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Biochemical analyses of plant mitochondrial NAD(P)H dehydrogenases

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BIOCHEMICAL ANALYSES OF PLANT MITOCHONDRIAL NAD(P)H DEHYDROGENASES

by

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GRADUATE COLLEGE
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Plant mitochondria possess the unique ability to oxidize cytosolic reducing equivalents in the form of NADH and NADPH. In this dissertation, the enzymes responsible for exogenous NADH and NADPH oxidation in red beetroot (*Beta vulgaris* L.) and maize (*Zea mays* L.) mitochondria were partially purified and characterized. Three NAD(P)H dehydrogenase activities were found in the soluble protein fraction of "aged" red beetroot mitochondria. One of these dehydrogenases was purified to a single polypeptide with an apparent molecular weight of 42 kilodaltons. This dehydrogenase was capable of oxidizing both NADH and NADPH. This enzyme represents greater than 90% of the recoverable NADPH dehydrogenase activity and, therefore, the 42 kilodalton dehydrogenase is likely an exogenous NAD(P)H dehydrogenase. A second dehydrogenase was partially purified to a major polypeptide of 55 kilodaltons and a minor protein of 40 kilodaltons. This dehydrogenase oxidized only NADH, and was sensitive to a potent and specific inhibitor of the exogenous NADH dehydrogenase, platanetin, a dihydroxyflavone isolated from the bud scales of plane (*Plantanus occidentalis*) trees. A third NADH dehydrogenase was purified from red beetroot mitochondria that specifically oxidized NADH and consisted of a 32 kilodalton protein. All three dehydrogenase activities were insensitive to inhibition by rotenone, a specific inhibitor of the endogenous NADH dehydrogenase (Complex I of the mitochondrial electron transport chain).

Two NAD(P)H dehydrogenase activities were partially purified from the soluble protein fraction of maize mitochondria isolated from etiolated seedlings. One of these dehydrogeases oxidized both NADH and NADPH, and represented greater than 70% of the recoverable NADPH dehydrogenase activity, implicating that this enzyme likely represents the exogenous NADH and NADPH dehydrogenase. Furthermore, the NAD(P)H dehydrogenase activities of this enzyme were sensitive to inhibition by the plane tree bud extract containing platanetin. This exogenous NAD(P)H dehydrogenase was partially purified and appears to be comprised of three major polypeptides with apparent molecular weights of 60, 58 and 42 kilodaltons. The maize exogenous NAD(P)H dehydrogenase was found to be immunologically related to the red beetroot 55 kilodalton NADH dehydrogenase.

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LIST OF ABBREVIATIONS

NADH:	nicotinamide adenine dinucleotide (reduced)
NADPH:	nicotinamide adenine dinucleotide phosphate (reduced)
DH:	dehydrogenase
M_r:	relative molecular weight
DCPIP:	2,6-dichlorophenol-indophenol
pCMB:	*p*-chloromercuribenzoic acid
NEM:	*N*-ethylmaleimide
CMS:	*p*-chloromercuriphenylsulfonic acid
nBT:	nitro blue tetrazolium
BCIP:	5-bromo-4-chloro-3-indolyl phosphate
SMP:	sub-mitochondrial particles
UQ:	ubiquinone
9-AA:	9-aminoacridine
CTC:	chlorotetracycline
LDAO:	lauryl dimethylamine *N*-oxide
FeCN:	ferricyanide
SDH:	succinate dehydrogenase
alt. ox.:	alternative oxidase
SHAM:	salacyclic hydroxamic acid
KCN:	potassium cyanide
platan.:	platanetin
anti a:	antimycin a
2D:	two-dimensional
MAb:	monoclonal antibody

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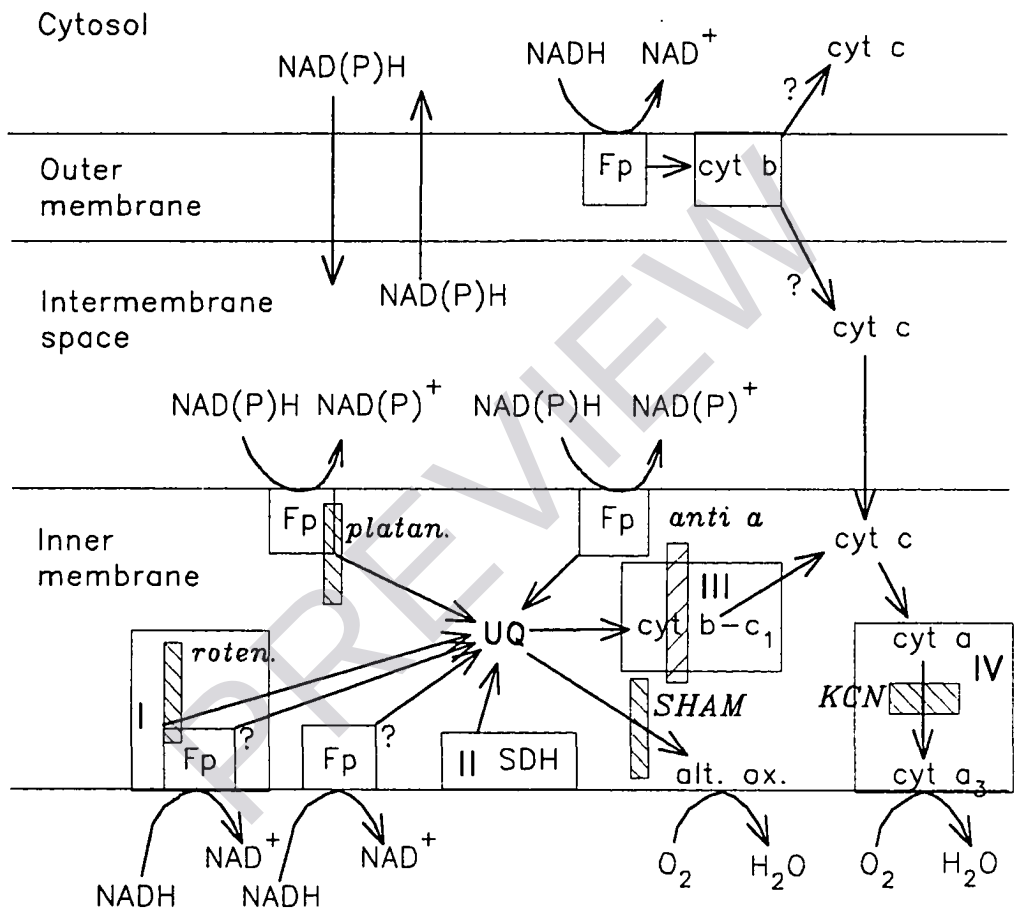
Chapter I. Introduction

Plant mitochondria are remarkably flexible in their ability to oxidize NAD(P)H. In all, there are five NADH oxidizing systems proposed to be operational in plant mitochondria and at least one pathway for NADPH oxidation. Figure 1 is a diagram of these membrane bound NAD(P)H dehydrogenases and the mitochondrial respiratory chain.

The outer membrane NADH dehydrogenase (DH) is present in both plant and animal mitochondria and is specific for the 4α-proton of NADH (Douce et al., 1973). It can donate electrons effectively to ferricyanide (FeCN), dichlorophenol-indolphenol (DCPIP), and cytochrome c, but not to duroquinone. The function and protein composition of this outer membrane NADH DH is not known, although it has been shown to contain a flavin dehydrogenase and a cytochrome b (Douce et al., 1973). The outer membrane NADH DH is not sensitive to inner membrane electron transport chain inhibitors such as rotenone, antimycin a, and cyanide (Douce et al., 1973). Specific inhibitors include the herbicides 2,4-di- and 2,4,5-trichlorophenoxyacetic acids (Mannella and Bonner, 1978). The outer membrane NADH DH is unaffected by bivalent cations and cation chelators (Palmer and Moller, 1982), a property which is somewhat uncharacteristic of the other exogenous NAD(P)H oxidizing pathways.

There are at least four NAD(P)H dehydrogenase activities associated with the inner mitochondrial membrane, all of which reduce the membrane pool of quinone (see Figure 1). These dehydrogenases are specific for the 4ß-hydronium ion of NAD(P)H, distinguishinging themselves from the outer membrane dehydrogenase, which specifically oxidizes the 4α-hydronium ion. There are two endogenous NADH oxidizing activities which can be discerned based upon their differing apparent affinities for NADH and differing sensitivities to rotenone, a commonly used respiratory chain inhibitor which acts at complex I. The traditional endogenous NADH DH activity (complex I) is present in plant and animal mitochondria and is

Figure 1. Schematic representation of the membrane-bound NAD(P)H dehydrogenase systems and electron transport chain of plant mitochondria. The outer membrane is permeable to molecules with M_r of 10 kDa or less and is permeable to NAD(P)H as indicated. The route of electrons from the outer membrane dehydrogenase is unclear, the question marks indicate possible, but unproven pathways. Several of the electron transport chain components are indicated by the roman numerals I through IV. These complexes represent the endogenous NADH DH (Complex I), succinate DH (SDH, Complex II), the b-c_1 complex (Complex III), and cytochrome oxidase (Complex IV). Proton translocation occurs at Complexes I, III, and IV. Proteins that possibly bind flavin molecules are indicated by the abbreviation Fp (flavoprotein). The following abbreviations are also used: cyt a, a_3, b, c, and b-c_1 (cytochromes a, a_3, b, c, and b-c_1); UQ (ubiquinone); alt. ox. (alternative oxidase); SHAM (salacyclic hydroxamic acid, an alternative oxidase inhibitor); KCN (potassium cyanide, a cytochrome oxidase inhibitor); roten. (rotenone, a Complex I inhibitor); anti a (antimycin a, a Complex III inhibitor); platan. (platanetin, an exogenous NAD(P)H DH inhibitor). The question marks in the inner membrane indicate a lack of concensus on the existence of either a rotenone-insensitive site of ubiquinone reduction on Complex I or a distinct rotenone-insensitive DH. The arrows designate the flow of electrons.



the major pathway for entrance of matrix-derived NADH into the electron transport chain. A rotenone-insensitive NADH dehydrogenase activity may also be present on the inside of the inner membrane which has a low affinity (high apparent K_m) for NADH (Marx and Brinkman, 1978; Moller and Palmer, 1982). Recent evidence has indicated that a rotenone-insensitive NADPH dehydrogenase functions on the matrix side of the inner mitochondrial membrane (Moller and Rasmusson, 1990; Rasmusson and Moller, 1991a). There are NADH and NADPH dehydrogenase activities present on the outer surface of the inner membrane which oxidize cytosolic NAD(P)H. These activities are termed exogenous dehydrogenases because they oxidize NAD(P)H that is exogenous to the mitochondria and link this oxidation to the respiratory chain. Both of these activities are insensitive to rotenone and have characteristic K_m values that can be used to distinguish the activities from the endogenous dehydrogenases. In many cases, the kinetic parameters can also be used to distinguish between the activities located on the same side of the inner mitochondrial membrane, as has been attempted in dissecting the endogenous NADH oxidizing systems.

The endogenous NADH dehydrogenases have been investigated primarily through the use of submitochondrial particles (SMPs). Complex I has a high affinity for NADH (K_m of 8 μM) and is sensitive to rotenone (Moller and Palmer, 1982). The rotenone-insensitive endogenous NADH DH activity displays a K_m of 80 μM for NADH (Moller and Palmer, 1982) and does not conserve energy (Brunton and Palmer, 1973; Day and Wiskich, 1974a; Day and Wiskich, 1974b). Soole et al. (1990a) have reported K_m values of 145 $\pm$ 37 and 24 $\pm$ 9 μM for the rotenone-insensitive and rotenone-sensitive dehydrogenase activities, respectively. Due to the differences in affinity for NADH, Moller and Palmer (1982) proposed two separate dehydrogenase activities, attributable to a single enzyme with a branch point, or the presence of two independent enzymes.

The results of Soole et al. (1990a) indicate an enzyme with a single NADH binding site with two sites of UQ-reduction which utilize a common intermediate "P". The accessibility of UQ to each of these sites and the reduction level of the intermediate "P" can influence NADH binding and thus create the kinetic differences observed for the two pathways. Marx and Brinkman (1979) performed temperature dependency studies of malate oxidation, highlighting differences in electron flow of the rotenone sensitive and insensitive pathways. These data could be interpreted to suggest the existence of two dehydrogenase activities.

Evidence that supports the existence of two separate enzymes on the martrix surface of the inner mitochondrial membrane has come from several observations. Rasmusson and Moller (1991c) reported that the oxidation of deamino-NADH (nicotinamide hypoxanthine dinucleotide) could be almost completely inhibited by rotenone. Oxygen uptake that resumed upon addition of NADH indicated the presence of two enzymes since only complex I type enzymes are capable of oxidizing deamino-NADH (Matsushita et al., 1987). The observation that inside-out SMPs are capable of NADPH oxidation has also supported the hypothesis that two separate matrix dehydrogenases exist (Rasmusson and Moller, 1991a, 1991b; Petit et al, 1991). The most recent publication concerning matrix NADH dehydrogenases has concluded that both hypotheses are correct. Based on deamino-NADH oxidation and sensitivity towards rotenone, Menz et al. (1992) support the theory that two separate dehydrogenases exist. However, in line with work by Soole et al. (1990a), Menz et al (1992) also propose that complex I contains two sites of quinone reduction, one is sensitive to rotenone and the second is insensitive.

Complex I has been purified in an active form from animal and fungal mitochondria (Chen and Guillory, 1981; Ise et al., 1985) and consists of a number of proteins. Cottingham et al. (1986) have attempted immunological analyses of plant

mitochondrial complex I using antisera raised against mammalian complex I. Cottingham and Moore (1988) have performed gel excision experiments to address the nature of the polypeptide composition of the plant mitochondrial complex I. Soole et al. (1991) have partially purified complex I from beetroot mitochondrial inner membranes and have also found that it is composed of numerous polypeptides. The complex I preparation was found to cross-react with polyclonal antisera raised against bovine heart complex I. The Western blots are not very convincing since the antisera apparently react with one of the ATPase subunits which is present in the beetroot mitochondrial complex I preparation. The recognition of an ATPase subunit by the antiserum indicates that the antiserum, which contains antibodies against an ATPase subunit, and the beetroot mitochondrial complex I preparation, which is apparently contaminated with ATPase, are questionable. The partially purified enzyme was not sensitive to rotenone and was capable of oxidizing NADPH at a rate approximately 10 % of the rate of NADH oxidation.

The respiratory chain-linked exogenous NADH DH is located on the outer surface of the inner membrane. This exogenous NADH DH is unique to plant and fungal mitochondria, and as a result, has received the most attention. This enzyme provides direct access of cytosolic NADH pools to the respiratory chain. Although the dehydrogenase itself does not conserve energy, the electrons are contributed to the quinone pool and thus can be potentially shuttled through the cytochrome pathway and be linked to phosphorylation via complexes III and IV (Moller and Lin, 1986). The oxidation of exogenous NADH may provide an important avenue for maintaining a balance between the redox pools of the cytosol and the mitochondrial matrix.

Fairly extensive kinetic studies of exogenous NADH oxidation have been done using intact mitochondrial preparations. A variety of plant tissues have been investigated, with pH optimum values in the range of 6.8 to 7.6 being reported (Arron and Edwards, 1979; Edman et al., 1985; Elthon et al., 1986; Moller and

Palmer, 1981a; Moller and Palmer, 1981b; Moller et al., 1983; Moller et al., 1982). The K_m of the exogenous NADH dehydrogenase also varies among plant species, falling in a range from 10 to 33 μM (Aaron and Edwards, 1980; Soole et al., 1990a).

Several groups have identified specific responses of the exogenous NADH DH to a variety of factors which influence or affect net charge at the membrane surface and at the site of the enzyme. Edman et al. (1985) report that the K_m of the exogenous DH for NADH is subject to change as the surface potential of the inner mitochondrial membrane changes. A membrane potential near zero allows the K_m to remain low, while a very negative membrane potential results in a high K_m. It follows that changes in the membrane potential (while maintaining a constant NADH concentration) would affect the rate of NADH oxidation. Cations have been found to stimulate exogenous NADH oxidation by a general screening of membrane-associated charges (Aaron and Edwards, 1979; Coleman and Palmer, 1971; Earnshaw, 1975; Edman et al., 1985; Hackett, 1961; Johnston et al., 1979; Miller et al., 1970; Moller et al., 1981; Moller et al., 1984; Moller and Palmer, 1981c; Moller et al., 1982; Moore and Akerman, 1982). This effect of cations appears to be a function of the valency of the cation and not of the chemical nature of a given cation (Hackett, 1961; Johnston et al., 1979; Moller and Palmer, 1981c; Moller et al., 1982). However, a specific requirement for Ca^{2+} has been reported by many groups (Aaron and Edwards, 1979; Coleman and Palmer, 1971; Moller et al., 1981; Moore and Akerman, 1982). Coleman and Palmer (1971) have concluded that calcium facilitates binding of the dehydrogenase flavoprotein to the membrane surface, linking the enzyme to the redox components of the electron transport chain. A calcium concentration of ≥ 0.1 μM is required to generate an optimal surface potential under which the dehydrogenase itself is not limiting (Moore and Akerman, 1982). Calmodulin appears to have no role in mediating the Ca^{2+} stimulation (Schwitzguebel

et al., 1985).

Along these same lines, Rugolo et al. (1991) have investigated the effects of polyamines and ionic strength on exogenous NADH oxidation. Addition of the polyamines spermine and spermidine in a low cation medium (2 mM K^+) caused a decrease in the apparent K_m of the exogenous DH for NADH and an increase in the apparent V_{max}. These polyamines also decreased the apparent K_m for Ca^{2+} required to activate the exogenous DH in a high cation medium (physiological concentrations of P_i, Mg^{2+}, and K^+). The polycationic polyamines apparently screened membrane bound charges in a manner which was dependent upon the valency of the cation, where spermine was more effective than spermidine. Using 9-aminoacridine (9-AA) as an indicator of the extent of the screening of electrostatic charges at the membrane surface, several reports have made direct correlations between charge screening and exogenous NADH DH activity (Moller et al., 1981; Rugolo et al. 1991). Chlorotetracycline (CTC) has also been used to investigate charges at the membrane surface. CTC will fluoresce only when chelated to a metal cation in an apolar environment such as methanol or in biological membranes (Moller et al., 1981b). Moller et al. (1981b) found CTC to inhibit exogenous NADH oxidation only in the presence of added Ca^{2+}, observing no effect on the activity when CTC was added alone. Moller et al. (1983) found a strong correlation between CTC fluoresence and the inhibition of exogenous NADH oxidation, reporting that CTC inhibited only when additional Ca^{2+} was present and only at pHs around neutrality. Moller et al. (1981b) also found CTC to be ineffective at acidic pHs, even in the presence of added Ca^{2+}. A theory has been proposed to explain why CTC inhibits only in the presence of additional Ca^{2+}, which is contrary to the inhibitory effects of cation chelators such as EDTA and EGTA whose inhibition is relieved upon addition of Ca^{2+}. This theory predicts that the CTC-Ca^{2+} complex exchanges for the Ca^{2+} bound at the exogenous NADH dehydrogenase, rendering the enzyme inactive

(Moller et al, 1983). Additional reports have found that CTC will inhibit exogenous NADH oxidation (Moller et al., 1984; Moller et al., 1986).

CTC and 9-AA have been used fairly extensively to monitor membrane potential and Ca^{2+} levels at the inner mitochondrial membrane. Moller et al. (1981) reported that *Arum maculatum* mitochondria contain fewer negative charges on a protein basis than do *Helianthus tuberosus* mitochondria, potentially explaining why *Arum* mitochondria oxidize NADH at rates which are 10-times faster than *Helianthus* mitochondria. The results of Rugolo et al. (1991) indicate that the polyamines spermine and spermidine are capable of affecting the access of NADH to the exogenous DH as well as the access of free Ca^{2+} to the catalytic site on the enzyme.

Among the inhibitors of exogenous NADH oxidation are chelators such as EDTA, EGTA, and citrate, which remove calcium and other cations from the membrane surface and from the enzyme itself (Aaron and Edwards, 1979; Coleman and Palmer, 1971; Cottingham and Moore, 1984; Cowley and Palmer, 1978; Moller et al., 1984; Moller and Palmer, 1981b; Schwitzguebel et al., 1985). Addition of excess calcium will reverse the inhibition caused by these chelators (Moller et al., 1984; Schwitzguebel et al., 1985). Interestingly, the binding of NADH to the dehydrogenase renders the Ca^{2+} inaccessible to the chelators (Moller et al., 1981).

The rate at which mitochondria oxidize respiratory substrates is dependant on various factors which influence the rate of transfer through the mitochondrial electron transport chain. These different rates of oxidation reflect the environment or state of the mitochondria. Mitochondrial inhibitors often differentially inhibit mitochondrial processes depending upon the mitochondrial state. The state 4 oxidation (electron transport is inhibited by the proton gradient generated during respiration across the membrane) of exogenous NADH is stimulated by rotenone and amytal, while the state 3 rate of oxidation (the proton gradient is not available to inhibit electron