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# Functional Molecular Aspects of the NADH Dehydrogenases of Plant Mitochondria

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There are multiple routes of NAD(P)H oxidation associated with the inner membrane of plant mitochondria. These are the phosphorylating NADH dehydrogenase, otherwise known as Complex I, and at least four other nonphosphorylating NAD(P)H dehydrogenases. Complex I has been isolated from beetroot, broad bean, and potato mitochondria. It has at least 32 polypeptides associated with it, contains FMN as its prosthetic group, and the purified enzyme is sensitive to inhibition by rotenone. In terms of subunit complexity it appears similar to the mammalian and fungal enzymes. Some polypeptides display antigenic similarity to subunits from *Neurospora crassa* but little cross-reactivity to antisera raised against some beef heart complex I subunits. Plant complex I contains eight mitochondrial encoded subunits with the remainder being nuclear-encoded. Two of these mitochondrial-encoded subunits, nad7 and nad9, show homology to corresponding nuclear-encoded subunits in *Neurospora crassa* (49 and 30 kDa, respectively) and beef heart CI (49 and 31 kDa, respectively), suggesting a marked difference between the assembly of CI from plants and the fungal and mammalian enzymes. As well as complex I, plant mitochondria contain several type-II NAD(P)H dehydrogenases which mediate rotenone-insensitive oxidation of cytosolic and matrix NADH. We have isolated three of these dehydrogenases from beetroot mitochondria which are similar to enzymes isolated from potato mitochondria. Two of these enzymes are single polypeptides (32 and 55 kDa) and appear similar to those found in maize mitochondria, which have been localized to the outside of the inner membrane. The third enzyme appears to be a dimer comprised of two identical 43-kDa subunits. It is this enzyme that we believe contributes to rotenone-insensitive oxidation of matrix NADH. In addition to this type-II dehydrogenases, several observations suggest the presence of a smaller form of CI present in plant mitochondria which is insensitive to rotenone inhibition. We propose that this represents the peripheral arm of CI in plant mitochondria and may participate in nonphosphorylating matrix NADH oxidation.

**KEY WORDS:** Plant; respiration; NADH dehydrogenases; rotenone; Complex I; protein purification; mitochondria.

## INTRODUCTION

Mitochondria are the site of ATP production in aerobic eukaryotic cells. These organelles play important roles with respect to energy production and anabolic metabolism in both photosynthetic and nonphotosynthetic plant cells. However, it is generally thought that the rate of respiration is regulated by the supply of ADP. During photosynthesis the ATP/ADP ratio is high; thus, respiration or electron transfer in the mitochondrion is not maximal. In addition to the normal phosphorylating complexes, NADH: ubiquinone oxidoreductase (Complex I, CI), ubiquinol cytochrome *c* reductase (Complex 3, CIII), and cytochrome oxidase (Complex 4, CIV), the plant mitochondrial respiratory chain contains pathways of substrate oxida-

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tion not present in mammalian mitochondria. These include the ability to oxidize cytosolic NAD(P)H, the oxidation of glycine in photosynthetic tissues, the oxidation of matrix NADH without energy coupling via a rotenone-insensitive NADH pathway, and an alternative oxidase which bypasses the proton translocation steps associated with both ubiquinol:cytochrome *c* reductase and cytochrome oxidase. It can be observed that together with succinate dehydrogenase (Complex 2, CII) and the alternative oxidase, plant mitochondria are capable of oxidizing substrates without any oxidative phosphorylation. Regulation of electron flow between the phosphorylating and nonphosphorylating pathways and the role these nonphosphorylating pathways play in plant cell metabolism are active areas of investigation.

In this review, we will cover the latest developments in the oxidation of NAD(P)H by plant mitochondria focusing on the composition and morphology of complex I (CI) and the "alternative dehydrogenases" involved in oxidizing cytosolic or external and matrix NAD(P)H in a nonenergy conserving manner. We will also speculate on the involvement of CI in a potential route of rotenone-insensitive matrix NADH oxidation. The kinetic parameters of these pathways have been most recently reviewed in detail (Møller *et al.*, 1993; Wiskich and Menz, 1993).

In addition to the NAD(P)H dehydrogenases of the inner membrane, plant mitochondria also have a dehydrogenase located on the outer membrane. The protein(s) responsible for this activity have not been isolated and its role *in vivo* is not clear; however, the details of this pathway may be found in a previous review (Møller and Lin, 1986) and will not be covered in this paper.

## COMPLEX I

### Composition, Morphology, and Assembly

Complex I (CI), the phosphorylating NADH dehydrogenase, is an oligomeric lipoprotein and has been well characterized in beef heart (Walker, 1992), *Neurospora crassa* mitochondria (Weiss *et al.*, 1991), *Paracoccus denitrificans* (Yagi, 1993), and recently in *E. coli* (Leif *et al.*, 1993) membranes. The bovine and fungal enzymes appear to be functionally similar, being composed of 42 and 32 nonidentical polypeptides, respectively (Walker, 1992, Schulte *et al.*, 1994) and are estimated to have molecular weights of around 880 kDa. In both organisms seven of these polypeptides are encoded in the mitochondrion. These are known as subunits ND1 to ND6 and subunit ND4L, all of which are hydrophobic membrane proteins.

CI contains FMN as the prosthetic group. It also contains three tetranuclear FeS centers, known as clusters N-2, N-3, N-4, and a binuclear cluster, N-1. In beef heart, two additional clusters have been detected, N-1b and N-5 (Walker, 1992). Both the beef heart and *Neurospora* CI have the ability to reduce ubiquinone analogues and FeCN. Only electron flowthrough to ubiquinone is sensitive to rotenone inhibition in mitochondria, and only one preparation of purified CI from beef heart mitochondria demonstrated rotenone-sensitive NADH:ubiquinone reductase activity (Hatefi *et al.*, 1962). Complex I isolated from *Neurospora* mitochondria does show sensitivity to rotenone when reinserted into artificial liposomes (Wiess *et al.*, 1991).

Electron microscopic analysis of *N. crassa* CI crystals have shown that the enzyme has two arms: one peripheral and one membrane spanning (Wiess *et al.*, 1991). The complexity of CI has hampered progress in the study of its function; therefore, attempts were made to find simpler forms. Many studies have turned to bacterial enzymes which are functionally similar but much smaller, containing 14 polypeptides in the case of *P. denitrificans* (Yagi, 1993) and *E. coli.* (Leif *et al.*, 1993). However, when *Neurospora* cells were grown in the presence of chloramphenicol, a mitochondrial protein synthesis inhibitor, a smaller form of CI was assembled that contained only nuclear-encoded polypeptides, FMN, and clusters N-1, N-3, and N-4. It catalyzed NADH oxidation to FeCN and ubiquinone analogues but was not affected by rotenone. It was found that this form represented the peripheral arm of the complex that protrudes into the matrix (for a review of this work, see Friedrich *et al.*, 1993) and the fact that it catalyzes UQ reduction suggests the presence of an internal site for UQ reduction within CI. The membrane arm contains all the mitochondrial encoded polypeptides plus cluster N-2 and the ubiquinone-binding site.

The existence of separate assembly pathways has been shown for the peripheral and membrane parts of CI. This comes from the observations that: (1) in chloramphenicol-treated cells only a smaller form of CI is present; thus, the peripheral arm can form in the absence of the membrane arm (Friedrich *et al.*, 1989), (2) pulse-labelling experiments have shown that some CI subunits are found transiently in intermediate complexes that resemble the membrane arm (Tuschen *et*

*al.*, 1990), (3) in manganese deficient growth conditions, the peripheral form of CI was not formed but the membrane arm could be detected (Schmidt *et al.*, 1992). More sophisticated experiments with *Neurospora*, using gene disruption, where specific nuclear genes were interrupted, has led to the development of cell lines where either only the peripheral or membrane arm are formed, confirming the initial observations. Survival of these altered cells demonstrates that *Neurospora* cells can exist without a functional CI and the "alternative" nonphosphorylating dehydrogenase compensates. The respiratory activities of these mutant cells were normal except that pyruvate/malate oxidation was insensitive to piericidin A which acts specifically at the ubiquinone-binding site of the membrane form of CI (Friedrich *et al.*, 1994).

Of the many polypeptides associated with CI, only a few have been assigned a function (see Walker, 1992 for a review); a 51-kDa subunit contains the NADH and FMN binding sites and a tetranuclear iron sulfur cluster; the ND1 mitochondrial gene product has the rotenone-binding site (Earley *et al.*, 1987), which has been confirmed by sequence homology to a bacterial dehydrogenase that uses ubiquinone as a substrate (Friedrich *et al.*, 1990). In *Neurospora crassa*, recent studies have also identified a 9.6-kDa subunit as being an acyl-carrier protein and a 75-kDa unit that has chaperone-like properties but is not part of the mature CI complex (Schulte *et al.*, 1994). Recently, a rotenone-resistant cell line was shown to have a mutation in the ND4 gene. CI in these cells was rotenone-insensitive and the mitochondrial subunits did not assemble, suggesting that ND4 is important in the assembly of mature CI (Hofhaus and Attardi, 1993). The identification of only 14 polypeptides in bacterial forms of CI suggests that this number represents the minimal number for proton tranlocation. It remains to be seen what role the many other "accessory" polypeptides play in mammalian and fungal CI.

## PLANT MITOCHONDRIAL COMPLEX I

The study of plant NADH dehydrogenases has been difficult because specific inhibitors are not available for them and they tend to lose their specific identity when solubilized, such as orientation in the inner membrane and sensitivity to rotenone. CI has been distinguished from the "alternative" dehydrogenases on a size basis as the beef heart and fungal enzymes are around 880 kDa in size.

Plant CI was first isolated from inner-membrane-enriched fractions of beetroot mitochondria (Soole *et al.*, 1992). This tissue was chosen as it has only low levels of the external-facing dehydrogenase (Day *et al.*, 1976) and so minimized the number of NADH dehydrogenases released from the membrane. This preparation used deoxycholate to solubilize the protein and a form of CI was isolated that had at least 24 polypeptides, was about 400 kDa in total size, had NADH:ubiquinone reductase activity, but was not sensitive to rotenone. However, this preparation was contaminated by ATPase. More recently, CI has been purified using other detergents and chromatography techniques from beetroot (Rasmusson *et al.*, 1994), broad bean (Leterme and Boutry, 1993), and potato (Herz *et al.*, 1994) mitochondria. The purification protocol from beetroot used high levels of Triton X-100 to release CI and showed that reinsertion into liposomes regained some sensitivity to rotenone but the preparation was only 40% inhibited (Rasmusson *et al.*, 1994). It contained FMN as its prosthetic group and approximately 30 polypeptides. The large molecular band present at 100 kDa present in the previous beetroot preparation (Soole *et al.*, 1992) was not present when the enzyme was extracted at this high Triton concentration. This had previously been assumed to be a contamination by a transhydrogenase that has been shown to be present in plant mitochondria (Carlenor *et al.*, 1988). The purity of this preparation was not fully addressed, but the presence of strong bands at 54, 53, and 51 kDa suggests from our experience that ATPase contamination was present.

In an attempt to remove the ATPase contamination present in the early preparations of plant CI and beef heart CI, Leterme and Boutry (1993) used additional detergent stripping of the mitochondria using low concentrations of Triton X-100 and CHAPS. This approach has also been used for CI isolated from potato and when purified using a sucrose gradient and ion-exchange chromatography, any trace of the ATPase contaminant was removed (Herz *et al.*, 1994). The preparation from potato had FMN as its prosthetic group, 32 polypeptides, and total molecular weight of about 900 kDa, suggesting that this preparation (and the broad bean CI) is the most pure and complete form of CI isolated to date. Therefore in terms of subunit complexity and size, plant CI resembles the mammalian and fungal enzymes.

All of these preparations of CI have NADH:ubiquinone and NADH:FeCN reductase activities but very little activity with NADPH as a substrate. Preliminary

investigations of EPR signals from beetroot submitochondrial particles (SMP) detected the presence of centers with similar, if not identical, *g* values to those of *Neurospora* CI, suggesting that clusters N-1, N-2, N-3, and N-4 are present in plant CI and indicates a similar pattern of electron flow in the complex (R. I. Menz, personal observation).

In all the studies of plant CI, the immuno-cross-reactivity with antisera raised against beef heart and *Neurospora* polypeptides was investigated. The results are summarized in Table I. The deoxycholate preparation cross-reacted with antisera raised against whole CI from beef heart but picked up the ATPase contamination (Soole *et al.*, 1992). Some subunits of CI from beetroot and potato cross-reacted strongly with antisera raised against whole *Neurospora* CI and individual subunits (Table I). Interestingly, we have screened beetroot inner membrane fragments with antisera raised against specific subunits of beef heart CI and found very little cross-reactivity. However, anti-beef heart 19-kDa antisera cross-reacted strongly with a 44-kDa polypeptide from both beetroot and pea leaf inner membrane preparations (Table I).

Recently the mitochondrial genome has been completely sequenced in *Marchantia polymorpha*

**Table I.** Summary of Cross-Reactivity of Plant CI with Antisera Directed against Various Subunits and Preparation of CI

| Antiserum | Origin of antiserum | Immuno-reaction | Polypeptides (kDa) |
|---|---|---|---|
| anti-CI | *Neurospora* | + | 72, 18, 61[a]; +[2] 80, 54, 53, 51, 27, 25, 22[c] |
| anti-70 kDa | *Neurospora* | + | 75[b] |
| anti-49 kDa | *Neurospora* | + | 42.5[b] |
| anti-42 kDa | *Neurospora* | —[b] | |
| anti-32 kDa | *Neurospora* | —[b] | |
| anti-21.3 kDa | *Neurospora* | —[b] | |
| anti-acyl carrier protein | *Neurospora* | —[b] | |
| anti-nad 9 | Wheat | + | 27[b] |
| anti-CI | Beefheart | + | 55, 54, 51, 41.5, 37.5, 30[d] |
| anti-75 kDa | Beefheart | —[b] | |
| anti-51 kDa | Beefheart | —[b] | |
| anti-24 kDa | Beefheart | —[b] | |
| anti-19 kDA | Beefheart | + | 44[b] |

[a] K. L. Soole, personal observations.
[b] Herz *et al.* (1994).
[c] Rasmusson *et al.* (1994).
[d] Soole *et al.* (1992).

(Oda *et al.*, 1992) and it was found that all the nad genes (i.e., ND genes, in mammalian terminology) present in mammalian mitochondrial genome, that is, nad1, nad2, nad3, nad4, nad5, and nad6, are present in the plant genome, including two extra putative CI subunits, nad7 (Oda *et al.*, 1992) and nad9 (Lamattina *et al.*, 1993, Kubo *et al.*, 1993). Antisera raised against the wheat mitochondrial nad9 gene product produced in *E. coli* cross-reacted with a 27-kDa polypeptide in purified beef heart CI (Lamattina *et al.*, 1993), confirming it to be a subunit of NADH dehydrogenase.

The comparison of similar antigenicity between subunits reveals only limited information, as it is often difficult to compare sizes between different labs using different gel systems. More information can be gained from subunit sequences. Currently, only N-terminal sequences are available for bean and potato CI subunits. Leterme and Boutry (1993) found that a 42-kDa subunit showed a level of N-terminal sequence homology with a 49-kDa subunit from beef heart and *Neurospora* CI, as well as homology with the nad7 gene from the *Marchantia polymorpha* mitochondria genome, confirming that the nad7 gene product is a CI subunit. Herz *et al.* (1994) also found that a 42.5-kDa subunit in potato CI had homology to the nad7 gene product, an observation further confirmed by Gäbler *et al.* (1994). Therefore, N-termini for this polypeptide are conserved between potato, broad bean, nad7 of *Marchantia*, and the *Neurospora* and beef heart 49-kDa subunit. This polypeptide is also conserved in bacteria (Yagi, 1993) and so must be one of the essential subunits for CI activity. In beef heart, the 49-kDa subunit is localized in the IP fraction (iron-sulfur protein fraction of CI) and may bear one or more Fe-S clusters.

Further, a 27-kDa polypeptide from potato CI showed N-termini homology with the 31- and 30-kDa polypeptides from beef heart and *Neurospora* CI, respectively, and orf 212 of *Marchantia* (Herz *et al.*, 1994). This polypeptide was cross-reactive with antisera raised against the wheat nad9 gene product and so confirms that this mitochondrial gene product is part of CI. Again the 30-kDa beef heart polypeptide is associated with the IP fraction of CI and bears Fe-S clusters. The most interesting fact in this work is that both these polypeptides, the 49-kDa and the 31-kDa subunits, are nuclear-encoded in beef heart and *Neurospora* cells and are part of the peripheral arm of CI (Schulte *et al.*, 1994); however, in plant cells they are mitochondrial gene products, nad7 and nad9. Consequently, it appears that two mitochondrial gene prod-

ucts are present in the peripheral arm of plant CI, assuming the plant enzyme has a similar structure. This would imply that the assembly pathway for plant CI differs from *Neurospora* or that assembly is independent of the origin of the polypeptide as suggested by Lamattina *et al.* (1993). This area is worthy of further investigation to distinguish between these two possibilities. The presence of a peripheral and membrane arm to plant CI has yet to be demonstrated. However, several observations suggest the presence of a putative smaller form of CI in plant mitochondria (discussed in the next section).

## NATURE OF THE ROTENONE-INSENSITIVE MATRIX NADH OXIDATION

The observation that rotenone or piericidin A did not cause a complete inhibition of the oxidation of NAD-linked substrates was first observed by Wiskich and Bonner (1963). Brunton and Palmer (1973) further investigated this activity and concluded it was catalyzed by a rotenone-insensitive NADH dehydrogenase that did not have access to external NADH, was linked to only two sites of phosphorylation, and was unique from the external-facing NADH dehydrogenase.

Møller and Plamer (1982) demonstrated that the rotenone-insensitive activity had a tenfold lower affinity for NADH compared to the rotenone-sensitive component of matrix NADH oxidation in Jerusalem artichoke mitochondria (80 μM versus 8 μM). Soole *et al.* (1990) further investigated the kinetics of NADH oxidation in beetroot SMP prepared from fresh beetroot tissue and found that there was a tenfold difference in the affinity for NADH between rotenone-sensitive and -insensitive oxidation. However, when using an artificial electron acceptor such as the UQ analogue, UQ-1, to examine the kinetics of the NADH dehydrogenase, the high $K_m$ for rotenone-insensitive NADH oxidation was not detected. Further, when the $K_m$ for NADH was determined using the complete respiratory chain in the presence of low levels of UQ-1, then the rotenone-insensitive NADH oxidation had a similar $K_m$ (NADH) as the rotenone-sensitive oxidation (on the order of 10 μM). From these observations, we suggested that rotenone-insensitive oxidation may not be catalyzed by a separate dehydrogenase but that it results from an alternative route of electron flow through CI. It was hypothesized that this route of electron flow had restricted access to the endogenous UQ pool and thus the apparent affinity of this activity for NADH was lower.

Further work from Rasmusson and Møller (1991a) showed that an analogue of NADH, deamino-NADH, when oxidized by plant SMP was completely sensitive to inhibition by rotenone, that is, the rotenone-insensitive activity could not use this substrate. This was confirmed in beetroot SMP (Menz *et al.*, 1992). The simplest explanation for this observation is that two separate NADH-binding sites exist for rotenone-sensitive and -insensitive oxidation and is the most compelling evidence for two separate dehydrogenases.

However, the potential for a rotenone-insensitive-like activity with CI cannot be discounted. Since Soole *et al.* (1990) first proposed this possibility, several observations suggest that either a form of CI or an alternative route of electron flow through CI may exist that is insensitive to rotenone inhibition. These are:

1. The ability of exogenous UQ-1 to stimulate both rotenone-insensitive NADH and deamino-NADH oxidation in beetroot SMP (Soole *et al.*, 1990, Menz *et al.*, 1992). This observation, coupled with the fact that the peripheral arm assembly intermediate that catalyzes rotenone-insensitive NADH:UQ reductase activity in *Neurospora* (Schulte *et al.*, 1994), suggests the presence of an internal UQ site within CI prior to the site of rotenone inhibition.
2. A mild protease treatment of beetroot SMP eliminates the rotenone inhibition of NADH:UQ reductase (Soole, 1989), suggesting that only a mild treatment of CI will result in a loss of sensitivity to rotenone. However, it has not been demonstrated that this protease treatment eliminates the proton pumping activity of CI. The protease effect on CI has also been reported for beef heart CI (Crowder and Ragan, 1977).
3. The CHAPS stripping of the mitochondrial membranes, developed by Leterme and Boutry (1993) for reducing ATPase contamination, resulted in the release of an NADH dehydrogenase from the inner membrane which represented 37% of the activity. Enzymatically, it did not represent CI but contained corresponding CI polypeptides and resembled the form of CI isolated by Soole *et al.* (1992). The CHAPS extract and purified CI were not assessed for their rotenone sensitivity in this study; however, Leterme and Boutry (1993) con-

cluded that plant mitochondria contain two large protein complexes capable of oxidizing NADH.

Further to this observation is that analysis of purified beetroot CI by Blue native gel electrophoresis revealed the presence of NADH dehydrogenase bands at both 670 kDa and 400 kDa (Rasmusson *et al.*, 1994), again suggesting the presence of a smaller form of CI in plant mitochondria.

4. Mitochondria isolated from the yeast *Saccharomyces cerevisiae* in stationary phase have a NADH dehydrogenase that is rotenone-insensitive and has a smaller molecular weight than the CI-like protein but is much more complex than the smaller non-phosphorylating NADH dehydrogenases present in other aerobic yeast (Büschges *et al.*, 1994). Therefore, the presence of a smaller rotenone-insensitive form of CI present during a normal physiological state is not without precedent.

5. From sequence analyses of beef heart CI polypeptides, a putative additional NADH binding site may exist in CI in both the 51-kDa and 39-kDa polypeptides (Walker, 1992). This may represent a secondary NADH-binding site in CI, providing an alternative hypothesis to the separate dehydrogenase theory to explain two separate binding sites responsible for rotenone-sensitive and -insensitive oxidation.

From these observations, we propose than in plant mitochondria, an assembly intermediate of CI exists, which is insensitive to inhibition by rotenone. Although the half-life of this intermediate (the peripheral arm) is quite short in *Neurospora* mitochondrial membrane, it may have a longer life span in the plant membrane due to its appearance in the purification procedure for CI. Alternatively, a separate NADH binding site which does not accept deamino-NADH as a substrate may exist as part of CI. Although this is suggested by sequence homology from beef heart CI (see point 5 above), oxidation of NADH was as sensitive to rotenone inhibition as the deamino-NADH activity in the purified plant CI (Herz *et al.*, 1994), making this proposal unlikely.

Therefore we propose that the plant mitochondrial inner membrane contains an FAD-dependent NADH dehydrogenase which donates electrons directly to the UQ pool, and a smaller, less complex form of CI exists which does not pump protons and can participate in rotenone-insensitive oxidation of NAD-linked substrates (Fig. 1). When and if this assembly intermediate of CI is present in the plant system is a question being investigated in our laboratory.

## ALTERNATIVE CYTOSOLIC NAD(P)H OXIDATION

Most plant mitochondria have the ability to oxidize cytosolic NADH; the only exception is mitochondria isolated from the tubers of some cultivars of red beetroot (Day *et al.*, 1976); however, upon slicing and aging (Rayner and Wiskich, 1983) or cold storage (Fredlund *et al.*, 1991), this activity is induced. In contrast, not all plant mitochondria have the capacity for respiration-linked NADPH oxidation; for example, mitochondria isolated from pea leaves can oxidize NADPH while those isolated from spinach leaves cannot (Kromer *et al.*, 1992).

Kinetic and inhibitor analyses of mitochondria that oxidize both substrates have shown that NADH and NADPH oxidation display different pH profiles (Møller and Palmer, 1981), sensitivities to the inhibitors amytal (Koeppe and Miller, 1972), mersalyl, and different requirements for calcium (Arron and Edwards, 1980; Nash and Wiskich, 1983), suggesting different enzymes are responsible. However, the most convincing evidence for NADH and NADPH oxidation to be mediated by separate enzymes comes from work by Zottini *et al.* (1993), who showed that mitochondria isolated from sugarbeet root could only oxidize cytosolic NADH. However, mitochondria isolated from undifferentiated sugarbeet callus could oxidize both NADH and NADPH, suggesting that sugarbeet encodes two enzymes.

The other side of the argument is that NADH and NADPH oxidation are mediated by the same enzyme. Rugolo and Zannoni (1992), using Jerusalem artichoke mitochondria, found that under high salt (physiological) conditions NADH oxidation exhibited a biphasic response to platanetin, a specific inhibitor of external NADH oxidation (Ravanel *et al.*, 1986), suggesting a high- and low-affinity platanetin binding site. However, under low salt conditions with NADH or when NADPH was being oxidized, only inhibition at the low affinity site was observed. They also demonstrated that changing the ionic strength changed the $K_m$ (UQ) and that both NADH and NADPH are inhibited by mersalyl in a similar manner. From these observations they concluded that NADH and NADPH oxidation occurred via a single nonspecific enzyme with two UQ binding sites, one of which was only operable at

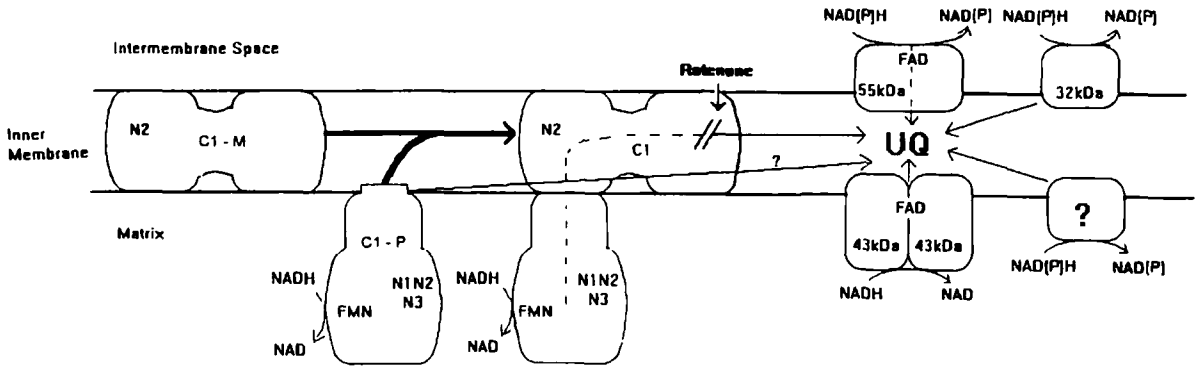


**Fig. 1.** A diagrammatic representation of the NADH dehydrogenases associated with the inner membrane of plant mitochondria. C1—complex I; assembly intermediates of C1 are represented as C1-M, membrane arm and C1-P peripheral arm. N1 to N3 represent the iron-sulfur clusters of known location, FMN, flavin mononucleotide, FAD, flavin adenine dinucleotide, UQ, ubiquinone. The bold arrows represent the assembly pathway of C1 (→), the other arrows (→,- - -›) represent the routes of electron flow. The question marks signify enzymes and routes of electron flow that have yet to be shown in the plant inner membrane. The NADH or NADPH-oxidizing activity of the externally facing dehydrogenases may be species-dependent (see text for details).

high ionic strength with NADH. This view has also been supported by the fact that nonspecific NAD(P)H activities have been isolated from plant mitochondria (Møller *et al.*, 1993).

It becomes apparent that the most likely scenario is that there are two classes of dehydrogenase capable of mediating cytosolic NAD(P)H oxidation; a specific NADH dehydrogenase and a nonspecific NAD(P)H dehydrogenase. In some tissues such as sugarbeet, NAD(P)H oxidation is mediated by both classes of enzyme. However, the possibility exists that some species capable of oxidizing NADPH may only contain a nonspecific NAD(P)H dehydrogenase.

## ALTERNATIVE MATRIX NAD(P)H OXIDATION

Since it has become apparent that plant mitochondria contain alternative rotenone-insensitive matrix NAD(P)H dehydrogenase(s) (Rasmusson and Møller, 1991a; Menz *et al.*, 1992), the possibility that this activity could be mediated by separate NADH and NADPH-oxidizing enzymes must be considered. Rasmusson *et al.* (1991b) used inside-out potato SMP to demonstrate that rotenone-sensitive internal NADPH oxidation is highly dependent on $Ca^{2+}$ with the rate being stimulated fifteenfold in the presence of $Ca^{2+}$. In contrast, NADH oxidation is only affected slightly by $Ca^{2+}$. They also showed differences in the mersalyl sensitivity of this activity, with NADH oxidation inhibited 50% and NADPH oxidation inhibited 80%. This difference in calcium sensitivity has also been seen in beetroot although not as dramatic as reported for potato; NADH oxidation is stimulated 9.5% by 1 mM calcium, while NADPH oxidation is stimulated by 48% (R. I. Menz, personal observation). We have also shown a difference in beetroot insensitivity to the inhibitor *p*-CMB, with NADPH oxidation being inhibited by 64%, while NADH oxidation is not inhibited (R. I. Menz, personal observation).

These observations suggest that there is a possibility that alternative rotenone-insensitive oxidation of matrix NADH and NAD(P)H could be mediated by different enzymes. However, direct evidence to support this theory could be difficult to obtain, due to the low levels of the NADPH activity.

## PURIFICATION OF ALTERNATIVE NAD(P)H DEHYDROGENASES

Several factors complicate the purification of the alternative NAD(P)H dehydrogenases; these are: (1) There is no specific assay (or inhibitor) for the individual dehydrogenases, (2) the plant mitochondria contain several enzymes capable of catalyzing oxidation of NAD(P)H in the presence of artificial electron acceptors, (3) these enzymes have very high specific activities, which results in low protein abundance, and (4) the substrate specificity may change depending upon the solubilization state or electron acceptor used.

Therefore, although several attempts have been made to purify the alternative NAD(P)H dehydrogenase (Cook and Cammack, 1984; Cottingham and Moore, 1984; Klein and Burke, 1984; Cook and Cammack, 1985; Chauveau and Lance, 1990; Luethy *et al.*, 1992; Rasmusson *et al.*, 1993; Knudten *et al.*, 1994), most of these have resulted in only partial purifications of enzymes that could not be assigned to a particular pathway. The exceptions are recent preparations by Leuthy *et al.* (1991), (1992), Knudten *et al.* (1994), and Rasmusson *et al.* (1993) in which activities were resolved to single polypeptides. Fortunately, both groups purified enzymes from the soluble fraction of red beetroot (Leuthy *et al.*, 1991; Rasmusson *et al.*, 1993). However, there are inconsistencies between these two groups.

Luethy *et al.* (1991) resolved the beetroot soluble fraction into three separate activities. Hydrophobic interaction chromatography resolved the first two activities to single polypeptides. The first peak activity was found to be a associated with a 43-kDa polypeptide; this peak oxidized both NADH and NADPH with DCPIP as an acceptor. The second peak activity was associated with a 32-kDa polypeptide and would only oxidize NADH with DCPIP as acceptor. The third activity was found to be enriched in two polypeptides peptides (40 and 55 kDa) and was inhibited by plane tree bud extract which contains platanetin, a specific inhibitor of the external NADH dehydrogenase (Ravanel *et al.*, 1986). They concluded that the peak-3 activity was responsible for oxidation of cytosolic NADH.

Rasmusson *et al.* (1993) also used anion exchange chromatography to separate three activities from the soluble fraction of red beet. The first of these they resolved to one polypeptide of 26 kDa using affinity chromatography. This peak oxidized both NADH and NADPH with duroquinone and FeCN as acceptors. The second peak was found to only have NADH: FeCN reductase activity and was associated with a protein with a molecular weight of 35–40 kDa (determined by gel filtration). The third and final peak, which had high NADH:FeCN reductase activity, also had lipoamide dehydrogenase activity and was therefore attributed to the L-protein. As the mitochondria used had little or no capacity for external NADH oxidation, and with the assumption that the rotenone-insensitive internal enzyme must be able to reduce ubiquinone analogues, they concluded that their peak 1 was the rotenone-insensitive internal enzyme. They also proposed that the peak-3 activity seen by Luethy *et al.* (1991) was lipoamide dehydrogenase, as it has a subunit molecular weight of approximately 55 kDa (Barrera *et al.*, 1972)

Recent work by Menz (unpublished results) with beetroot mitochondria has shown that sonication releases 40–50% of the rotenone-insensitive-internal NADH oxidoreductase activity, and hence the resulting soluble fraction is suitable for purification of this enzyme. When this fraction was subject to ion exchange chromatography, three separate activities could be resolved. Using affinity chromatography, peak 1 was purified to a single polypeptide with a molecular weight of 43 kDa; however, the native molecular weight was determined to be 75–80 kDa. This peak oxidized both NADH and NAD(P)H peak and was very stable. The molecular weight and stability of this dehydrogenase showed that it was similar to the one purified by Leuthy *et al.* (1991). The second peak only had NADH:FeCN reductase activity and was determined to have a native molecular weight of approximately 32 kDa and was most certainly a common component in all three purification protocols (Leuthy *et al.*, 1991; Rasmusson *et al.*, 1993; R. I. Menz, personal observation). The third peak exhibited high NADH:FeCN reductase activity and lipoamide dehydrogenase activity, confirming that this activity is attributed to lipoamide dehydrogenase. However, the mitochondria used could not oxidize external NADH, unlike those used by Leuthy *et al.* (1991). Therefore, mitochondria from aged beetroot were prepared and the anion exchange profiles of these mitochondria were compared with the profiles of mitochondria without this activity. Subtraction of the two profiles revealed an extra peak present on the leading shoulder of the lipoamide dehydrogenase peak. It was found that this shoulder could be resolved by affinity chromatography to a single polypeptide with a molecular weight of approximately 55 kDa, which had no lipoamide dehydrogenase activity. It appeared that the protocol used by Luethy *et al.* (1991) somehow missed the lipoamide dehydrogenase activity. Investigation of lipoamide dehydrogenase purified from yeast (Sigma Chemical Company, USA) revealed that NADH:FeCN reductase activity was 20 times greater than the NADH:DCPIP reductase activity of this enzyme, explaining why it was not detected by Luethy *et al.* (1991). More recently, Elthon's group has isolated two dehydrogenases with molecular weights 32 kDa (Knudten *et al.*, 1994) and 58 kDa (Luethy *et al.*, 1995) from maize mitochondria. These appear very similar to the beetroot counterparts (32 kDa and 55 kDa) except that the 32-kDa protein has the ability to oxidize NADH and

NADPH and may have a higher capacity to use quinone analogues as electron acceptors. Antibody cross-linking experiments have localized both of these proteins to the outside of the inner membrane or the intermembrane space, suggesting that these two proteins are involved in the oxidation of cytoplasmic NAD(P)H (Knudten *et al.*, 1994; Luethy *et al.*, 1995). It is also interesting that polypeptides of these molecular weights are common features of several of the partial purifications mentioned. Therefore, we conclude that both the 32-kDa and 55-kDa enzymes are associated with the oxidation of cytosolic NAD(P)H; however, the specific role, NADH or NADPH oxidation, could be species-dependent (Fig. 1).

What enzyme is responsible for the rotenone-insensitive oxidation of matrix NAD(P)H oxidation is less clear. The question remains as to whether Rasmusson *et al.* (1993) has isolated the same activity as Luethy *et al.* (1991) and us, or whether all groups have missed an activity. Further, Rasmusson *et al.* (1993) report the same native molecular weight, 70–80 kDa, as we find with our peak 1 activity. The reported lack of stability of the 26-kDa protein makes it possible that this protein could be missed; however, the 43-kDa activity appears very stable. Interestingly the 26-kDa protein has a lot in common (molecular weight and stability) with DT diaphorase (Ernster, 1987). This enzyme has been reported from a variety of tissues and its expression is often correlated with stress response (Ernster, 1987). Perhaps the tissue used by Rasmusson *et al.* (1993) was somehow stressed and thus contained DT diaphorase, which was purified. If either the 26-kDa or 43-kDa protein is the internal rotenone-insensitive NAD(P)H, it poses the question of why polypeptides of this size and activities are not seen in maize. It could be possible that the high level of cytoplasmic NAD(P)H oxidation obscures the lower activities of the internal enzyme(s). In conclusion, we feel that the rotenone-insensitive internal NADH enzyme is most likely a dimer of two identical 43-kDa subunits. It is also possible that rotenone-insensitive internal NADPH oxidation is catalyzed by another enzyme which is present in low amounts and therefore has not been detected in purification attempts (Fig. 1).

## PHYSIOLOGICAL ROLE

An area of intense interest is how electron flow is regulated between the energy-producing or the phosphorylating and the nonphosphorylating or "wasteful" pathways of oxidation, as the bioenergetic efficiency of the mitochondrion is dependent on flux through these pathways. A balance between the phosphorylating and nonphosphorylating NADH oxidation severely effects growth of *E. coli* cells. Cells with NDH-II (the nonphosphorylating dehydrogenase) as the major route of oxidation lack a competitive advantage for growth compared with cells with functional NDH-I (Zambrano and Kolter, 1993). It is not yet clear what physiological role the nonphosphorylating pathways of NADH oxidation play in plant physiology. However, alternative oxidase, which is a major potential route of nonphosphorylating electron flow, has been shown to be induced when synthetic demands are high in the cell and under stress treatments such as cold temperatures, wounding, or slicing of tissues, and parasite attack (for a review, see Day *et al.*, 1995), pointing to a role for the nonphosphorylating pathways in adaptation, resistance, survival, and growth.

Although these observations have been made for alternative oxidase, very few observations have been made about the participation of rotenone-insensitive NADH oxidation and most of these relate to the increase of cytosolic NADH oxidation. There have been reports on the increase of the cytosolic-facing NADH dehydrogenase upon cold storage and calcium aging of beetroot tissue (Fredlund *et al.*, 1991; Rayner and Wiskich, 1983). Developmental studies of soybean mitochondria show that matrix rotenone-insensitive oxidation and the alternative oxidase develop in parallel (Bryce *et al.*, 1990). More recently, a study on Pi limitation in roots suggested that both the alternative oxidase and the internal rotenone-insensitive oxidation pathway increase in activity (Rychter *et al.*, 1992). All these imply that the alternative pathways of NADH oxidation react similarly to changes in a plant cell's metabolism as the alternative oxidase, but further work on these pathways is required.

## CONCLUSION

In summary, numerous pathways of NADH are associated with plant mitochondria. The structure of the enzymes responsible for the alternative pathways for NADH oxidation is becoming clearer, but further work is required. From the current knowledge available, Fig. 1 represents a summary of the dehydrogenases associated with the inner membrane of plant mitochondria.

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