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Purification, Characterization, and Submitochondrial Localization of a 58-Kilodalton NAD(P)H Dehydrogenase[1]

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An NADH dehydrogenase activity from red beet (*Beta vulgaris* L.) root mitochondria was purified to a 58-kD protein doublet. An immunologically related dehydrogenase was partially purified from maize (*Zea mays* L. B73) mitochondria to a 58-kD protein doublet, a 45-kD protein, and a few other less prevalent proteins. Polyclonal antibodies prepared against the 58-kD protein of red beet roots were found to immunoprecipitate the NAD(P)H dehydrogenase activity. The antibodies cross-reacted to similar proteins in mitochondria from a number of plant species but not to rat liver mitochondrial proteins. The polyclonal antibodies were used in conjunction with maize mitochondrial fractionation to show that the 58-kD protein was likely part of a protein complex loosely associated with the membrane fraction. A membrane-impermeable protein cross-linking agent was used to further show that the majority of the 58-kD protein was located on the outer surface of the inner mitochondrial membrane or in the intermembrane space. Analysis of the cross-linked 58-kD NAD(P)H dehydrogenase indicated that specific proteins of 64, 48, and 45 kD were cross-linked to the 58-kD protein doublet. The NAD(P)H dehydrogenase activity was not affected by ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid or $CaCl_2$, was stimulated somewhat (21%) by flavin mononucleotide, was inhibited by *p*-chloromercuribenzoic acid (49%) and mersalyl (40%), and was inhibited by a bud scale extract of *Platanus occidentalis* L. containing platanetin (61%).

In contrast to their mammalian counterparts, plant mitochondria are capable of oxidizing cytoplasmic NAD(P)H directly, coupling this oxidation to the electron transport chain (Moller and Lin, 1986). This occurs via exogenous NAD(P)H DHs located on the cytosolic face of the inner mitochondrial membrane (Palmer and Moller, 1982; Moller, 1986; Moller and Lin, 1986; Douce and Neuberger, 1989). The oxidation of endogenous mitochondrial matrix substrates (NADH and succinate) has been shown to take precedence over the oxidation of exogenous NAD(P)H (Dry et al., 1983; Day et al., 1985). Thus, it has been suggested that the exogenous NAD(P)H DHs may function in balancing the redox levels between NAD(P)H pools in the cytosol and the mitochondrion (Moller and Lin, 1986; Douce and Neuberger, 1989). As a result, the exogenous NAD(P)H DH could coordinate glycolytic flux with flow through the Krebs cycle. If this is true, then regulation of exogenous NAD(P)H DH activity could greatly influence plant metabolism. Consistent with this hypothesis, Krömer and Heldt (1991) have shown that the oxidation by mitochondria of reducing equivalents generated during photosynthesis is vital for obtaining maximum photosynthetic rates.

Exogenous NAD(P)H DH activity can be distinguished from the other mitochondrial NAD(P)H DH activities by its insensitivity to rotenone (Wilson and Hanson, 1969), stimulation by Ca^{2+} and inhibition by EGTA (Coleman and Palmer, 1971), and sensitivity to platanetin (Ravanel et al., 1986). Although these characteristics can distinguish exogenous NAD(P)H DH activity in situ, most of these characteristics are lost when the enzymes are released from the mitochondrial membrane (Moller and Lin, 1986). There have been many efforts to purify exogenous NAD(P)H DHs (Cook and Cammack, 1984, 1985; Cottingham and Moore, 1984, 1988; Klein and Burke, 1984; Cottingham et al., 1986; Chauveau and Lance, 1991; Luethy et al., 1991), which have led us to propose that exogenous NAD(P)H DH activity can result from up to three different enzymes depending on the species (Luethy et al., 1992). We had previously purified a 32-kD NADH DH and a 42-kD NAD(P)H DH from red beet root (*Beta vulgaris* L.) mitochondria (Luethy et al., 1991). Recently we purified, characterized, and determined the submitochondrial location of a 32-kD NADH DH from maize (*Zea mays* L.; Knudten et al., 1994). The 32-kD DH was found to be a strong candidate for an exogenous NADH DH. In this paper we report the isolation, characterization, and submitochondrial location of a 58-kD NAD(P)H DH from maize. The results support the view that the 58-kD DH is also a strong candidate for an exogenous NAD(P)H DH.

MATERIALS AND METHODS

Red beet roots (*Beta vulgaris* L.) were purchased at local markets. Maize (*Zea mays* L.), inbred line B73, seeds were

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Abbreviations: βME, β-mercaptoethanol; DCPIP, 2,6-dichlorophenol-indophenol; DH, dehydrogenase; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); NEM, *n*-ethylmaleimide; pCMB, *p*-chloromercuribenzoic acid; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone.

obtained from the Nebraska Seed Foundation (Lincoln, NE). Mitochondria were isolated from fresh beet roots and 4- to 5-d-old etiolated maize seedlings at 4°C according to previously published protocols (Hayes et al., 1991; Luethy et al., 1991). Purified mitochondria were resuspended in a medium containing 250 mM Suc and 30 mM Mops (pH 7.2). Total mitochondrial protein was estimated with a modified Lowry assay (Larson et al., 1977) using BSA as the protein standard. Isolated mitochondria were fractionated into three distinct protein fractions, yielding soluble proteins, membrane proteins, and soluble complex protein fractions, as previously described (Hayes et al., 1991). All enzyme purification steps were carried out at 4°C unless otherwise indicated. The isolation of outer membranes and cross-linking of mitochondrial proteins were performed as previously described (Knudten et al., 1994).

Assays

NAD(P)H DH activities were measured as 1 mM NAD(P)H-dependent DCPIP reduction in 30 mM Mops, pH 7.0. DCPIP (60 μM) reduction was monitored at 600 nm (extinction coefficient at 600 nm = 21.0 mM^{-1} cm^{-1}). Nonenzymatic reduction of DCPIP was subtracted from all measurements. When Q_0 was used as the electron acceptor the oxidation of NAD(P)H was followed at 340 nm (extinction coefficient at 340 nm = 6.22 mM^{-1} cm^{-1}). Assays were conducted at 25°C. Results are the means of at least three experiments.

SDS-PAGE, Antibody Production, Immunoblotting, and Immunoprecipitation

Separation of mitochondrial proteins was best achieved using 13 to 16% (w/v) acrylamide gradient slab gels with a 10% (w/v) acrylamide stacking gel. The general techniques used were as reported by Elthon and McIntosh (1986). Two-dimensional gels were run using pH 3 to 10 ampholytes according to the method of Barent and Elthon (1992). Protein was detected using Coomassie brilliant blue. Bio-Rad low molecular weight standards were used to estimate molecular mass. Protein gels shown are representative of at least three similar experiments. Polyclonal antibodies were generated against the partially purified exogenous DH from aged beet root mitochondria (Luethy et al., 1991) using the protocol described by Elthon et al. (1989) with BALB/c mice. Immunoblotting was performed as described by Hayes et al. (1991). Immunoprecipitation experiments were conducted as described by Knudten et al. (1994).

RESULTS

Purification of the 58-kD NAD(P)H DH

The exogenous NAD(P)H DHs can be easily removed from mitochondrial membranes by osmotic swelling and sonication (Douce et al., 1973; Cook and Cammack, 1985). Purification of mitochondrial NAD(P)H DHs was initiated by a mitochondrial fractionation that involved an osmotic shock and sonication of the mitochondria (Hayes et al., 1991). The fractionation procedure results in separation of the mitochondrial constituents into three distinct fractions consisting of membrane proteins, soluble proteins, and large soluble protein complexes (Hayes et al., 1991; Lund et al., 1992). Hayes et al. (1991) found that only 55% of the NADH DH activity remained in the membrane fraction when maize mitochondria were fractionated, and Luethy et al. (1991) reported that less than 30% of the NADH DH activity remained with red beet root mitochondrial membranes. These results are consistent with the literature in suggesting that the exogenous NAD(P)H DH is a loosely associated membrane protein.

A summary of the NAD(P)H DH activities recovered after maize mitochondrial fractionation is given in Table I. Most of the NADH and NADPH DH activity was found in the soluble fraction. Since the exogenous DH had previously been reported to be easily removed from the mitochondrial membranes, it was reasonable to assume that some of the activity found in the soluble protein fraction was due to the exogenous DH. In addition, since the specific activities of NAD(P)H DH in the soluble protein fraction were substantially higher that those found in the membrane fraction, the soluble fraction constituted excellent starting material for purification of the exogenous DH. We previously reported similar results with red beet root mitochondria (Luethy et al., 1991).

Anion-exchange chromatography (Mono Q, Pharmacia) was previously used to analyze the NAD(P)H DHs contained in the soluble protein fraction from both maize and red beet root mitochondria. Red beet root mitochondria were isolated from root tissue that had been "aged" (Luethy et al., 1991), and the soluble fraction was found to contain three NAD(P)H DH activities (Luethy et al., 1991). Only two NAD(P)H DH activities have been found in the soluble fraction from maize (Hayes et al., 1991; Luethy et al., 1992). Based on SDS-PAGE analyses of partial purifications, we proposed that the last NAD(P)H DH activity eluted from the Mono Q column with both red beet root and maize was due to a 58-kD protein (Luethy et al., 1992).

In this paper we report the purification of the last Mono Q peak of NADH DH activity from beet root mitochondria to homogeneity. Using the same protocols as before but with "fresh" red beet roots, we purified the NADH DH activity from the soluble fraction with the Mono Q column. Figure 1 shows that the activity purified to a 58-kD protein that typically appears as a doublet. Two-dimensional gel analysis of the 58-kD doublet is presented to the right in

Table I. *NAD(P)H-DCPIP oxidoreductase activity of maize submitochondrial fractions*

The results are the means of three experiments.

Mitochondrial Subfraction	Total Protein	Specific Activity (Percentage of Total Activity)	
		NADH	NADPH
	%	μmol min^{-1} mg^{-1} protein	
Soluble	18.8	4.29 (55.7)	7.75 (74.1)
Complexes	8.1	1.04 (5.8)	1.37 (5.7)
Membranes	73.1	0.76 (38.5)	0.54 (20.2)

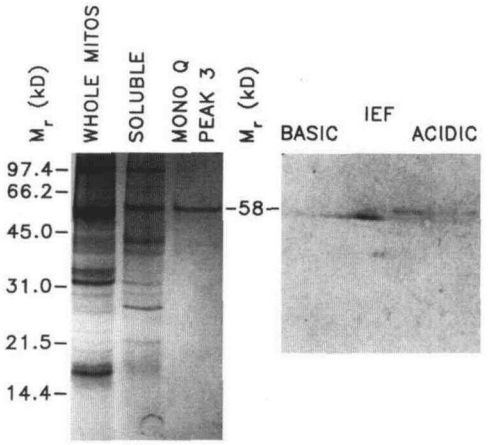

Figure 1. Purification of the red beet root 58-kD NADH DH. The soluble fraction of mitochondria isolated from fresh red beet roots was separated on Mono Q as previously described (Luethy et al., 1991). The third peak of NADH DH activity was purified to a 58-kD protein doublet based on SDS-PAGE (left). Approximately 15 μg of protein were loaded in the mitochondria (mitos) and soluble lanes, and about 1 μg of protein was loaded in the Mono Q peak 3 lane. Proteins were stained with Coomassie brilliant blue. Right, Two-dimensional protein blot of the purified protein. The blot was stained with Amido black.

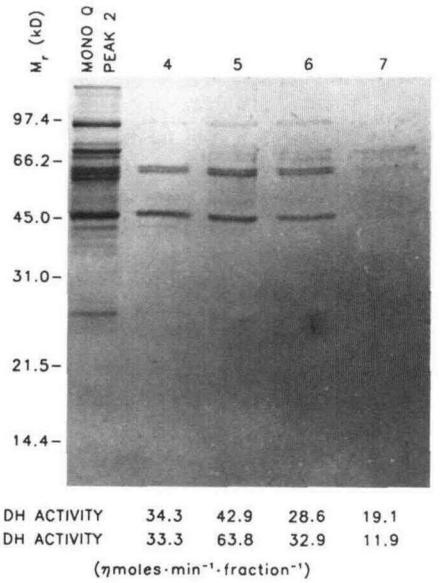

Figure 2. The soluble fraction from maize mitochondria was separated on Mono Q as described by Luethy et al. (1991). The second peak of NADH DH activity was collected and its NaCl concentration adjusted to 2 M, and it was applied to a phenyl-Superose column. A single peak of activity eluted from phenyl-Superose and the activity and surrounding fractions were analyzed by SDS-PAGE. Lane 1 contains approximately 15 μg of the Mono Q peak 2 activity that were applied to the phenyl-Superose column. Lanes 2 through 5 contain approximately half of the protein present in each phenyl-Superose activity fraction (4–7). NAD(P)H-DCPIP oxidoreductase activities are indicated below the lanes. Proteins were visualized by staining with Coomassie brilliant blue.

Figure 1. The lower molecular mass protein in the 58-kD doublet often appears more prevalent, as in the two-dimensional gel of Figure 1. The purified activity from red beet root mitochondria had an average specific activity of 2.17 μmol DCPIP min^{-1} mg^{-1} protein.

In maize, we have not been able to purify the same activity to homogeneity. The second peak of NAD(P)H DH activity from Mono Q contains several proteins (left lane of Fig. 2). The NaCl concentration of the peak activity fractions was then adjusted to 2.0 M NaCl and applied to a phenyl-Superose column. Higher concentrations of salt were found to inactivate the enzyme. This phenomenon was previously noted with the homologous enzyme from red beet root mitochondria (Luethy et al., 1991). The column was eluted with a 2.0 to 0 M NaCl gradient, and 0.5-mL fractions were collected. The activity did not bind to the column and eluted in the first few fractions. The right four lanes of Figure 2 show the protein and activity profiles obtained. The presence of the 58-kD protein doublet and a 45-kD protein strongly correlated with the activity. The weak protein at 25 kD had previously been shown not to correlate with NAD(P)H DH activity (Luethy et al., 1992). The specific activity of fraction 5 was 7.15 μmol DCPIP min^{-1} mg^{-1} protein with NADH as the substrate.

Immunological Characterization of the 58-kD NAD(P)H DH

Antibodies were prepared against the 58-kD protein from red beet roots and used in immunoprecipitation experiments under conditions as described by Knudten et al. (1994). The antibodies were incubated with maize Mono Q fractions containing the 58-kD NAD(P)H DH activity and found to immunoprecipitate 44% of the activity. The antibodies were further used to evaluate the distribution of the 58-kD DH between submitochondrial fractions obtained by disruption of maize mitochondria (Fig. 3). The results indicate that the 58-kD protein is found in significant

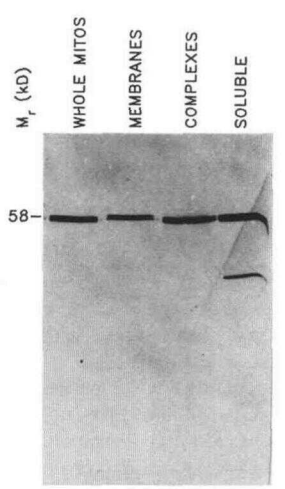

Figure 3. Immunoblot analysis of maize submitochondrial fractions probed with polyclonal antibodies raised against the red beet root 58-kD DH. Each lane contained approximately 15 μg of protein. MITOS, Mitochrondria.

amounts in the membrane, complex, and soluble fractions. These results suggest that the 58-kD protein may be part of a protein complex that is loosely associated with the membrane. A lower band was present in the soluble fraction, which may represent a degradation product of the 58-kD protein, since it was not present in whole mitochondria.

The polyclonal antibodies have also been used to assess whether the 58-kD DH protein is present in other species. Figure 4 is an immunoblot in which mitochondria from various plant species and from rat liver were probed with the 58-kD DH polyclonal antibodies. The antibodies recognized proteins in the 58-kD range in each of the plant species tested. Notably, there appeared to be no immunologically related protein species in rat liver mitochondria. Animal mitochondria are known not to contain exogenous NADH DH activity. The 58-kD NADH DH is thus reasonably conserved among a diverse group of plant species, including monocot and dicot plants as well as actively growing and dormant tissues. The 58-kD protein is prevalent in mitochondria isolated from the thermogenic appendices of *Arum italicum* and *Sauromatum guttatum*, which are known to have high levels of exogenous NAD(P)H DH activity (Lance, 1974; Elthon and McIntosh, 1986).

Submitochondrial Localization of the 58-kD NAD(P)H DH from Maize

We previously used the membrane-impermeable protein cross-linking agent DTSSP (Pierce, Rockford, IL) to determine the submitochondrial location of the 32-kD NADH DH from maize (Knudten et al., 1994). In freshly isolated intact mitochondria, DTSSP can pass through the outer membrane, presumably through porin, and thus cross-link proteins on both surfaces of the outer membrane, proteins in the intermembrane space, and proteins on the outer surface of the inner membrane. DTSSP is cleavable with βME, and thus cross-linking can be followed by the migration of cross-linked proteins as high mol wt complexes in SDS-PAGE run in the absence of βME. When SDS-PAGE of the cross-linked proteins is performed in the presence of βME, the proteins migrate to their usual mol wt. The cross-linking of specific proteins can be followed on protein blots with antibodies (Knudten et al., 1994). In Figure 5 (top) similar experiments were performed, and the protein blot was probed with polyclonal antibodies to the 58-kD DH. The results show that nearly all of the protein became cross-linked; however, some was inaccessible. Most of the 58-kD protein was cross-linked to such an extent that it remained in the well of the gel and is thus not visible in Figure 5. Two distinct bands of cross-linked 58-kD protein were small enough to enter the gel. The results indicate clearly that the majority of the 58-kD pro-

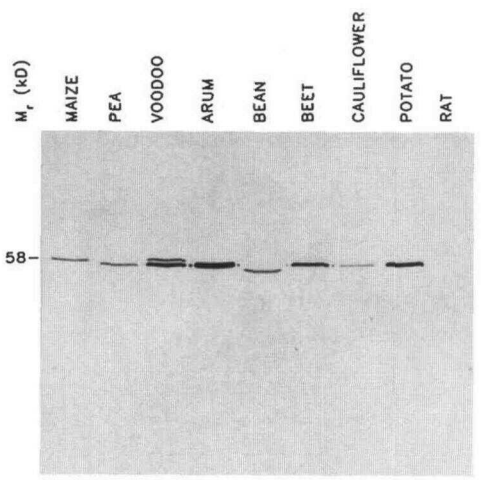

Figure 4. The presence of the 58-kD NAD(P)H DH in different species. The protein blot was prepared with 15 μg of each type of mitochondria and was probed with polyclonal antibodies to the 58-kD DH. The mitochondria were isolated from etiolated maize (inbred line B73) epicotyl tissue, green pea (*Pisum sativum* L. cv Homesteader) leaves, voodoo lily (*S. guttatum* Schott) appendix tissue on the day of heating, *A. italicum* Mill. appendices, etiolated bean (*Phaseolus vulgaris* cv Sprite) hypocotyls, aged red beet (*B. vulgaris* L.) roots, cauliflower (*Brassica oleracea* L.) inflorescences, potato (*Solanum tuberosum* L.) tubers, and rat (*Rattus norvegicus* Berkenhout var *albinus*) liver. All mitochondria were isolated with the same procedure used for maize mitochondria.

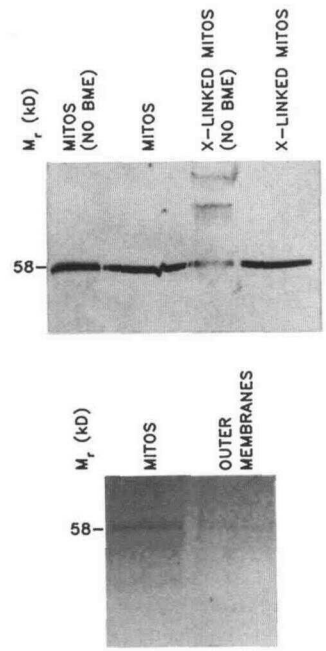

Figure 5. Submitochondrial localization of the 58-kD NAD(P)H DH from maize. The top panel is an immunoblot probed with polyclonal antibodies to the 58-kD DH. The left two lanes are control mitochondria in which the proteins were separated by SDS-PAGE in the absence and presence of βME. The right two lanes are mitochondria that were incubated with the membrane-impermeable protein cross-linking agent DTSSP. This cross-linking agent is homo-bifunctional and is cleavable by βME. Migration of proteins as higher mol wt complexes in the absence of βME indicates cross-linking. Approximately 20 μg of mitochondrial protein were loaded per lane. The bottom panel is a protein blot of whole mitochondria and isolated outer membranes of mitochondria probed with polyclonal antibodies to the 58-kD DH. Approximately 5 μg of protein were loaded per lane. Procedures for cross-linking and for isolation of outer membranes were as described by Knudten et al. (1994). MITOS, Mitochondria; X-LINKED MITOS, cross-linked mitochondria.

tein is located exterior to the inner membrane barrier. Controls similar to those previously published (Knudten et al., 1994) were performed to ensure that DTSSP did not penetrate the inner membrane (data not shown).

Outer membranes were isolated from maize mitochondria and used to prepare a protein blot comparing total mitochondrial proteins with those of the outer membrane (Fig. 5, bottom). The protein profile of the Coomassie stained gel was similar to that previously published (Knudten et al., 1994). The results show that the 58-kD protein is not enriched in the outer membrane preparation, indicating that the 58-kD NADH DH is not associated with the outer membrane.

Identification of Proteins Cross-Linked to the Maize 58-kD NAD(P)H DH

Proteins exterior to the inner membrane barrier were cross-linked in freshly isolated intact mitochondria as described above. These mitochondria were then fractionated into membrane, complex, and soluble proteins as previously described. The soluble fraction was separated on Mono Q and two peaks of NAD(P)H DH activity were observed (Fig. 6). The position of these peaks did not correlate with those of the 32- and 58-kD NAD(P)H DH observed in control mitochondria that were not cross-linked. The cross-linked peaks were identified using the polyclonal antibodies to the 32- and 58-kD DHs (data not shown) and are labeled in Figure 6.

The protein content of the cross-linked 58-kD DH Mono Q peak was evaluated using SDS-PAGE in an effort to determine what proteins become cross-linked to the 58-kD

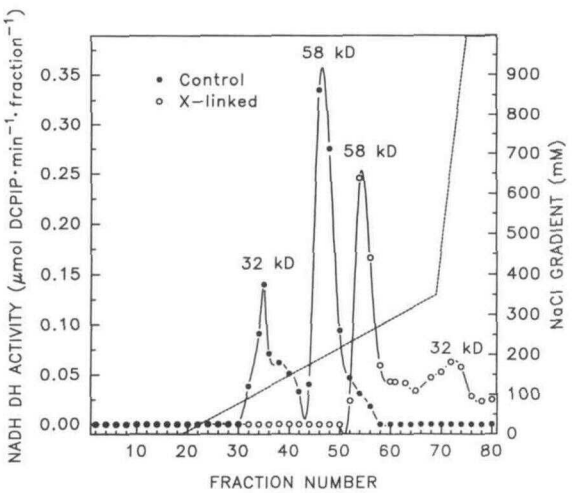

Figure 6. Separation of cross-linked maize NAD(P)H DH activities on Mono Q. Control and cross-linked maize mitochondria were fractionated and the soluble fractions applied independently to a Mono Q column. Each column was eluted with a 0 to 350 mM NaCl gradient and 0.5-mL fractions were collected. Two NAD(P)H DH activity peaks were observed in the control preparation as previously described (Hayes et al., 1991; Luethy et al., 1992). Two activity peaks were also observed in the cross-linked preparation and were identified using protein blots and polyclonal antibodies to the 32- and 58-kD DHs (data not shown).

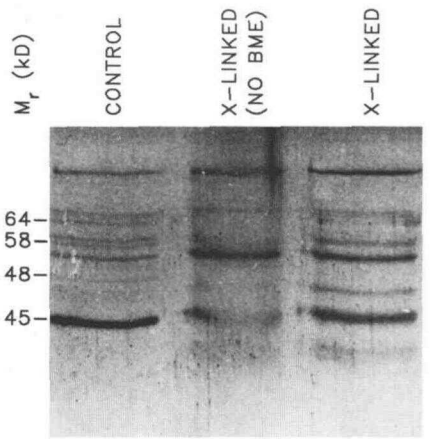

Figure 7. SDS-PAGE analysis of the control and cross-linked maize 58-kD NAD(P)H DH Mono Q activity peaks of Figure 6. Lane 1 corresponds to the 58-kD NAD(P)H DH Mono Q activity peak from control mitochondria subjected to SDS-PAGE in the presence of βME. Lanes 2 and 3 are of the 58-kD NAD(P)H DH activity from cross-linked mitochondria (X-LINKED), separated by SDS-PAGE in the absence (lane 2) and presence (lane 3) of βME. Approximately 3 μg of protein were loaded per lane. The gel was stained with Coomassie brilliant blue.

protein doublet (Fig. 7). Comparison of the cross-linked 58-kD peak separated in the presence and absence of βME showed that several proteins became cross-linked to the 58-kD DH as indicated by their absence in the no-βME lane. These proteins had apparent molecular masses of 64, 48, and 45 kD. Most of these proteins were also present in the 58-kD DH activity peak of control mitochondria that were not cross-linked (Fig. 7, lane 1); however, additional proteins were also present. Even though the cross-linked 58-kD activity was shifted significantly on the Mono Q profile, the protein profile was strikingly similar to the noncross-linked peak, suggesting that some association of these proteins normally exists in situ. These results are consistent with the submitochondrial fractionation data, which indicated that the 58-kD DH may be part of a complex. Difficulties observed in purifying the 58-kD DH to homogeneity from maize mitochondria and from aged beet root mitochondria are also consistent with the involvement of a protein complex.

Characterization of the Maize 58-kD NAD(P)H DH Activity

Figure 8 displays the pH profiles for the NADH and NADPH DH activities of the 58-kD DH of maize. NADH DH activity was optimal near pH 7.5 and the optimum for NADPH DH activity was somewhat higher at pH 8.0 to 8.5. The effect of various compounds on activity of the 58-kD NADH DH was investigated (Table II). EGTA amd $CaCl_2$ had no effect on the solubilized activity. NaCl and KCl at 1 M inhibited the activity significantly (about 40%). FAD had no effect on the activity, whereas FMN stimulated it 19%. NAD, NADP, ADP, and ATP did not affect the activity. The effect of sulfhydryl reagents on the activity was investigated: pCMB and mersalyl inhibited the activity, whereas

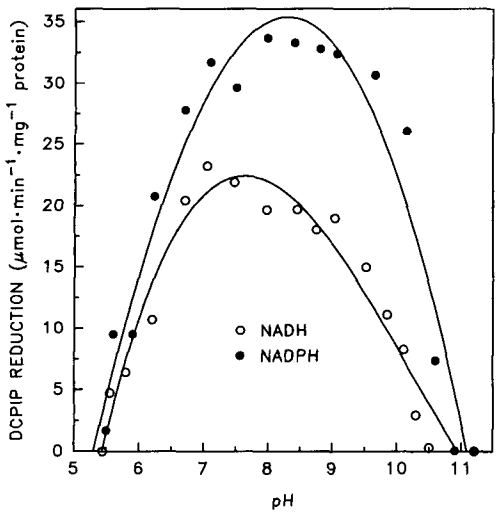

Figure 8. Determination of the pH optima for the NADH and NADPH DH activities of the 58-kD maize DH. Enzyme assays were performed in buffer containing 20 mM each of Mes, Tes, and Tricine buffers. The assays were conducted using 200 μM of either NADH or NADPH as substrate. The results are the means of three experiments.

NEM was ineffective. The activity was insensitive to 2,4-D, rotenone, and antimycin A. Of several flavin-like compounds evaluated, only the bud extract containing platanetin was effective (inhibited the activity 59%). Assays were also conducted with NADPH as substrate (Table II), and similar results were obtained. The 58-kD DH oxidized different co-factors as follows: NADH, 100%; deamino-NADH, 102%; NADPH, 94%; and deamino-NADPH, 111%. The 58-kD NAD(P)H DH was found to reduce 1 mM Q_0 at 240% of the rate observed with DCPIP (60 μM). The 58-kD NAD(P)H DH was not able to reduce oxygen.

DISCUSSION

In this paper we describe the purification of a 58-kD NADH DH activity from fresh red beet root mitochondria. To our knowledge, this represents the first purification of the 58-kD NADH DH to homogeneity. Previous characterization of the 58-kD NADH DH from aged red beet roots showed that it was readily solubilized from mitochondria by osmotic stress and sonication and was sensitive to inhibition by platanetin (Luethy et al., 1991), both of which are characteristics of the exogenous NAD(P)H DH (Douce et al., 1973; Ravanel et al., 1986). Because red beet root mitochondria are relatively inactive, we continued our investigation of this DH in maize. In maize, the 58-kD DH could not be purified to homogeneity; however, it was partially purified to a 58-kD protein doublet and a 45-kD protein. The activity from maize oxidized both NADH and NADPH. In maize the activity was also readily solubilized, was sensitive to platanetin, and was stimulated somewhat by FMN.

In this paper we have also shown that most of the maize 58-kD NAD(P)H DH protein is located on the outer surface of the inner mitochondrial membrane based on cross-linking studies, absence of the protein in outer membrane preparations, and behavior as a membrane-associated protein complex. Chauveau and Lance (1991) recently isolated a similar (as judged by the Mono Q elution profile and overall protein composition) activity from *Arum maculatum*. The *Arum* activity oxidized only NADH, was readily solubilized from mitochondria by freeze-thawing, and contained flavoprotein. They proposed that this activity was located on the outer surface of the inner membrane, based on several methodological assumptions. Taken together, the results of reported in this paper and those of Luethy et al. (1991) and Chauveau and Lance (1991) support the view that the 58-kD NAD(P)H DH protein likely functions as an exogenous NAD(P)H DH.

We have consistently found several NAD(P)H DH activities in solubilized preparations. A comparison of the protein profiles from different species suggests that up to three different NAD(P)H DHs may be released into the soluble fraction with sonication. We have for the first time been able to characterize in detail various NAD(P)H DH activities from more than one plant species. From red beet root mitochondria, three NAD(P)H DHs are released, a 42-kD

Table II. *Effect of various additions on activity of the maize 58-kD NAD(P)H DH*

NAD(P)H-DCPIP oxidoreductase activity was measured at pH 7.0 in 30 mM Mops using 1 mM NAD(P)H except for the 2,4-D assays, which were conducted with 100 μM NAD(P)H. Control rates for NADH-DCPIP activity varied between 10 and 20 μmol min^{-1} mg^{-1} protein and between 20 and 30 μmol min^{-1} mg^{-1} protein for NADPH-DCPIP activity. pCMB, 2,4-D, rotenone, flavone, phloretin, phloridzin, kaempherol, and apigenin were solubilized in 100% DMSO. Antimycin A was dissolved in 100% ethanol. The bud extract containing platanetin was prepared as previously described (Luethy et al., 1991). The results are the means of three experiments.

Addition	58-kD DH Activity	
	NADH	NADPH
	% of control	
None	100	100
5 mM EGTA	99	102
1 mM CaCl2	89	105
1 M NaCl	60	61
1 M KCl	58	56
1 mM FAD	95	97
1 mM FMN	119	123
200 μM NAD	87	94
200 μM NADP	88	98
200 μM ADP	96	95
200 μM ATP	98	99
150 μM NEM	96	91
150 μM pCMB	52	50
150 μM mersalyl	62	59
150 μM 2,4-D	89	92
8 μM rotenone	93	98
1 μM antimycin A	101	101
150 μM dicoumarol	90	92
150 μM flavone	85	96
150 μM phloretin	96	91
150 μM phloridzin	91	98
150 μM kaempherol	84	87
150 μM apigenin	94	99
Bud extract	41	38

NAD(P)H DH, a 32-kD NADH DH, and a 58-kD NADH DH (Luethy et al., 1991; this paper). We have not observed a 26-kD DH as reported by Rasmusson et al. (1993). We have found that only two DHs are released from maize mitochondria upon sonication, a 32-kD NADH DH (Knudten et al., 1994) and a NAD(P)H DH activity that contains a 58-kD protein doublet and a 45-kD polypeptide (Luethy et al., 1992; this paper). We have shown that the 58-kD protein from beet and maize are immunologically related. These studies indicate that the nature of exogenous NAD(P)H oxidation in plant mitochondria may vary depending on what DHs are expressed, the level of their expression, and their specificity toward NADH and NADPH.

Chauveau and Lance (1991) found that the *Arum* 32- and 58-kD DHs were both capable of reducing the quinones juglone and duroquinone. We have observed that the maize 32-kD DH (Knudten et al., 1994) and the maize 58-kD DH (this paper) are capable of reducing Q_0. This could mean that the purified DHs are capable of reducing the quinone pool directly but does not prove that other proteins are not involved. For the 58-kD protein, we have determined which proteins become cross-linked to it when freshly isolated intact mitochondria are incubated with the cross-linker DTSSP. The results showed that three proteins of 64, 48, and 45 kD became cross-linked to the 58-kD protein doublet (Fig. 7). These proteins are also found in activity fractions partially purified through Mono Q and phenyl-Superose (Fig. 2). These results indicate that the 58-kD protein doublet is in close association with these other proteins, but we have yet to evaluate their possible role in the NAD(P)H DH activity. Interestingly, the protein profile of the 58-kD DH from *Arum* is quite similar (Chauveau and Lance, 1991).

The pH profiles for NADH oxidation by three 58-kD DHs have now been determined. The *Arum* enzyme has an optimum near 5.5 (Chauveau and Lance, 1991), the red beet root enzyme has an optimum near 6.5 (Luethy et al., 1991), and the maize enzyme has an optimum near 7.5. pH profiles for NADH oxidation by three 32-kD DHs have also been established and were found to be near pH 7.0 for *Arum* (Chauveau and Lance, 1991), red beet root (Luethy et al., 1991), and maize (Knudten et al., 1994). The red beet root 58-kD NADH DH activity was previously shown to be inhibited by NAD^+, dicoumarol, mersalyl, NEM, and pCMB (Luethy et al., 1991). We have now observed that the maize 58-kD NAD(P)H DH is also inhibited by mersalyl and pCMB but not by dicoumarol, NEM, or NAD^+. The 32-kD NADH DH from red beet root was also found to be inhibited by mersalyl, pCMB, and dicoumarol (Luethy et al., 1991). Similar inhibition characteristics were observed for the activity from maize (Knudten et al., 1994). The pH optimum studies, inhibitor studies, and substrate specificity results indicate that differences between species are common and that these characteristics should not be used to correlate DH activities between species.

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