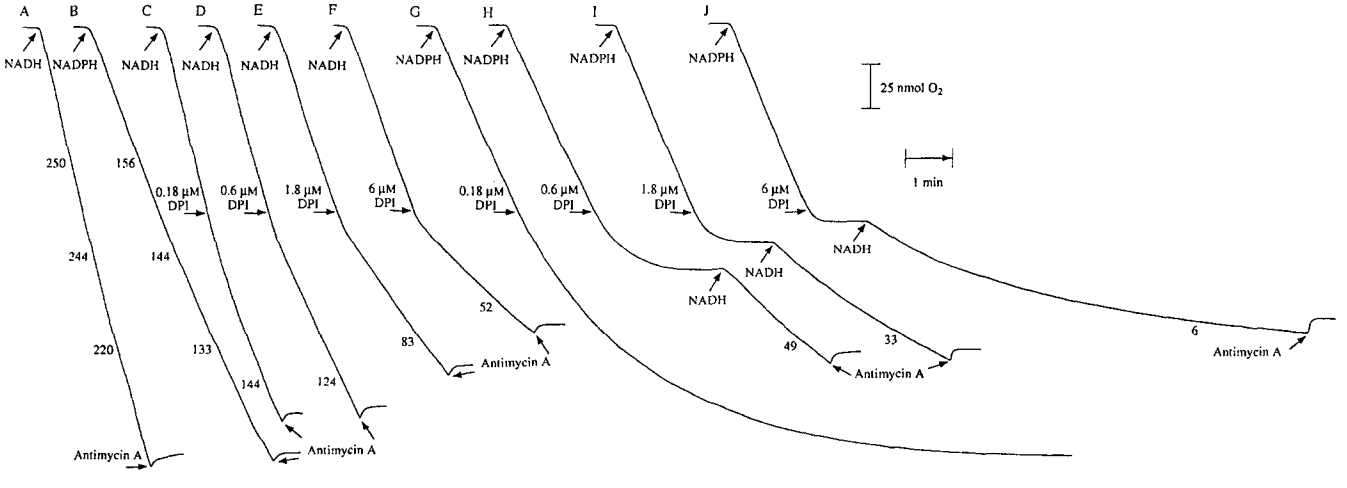

Fig. 3. Oxygen electrode traces showing the effect of DPI on the oxidation of exogenous NADH and NADPH by potato tuber mitochondria isolated in summer. The concentrations of NADH and NADPH were 1 mM; antimycin A, 1.0 μM; mitochondrial protein, 0.44 mg·ml^{-1}. Otherwise as in Fig. 1.

Table 1
The effect of preincubation of potato tuber mitochondria (isolated in summer) with DPI plus NADPH and with NADPH alone on the subsequent rates of NADH and NADPH oxidation

Preincubation	Oxygen consumption (nmol·min^{-1}·(mg protein)$^{-1}$)	
	NADH	NADPH
With NADPH only	241 (100%)	105 (100%)
With DPI + NADPH	143 (59%)	21 (20%)

The mitochondria (1.7 mg protein·ml^{-1}) were preincubated with 1.8 μM DPI in the reaction medium used for the oxygen electrode measurements (see section 2) for 5 min, centrifuged at 16,000 × g for 10 min, the pellet washed with medium three times and the mitochondria resuspended in 20 μl medium. Aliquots of 5 μl were removed for assay of NAD(P)H oxidation in the absence of DPI.

Table 2
The effect of 1.8 μM DPI on NAD(P)H oxidation by potato tuber mitochondria (isolated in summer) with oxygen as the electron acceptor.

Substrate	Inhibition (%)	
	Potato tuber mitochondria	*Arum maculatum* mitochondria
NADH	37	22
NADPH	100	93

Activity was determined as the decrease in absorbance at 340 nm. Since the rate of NAD(P)H oxidation was non-linear and the effect of DPI time-dependent, the inhibition was measured 2 min after DPI addition. Specific activities in nmol NAD(P)H oxidized·(mg protein)$^{-1}$·min^{-1} were: NADPH = *Arum spadix* mitochondria, 258; potato tuber mitochondria, 60; NADH = *Arum spadix* mitochondria, 860; potato tuber mitochondria, 156.

The existence of distinct external dehydrogenases with different substrate specificities suggests that these enzymes might be differentially expressed or regulated to allow different rates of cytoplasmic NADH and NADPH oxidation in different tissues and under different conditions. Oxidation of exogenous NAD(P)H is known to be Ca^{2+}-dependent [9,21]. In future experiments we will attempt to determine the Ca^{2+}-requirement of each of the two external dehydrogenases, and investigate other properties of these enzymes.

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