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Minireview

NAD(P)H-Ubiquinone Oxidoreductases in Plant Mitochondria

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Plant (and fungal) mitochondria contain multiple NAD(P)H dehydrogenases in the inner membrane all of which are connected to the respiratory chain via ubiquinone. On the outer surface, facing the intermembrane space and the cytoplasm, NADH and NADPH are oxidized by what is probably a single low-molecular-weight, nonproton-pumping, unspecific rotenone-insensitive NAD(P)H dehydrogenase. Exogenous NADH oxidation is completely dependent on the presence of free Ca^{2+} with a $K_{0.5}$ of about 1 μM. On the inner surface facing the matrix there are two dehydrogenases: (1) the proton-pumping rotenone-sensitive multisubunit Complex I with properties similar to those of Complex I in mammalian and fungal mitochondria. (2) a rotenone-insensitive NAD(P)H dehydrogenase with equal activity with NADH and NADPH and no proton-pumping activity. The NADPH-oxidizing activity of this enzyme is completely dependent on Ca^{2+} with a $K_{0.5}$ of 3 μM. The enzyme consists of a single subunit of 26 kDa and has a native size of 76 kDa, which means that it may form a trimer.

KEY WORDS: (Plant) mitochondria; NAD(P)H dehydrogenase; NAD(P)H-ubiquinone oxidoreductase; rotenone; Complex I, calcium; electrostatic interactions.

INTRODUCTION

Plant cells respire just like animal cells, that is, they consume oxygen and produce carbon dioxide. The central processes of respiration, the Krebs cycle and respiratory electron transport, take place in the mitochondria. Plant mitochondria have a similar structure and overall function as mammalian mitochondria (Douce, 1985). However, there are a number of distinct differences between plant and mammalian mitochondria, among them the presence of multiple NAD(P)H-ubiquinone oxidoreductases in the respiratory chain of plant mitochondria. It is the purpose of this review to give an overview of our present knowledge about these NAD(P)H dehydrogenases. During the past decade this subject has been reviewed repeatedly, either exclusively (Palmer and Møller, 1982; Palmer and Ward, 1985, Møller, 1986) or as part of a more general review (Douce, 1985; Moore and Rich, 1985; Møller and Lin, 1986; Douce *et al.*, 1987; Douce and Neuburger, 1989; Moore and Siedow, 1991, Møller *et al.*, 1993).

The basic properties of the NAD(P)H dehydrogenases are summarized in Table I and their connection with the rest of the electron transport chain illustrated schematically in Fig. 1.

EXTERNAL NAD(P)H DEHYDROGENASE(S)

External NAD(P)H Oxidation

Unlike mammalian mitochondria (Lehninger, 1955), fungal (von Jagow and Klingenberg, 1970) and virtually all plant mitochondria (Douce, 1985; Møller and Lin, 1986) oxidize NADH on the outer surface of the inner membrane. The only possible exception are red beetroots where mitochondrial oxidation of external NAD(P)H is very slow or missing, but even here the oxidation can be induced by treating the roots in various ways (Day *et al.*, 1976; Rayner and Wiskich, 1983, Fredlund *et al.*, 1991). NADH oxidation is linked to the electron transport chain via ubiquinone, is rotenone-insensitive, and bypasses

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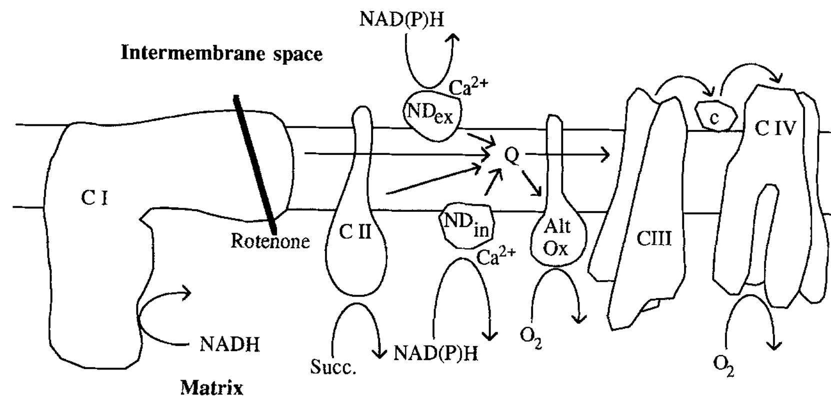

Fig. 1. Schematic drawing of the electron transport chain of plant mitochondria. CI–CIV denote the respiratory complexes: CI, NADH-ubiquinone oxidoreductase; CII, succinate-ubiquinone oxidoreductase; CIII, ubiquinol–cytochrome *c* oxidoreductase; CIV, cytochrome *c*–oxygen oxidoreductase; Alt Ox, alternative oxidase; *c*, cytochrome *c*; ND_{ex} and ND_{in}, external and internal rotenone-insensitive NAD(P)H dehydrogenase, respectively; Succ., Succinate; Q, ubiquinone.

Table 1. Properties of NAD(P)H-Ubiquinone Oxidoreductases in Plant Mitochondria[a]

Property	External dehydrogenase	Internal dehydrogenases	
		Complex I	Rotenone-insensitive
Native size, kDa	?	680 (11)	76 (17)
Structure	?	Heteromultimer, ca. 30 different polypeptides (11, 18)	Homotrimer? (17)
H^+ pumping	No (1)	Yes (1)	No (1)
Redox-active groups	?	FMN (11) 4 FeS centers (12, 13)	?
Sterospecificity (NADH)	β (2)	β (2)	β (17)
Specificity	NADH = NADPH (2)	NADH ≫ NADPH (11, 14, 18)	NADH = NADPH (17)
NADH			
K_m, μM	10–100 (3–6)	1–3 (11, 14)	14 (14)
pH optimum	6.8–7.2 (4, 8–10)	8.0 (14)	6.0–6.5 (14)
NADPH			
K_m, μM	16–1040 (4, 6, 9, 19, 20)	> 1000 (14)	25 (14)
pH optimum	6.0–6.6 (4, 8, 9)	5.8–6.0 (14)	6.5–7.0 (14)
Ca^{2+} dependence	Yes ($K_{0.5}$ = 0.3–1.0 μM) (5, 7)	No (15, 16)	Yes ($K_{0.5}$ = 3 μM) (15)

[a] Unless otherwise indicated, the values were determined at pH 7.2. References are given in parentheses and are as follows: (1) Douce, 1985; (2) K. M. Fredlund, A. G. Rasmusson, and I. M. Møller, unpublished data; (3) Møller and Palmer, 1981a; (4) Edman *et al.*, 1985; (5) Rugolo *et al.*, 1991; (6) Krömer and Heldt, 1991; (7) Moore and Åkerman, 1982; (8) Møller and Palmer, 1981b; (9) Møller and Palmer, 1981c; (10) Møller *et al.*, 1983; (11) Rasmusson *et al.*, 1993b; (12) Cammack and Palmer, 1973; (13) Brouquisse *et al.*, 1986; (14) Rasmusson and Møller, 1991a; (15) Rasmusson and Møller, 1991b; (16) Sjölin and Møller, 1991; (17) Rasmusson *et al.*, 1993a; (18) Soole *et al.*, 1992; (19) Koeppe and Miller, 1972; (20) Arron and Edwards, 1979.

the first coupling site (Douce, 1985; Møller and Lin, 1986). NADPH is also oxidized by plant (Koeppe and Miller, 1972; Arron and Edwards, 1979, 1980; Møller and Palmer, 1981b) and fungal mitochondria (Møller *et al.*, 1982) with properties similar to those of NADH oxidation.

The pH optimum is 6.8–7.0 for NADH oxidation and often lower for NADPH (Møller and Palmer, 1981b, c; Møller *et al.*, 1983; Edman *et al.*, 1985). Thus, the cytoplasmic pH of 7.0–7.5 (Kurkdjian and Guern, 1989) is close to the optimum for NADH oxidation and so high as to keep NADPH oxidation relatively low. The K_m (NADH) is 10–100 μM, whereas the K_m (NADPH) is more variable from 25 μM in pea leaf mitochondria in the presence of 2 mM Ca^{2+} (Krömer and Heldt, 1991) to more than 1000 μM in spinach leaf mitochondria in the absence of added Ca^{2+} (Edman *et al.*, 1985). Considering the concentration of free NAD(P)H in the cytoplasm, Krömer and Heldt (1991) have calculated that in pea leaf cells mitochondrial oxidation of cytoplasmic NADPH is 10 times faster than that of cytoplasmic NADH.

The oxidation of external NADH is dependent on Ca^{2+} at neutral pH (Møller *et al.*, 1981b) with a $K_{0.5}$ of about 0.3–1.0 μM for Jerusalem artichoke mitochondria (Moore and Åkerman, 1982: Rugolo *et al.*, 1991). In the presence of spermine the $K_{0.5}$ is reduced by a factor 2–3, which may be an important regulatory mechanism in the cell (Rugolo *et al.*, 1991) since the concentration of free Ca^{2+} in the cytoplasm of plant cells is on the order of 0.1–0.3 μM (Gilroy *et al.*, 1989). It would be interesting to see whether the pH dependence of NADH oxidation is also affected by polyamines. The Ca^{2+} dependence appears to disappear at lower pH (Møller and Palmer, 1981c; Møller *et al.*, 1983).

Ca^{2+} dependence for exogenous NADH oxidation is observed with oxygen, DQ, and UQ_1,[3] but not with ferricyanide, as electron acceptor (Soole *et al.*, 1990a). The Ca^{2+} dependence disappears when the dehydrogenase is released from the membrane either by sonication or by detergent treatment although the enzyme still reduces ubiquinone analogues (Cook and Cammack, 1984; Cottingham and Moore, 1984). It has therefore been proposed that "Ca^{2+} binding causes a conformational change in the enzyme, allowing it to interact better with UQ in its natural lipid environment. When the enzyme is solubilized, the reactivity with UQ analogues may be so enhanced that the Ca^{2+}-induced conformation has no effect" (Soole *et al.*, 1990a). A conformational change in the enzyme, in this case locking Ca^{2+} into place, has also been used to explain the slow inhibition of NADH oxidation by intact mitochondria after the addition of chelators to the medium (Møller *et al.*, 1981a). A possibility consistent with all of the above observations is that Ca^{2+} is involved in the binding of the enzyme to the inner membrane.

Exogenous NADPH oxidation also requires Ca^{2+} at neutral pH. Often this oxidation appears to require higher concentrations of Ca^{2+} than NADH oxidation (Arron and Edwards, 1979, 1980; Petit, 1984; Edman *et al.*, 1985). However, Rugolo and Zannoni (1992) found that both NADH and NADPH oxidation was saturated at 1 μM free Ca^{2+} in Jerusalem artichoke mitochondria.

In the presence of sufficient Ca^{2+} the oxidation of exogenous NAD(P)H by plant and fungal mitochondria is stimulated via electrostatic interactions (Johnston *et al.*, 1979; Møller and Palmer, 1981a; Møller *et al.*, 1981a, 1982, 1984; Edman *et al.*, 1985; Rugolo *et al.*, 1991). Part of the stimulation can be explained by the facilitated approach of the negatively charged NAD(P)H to the membrane with the decrease in the size of the surface potential at higher salt concentrations. However, since the V_{max} of electron transport from exogenous NAD(P)H to oxygen is stimulated, it has been suggested that electrostatic screening affects the lateral distribution of respiratory complexes in the inner membrane such as to increase their collision frequency (Kay *et al.*, 1985; Lidén *et al.*, 1987; Møller *et al.*, 1984, 1987).

The cytoplasm of a plant cell typically contains about 100 mM K^+ and 2–5 mM Mg^{2+} as well as other cations like polyamines. The outer membrane of isolated mitochondria appears to be permeable to all molecules below 5–10 kDa (Mannella, 1985) and the salt concentration in the intermembrane space is therefore likely to be close to that of the cytoplasm. This prediction has been confirmed by more direct measurements (Cortese *et al.*, 1991). So, unless the outer membrane under some physiological conditions becomes relatively impermeable to cations, the cation concentration is always high near the outer surface of the inner membrane and not likely to be a factor regulating the rate of electron transport.

[3] Abbreviations: Complex I, rotenone-sensitive NADH-ubiquinone oxidoreductase; DQ, duroquinone; SMP, submitochondrial particles; UQ, ubiquinone.

Purification of External NAD(P)H Dehydrogenase

There have been several attempts at purifying the external NADH dehydrogenase from plant mitochondria, but with a marked lack in consistent results (Cook and Cammack, 1984; Cottingham and Moore, 1984; Klein and Burke, 1984; Chauveau and Lance, 1991; Luethy *et al.*, 1991). The problems of purifying this enzyme are probably similar to those encountered when attempting to purify the internal rotenone-insensitive NAD(P)H dehydrogenase (see below).

An NADH dehydrogenase is released as a soluble protein from the outer surface of the inner membrane when the outer membrane of potato tuber mitochondria is selectively ruptured by swelling (Douce *et al.*, 1973). The enzyme, which has NAD(P)H-duroquinone reductase activity, has been partially purified by ion-exchange and affinity chromatography and shows the same activity with NADH and NADPH (K.M. Fredlund, A.G. Rasmusson, and I.M. Møller, unpublished data). This indicates that cytoplasmic NADH and NADPH are oxidized by the same enzyme, which is consistent with the conclusion of Rugolo and Zannoni (1992) based on the use of inhibitors.

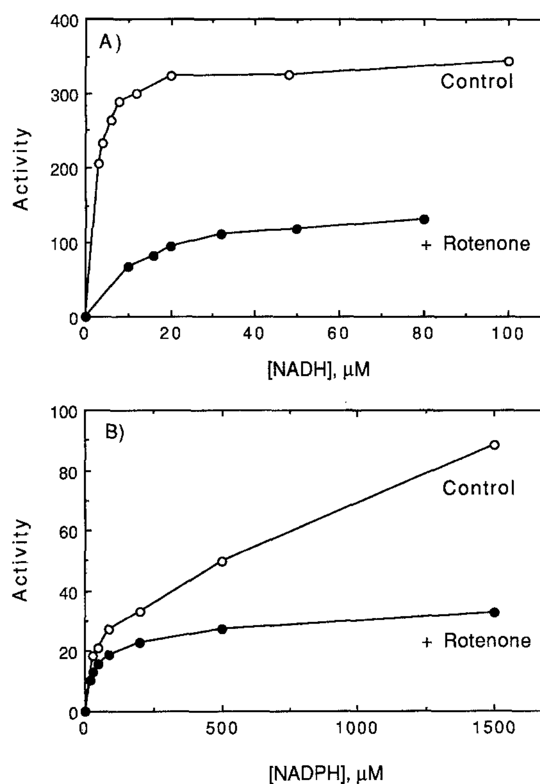

Fig. 2. Kinetics and rotenone-sensitivity of NADH (A) and NADPH (B) oxidation by inside-out submitochondrial particles isolated from potato tuber mitochondria. From Rasmusson and Møller (1991a), reproduced by the permission of *Physiologia Plantarum.*

OXIDATION OF MATRIX NAD(P)H

Much of our knowledge on the oxidation of matrix NAD(P)H comes from studies using inner membrane vesicles turned inside-out, the so-called inside-out submitochondrial particles (SMP), which are readily produced by sonicating plant mitochondria under high-salt conditions followed by differential centrifugation (Møller *et al.*, 1981c, 1987; Kay *et al.*, 1985; Petit *et al.*, 1987, 1991; Rasmusson and Møller, 1991a; Gardeström *et al.*, 1993).

In contrast to mammalian mitochondria, plant mitochondria can oxidize malate as the sole substrate due to the presence of malic enzyme in the matrix. This oxidation shows many odd properties depending on the source of the mitochondria and the assay conditions; one of these is the great variability in rotenone sensitivity (Palmer and Møller, 1982; Palmer *et al.*, 1982; Douce, 1985; Møller and Lin, 1986). This was finally explained by the detection of two NADH dehydrogenase activities on the inner surface of the inner membrane of plant mitochondria—a rotenone-sensitive activity with a low K_m (NADH) and a rotenone-insensitive activity with a much higher K_m (NADH) (Fig. 2) (Møller and Palmer, 1982; Palmer *et al.*, 1982). The presence of two dehydrogenase activities was confirmed by Soole *et al.* (1990b), and it was suggested that the rotenone-insensitive activity was due to a bypass within Complex I. However, the oxidation of deamino-NAD(P)H by inside-out SMP is completely rotenone-sensitive (Table II), indicating the presence of two active sites for NAD(P)H and thus of a separate rotenone-insensitive NAD(P)H dehydrogenase (Rasmusson and Møller, 1991a). This has since been confirmed by Menz *et al.* (1992).

The oxidation of NADH by inside-out SMP is stimulated by cations via electrostatic interactions just like the oxidation of exogenous NADH by intact mitochondria (Sjölin and Møller, 1991).

Matrix NADPH, possibly formed by the $NADP^+$-specific isocitrate dehydrogenase present in the matrix of plant mitochondria (Rasmusson and Møller, 1990), is also oxidized by the respiratory chain. The oxidation is completely inhibited by antimycin A and is linked to the formation of a membrane

Table II. Oxidation of Deamino-NAD(P)H by Inside-out Submitochondrial Particles from Potato Tuber Mitochondria (from Rasmusson and Møller, 1991a)

Experiment	Additions	Oxidation rate, nmol O_2 (mg protein)$^{-1}$ min^{-1}
1	deamino-NADH	429
	+ rotenone	10
	+ NADH	150
2	deamino-NADPH	22
	+ rotenone	3
	+ NAPH	45

potential similar in size to that observed with, for example, succinate (Rasmusson and Møller, 1991a). Only part of this NADPH oxidation is rotenone-sensitive, and the rotenone-insensitive part has a low K_m(NADPH) (Fig. 2B). The rotenone-insensitive NADPH oxidation has a lower pH optimum than NADH oxidation (Rasmusson and Møller, 1991a) and, in contrast to NADH oxidation by Complex I, it is almost completely dependent on Ca^{2+} at pH 7.0 with a $K_{0.5}$ of 3 μM (Rasmusson and Møller, 1991b).

In conclusion, the above results show that there are two NAD(P)H dehydrogenases oxidizing matrix NAD(P)H in plant mitochondria—the rotenone-sensitive Complex I, which primarily oxidizes NADH, and another rotenone-insensitive NAD(P)H-ubiquinone oxidoreductase which takes NADH and NADPH with equal affinity and which is Ca^{2+} dependent.

COMPLEX I

Purification

As described above, it has been known for a long time that plant mitochondria contain a Type I rotenone-sensitive proton-pumping NADH dehydrogenase. In addition. Moore and coworkers have used antibodies raised against Complex I from mammalian mitochondria to identify a number of cross-reacting polypeptides in plant mitochondria (Cottingham and Moore, 1988; Cottingham *et al.*, 1986). However, only recently the respiratory complex was isolated from plant mitochondria and its properties investigated. Soole *et al.* (1992) solubilized red beetroot mitochondria with deoxycholate and purified Complex I by gel filtration followed by affinity chromatography. We have been able to refine this purification after solubilizing with Triton X-100 using slightly different conditions. The apparent molecular mass of the complex was 680 kDa as determined by gel filtration chromatography (Rasmusson *et al.*, 1993b) compared to 400 kDa found by Soole *et al.* (1992). The higher molecular mass is consistent with the estimated size of Complex I in mammals and fungi (Ragan, 1987; Weiss *et al.*, 1991). The yield of purified enzyme was 10–20% and the specific activity was increased 10–15 fold compared to that of the inner membrane fraction (Soole *et al.*, 1992; Rasmusson *et al.*, 1993b).

Properties

The enzyme catalyzes NADH oxidation with the following electron acceptors: Ferricyanide > UQ_0 > UQ_1 > duroquinone > UQ_{10}. The NADPH-UQ_1 activity is about 10% of that of NADH-UQ_1 (Soole *et al.*, 1992; Rasmusson *et al.*, 1993b), consistent with data on mammalian Complex I (Hatefi and Hanstein, 1973).

The catalytic activity of the purified red beetroot Complex I is completely unaffected by rotenone (Soole *et al.*, 1992; Rasmusson *et al.*, 1993b) until the enzyme is reconstituted into phosphatidylcholine vesicles. Then both rotenone and DCCD sensitivity become apparent (Rasmusson *et al.*, 1993b).

Using submitochondrial particles, Soole *et al.* (1990b) and Menz *et al.* (1992) demonstrated the presence of two UQ binding sites on Complex I: One is rotenone-sensitive and binds both UQ_1 and UQ_{10}; the other is rotenone-insensitive and binds only UQ_1.

The purified enzyme contains FMN, but the stoichiometry is as yet unknown (Rasmusson *et al.*, 1993b). EPR spectroscopy of Jerusalem artichoke and potato mitochondria has allowed the detection of four iron-sulfur centers corresponding closely to centers N1b, N2, N3, and N4 in mammalian Complex I (Cammack and Palmer, 1973; Brouquisse *et al.*, 1986). No EPR spectroscopy has so far been done on purified plant Complex I.

Purified Complex I has been resolved into about 30 different polypeptides by one-dimensional SDS-PAGE (Soole *et al.*, 1992; Rasmusson *et al.*, 1993b). On Western blots, seven polypeptides show good cross-reactivity with antibodies raised against Complex I from beef heart (Soole *et al.*, 1992) or *Neurospora crassa* (Rasmusson *et al.*, 1993b). Three polypeptides in the 51–55 kDa region react equally well with both kinds of antibodies. Antibodies raised against the 49 and 30 kDa subunits of the beef heart Complex I cross-reacted with polypeptides of 46 and 27 kDa, respectively, in mung bean submitochondrial

particles (Cottingham *et al.*, 1986). With these exceptions, none of the polypeptides have been identified to date.

Mammalian and fungal Complex I consist of some 30 different subunits out of which seven are encoded in the mitochondrial DNA (Weiss *et al.*, 1991). Homologues of six of the mitochondrially encoded Complex I subunits of mammals and/or fungi have been found in the mitochondrial genome of higher plants (Stern *et al.*, 1986; Gualberto *et al.*, 1988; Wissinger *et al.*, 1988; Wintz *et al.*, 1989; Xue *et al.*, 1990; Haouazine *et al.*, 1992). A substantial interest in these genes has come from the discovery of RNA editing in plant mitochondria (Lamattina *et al.*, 1989). The transcripts of nad genes 1, 3, and 4, are edited (Bonnard *et al.*, 1992), resulting in as much as 14% amino acid exchange in the nad 3 gene product of wheat (Gualberto *et al.*, 1991). RNA editing leads to a greater homology between plants and other kingdoms for the amino acid sequence than for the DNA sequence (Bonnard *et al.*, 1992). There is no evidence whether partially edited transcripts can be translated or not, though polysomal m-RNA seems to be more edited than total m-RNA (Gualberto *et al.*, 1991).

In conclusion, plant mitochondria contain a Complex I similar to that of mammalian and fungal mitochondria.

INTERNAL ROTENONE-INSENSITIVE NAD(P)H DEHYDROGENASE

We have purified what we believe is the rotenone-insensitive dehydrogenase responsible for the oxidation of matrix NADH and NADPH (Rasmusson *et al.*, 1993a). Red beetroot mitochondria were disrupted, and the rotenone-insensitive NAD(P)H-DQ reductase released from the inner surface of the inner membrane was purified from the soluble fraction. Only one peak of NADPH-DQ reductase activity was detected after anion-exchange chromatography. When this peak was further purified by affinity chromatography, the activity eluted in a single peak at 100 nM NADPH. The purified NAD(P)H dehydrogenase shows equal activity with NADH and NADPH. As electron acceptor it prefers UQ_0 and DQ over UQ_1 and ferricyanide. It has an apparent molecular mass of 26 and 76 kDa as determined by SDS-PAGE and gel filtration, respectively, indicating that it may be a trimer in the native form (Rasmusson *et al.*, 1993a).

There are several possible reasons why previous attempts to identify and purify this enzyme and the external NAD(P)H dehydrogenase have failed: (a) use of too little starting material; (b) the presence of an external and an internal rotenone-insensitive NAD(P)H dehydrogenase with similar properties in most plant mitochondria (see above); (c) the fact that the soluble dihydrolipoamide dehydrogenase present in all mitochondria has both NADH-DQ and -ferricyanide reductase activities; (d) use of NADH-ferricyanide reductase activity, which is catalyzed by a number of enzymes, to detect the enzyme.

A rotenone-insensitive NADH dehydrogenase of 53 kDa, or twice the size of the NAD(P)H dehydrogenase purified from red beetroot mitochondria, has been isolated from the inner surface of the inner membrane of yeast mitochondria. It does not oxidize NADPH (de Vries and Grivell, 1988; Marres *et al.*, 1991). The enzyme is similar to an NADH dehydrogenase from *E. coli* and this enzyme has, in turn, been shown to contain two similar halves each with an NADH binding domain (de Vries *et al.*, 1992; Yagi, 1991, 1993). It is therefore possible that the plant enzyme is the product of a "basic" dehydrogenase gene.

The external and the internal rotenone-insensitive NAD(P)H dehydrogenases from plant mitochondria differ from the average ND2 dehydrogenase (Yagi, 1991) in several ways: (a) The internal one, and possible also the external, is smaller; (b) they are both readily released from the membrane in the absence of detergents and are therefore extrinsic proteins; (c) they both oxidize NADH and NADPH equally well; (d) Ca^{2+} appears to be involved in the regulation of their activity at least *in situ*.

PERSPECTIVES

We are now beginning to get glimpses of the molecular properties of the multiple NAD(P)H dehydrogenases in plant mitochondria, but we are still only at the beginning. We need to improve the purification procedures, reconstitute the enzymes and study their properties, identify the genes encoding the enzymes, in particular the low-molecular-mass rotenone-insensitive NAD(P)H dehydrogenases, and study their expression in different tissues and species. To obtain direct evidence for the physiological importance of these enzymes, their expression should be

manipulated selectively, e.g., by the anti-sense RNA technique, and the function of the mitochondria in the resulting plants studied.

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