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Purification, Characterization, and Submitochondrial Localization of the 32-Kilodalton NADH Dehydrogenase from Maize[1]

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Plant mitochondria have the unique ability to directly oxidize exogenous NAD(P)H. We recently separated two NAD(P)H dehydrogenase activities from maize (*Zea mays* L.) mitochondria using anion-exchange (Mono Q) chromatography. The first peak of activity oxidized only NADH, whereas the second oxidized both NADH and NADPH. In this paper we describe the purification of the first peak of activity to a 32-kD protein. Polyclonal antibodies to the 32-kD protein were used to show that it was present in mitochondria from several plant species. Two-dimensional gel analysis of the 32-kD NADH dehydrogenase indicated that it consisted of two major and one minor isoelectric forms. Immunoblot analysis of submitochondrial fractions indicated that the 32-kD protein was enriched in the soluble protein fraction after mitochondrial disruption and fractionation; however, some association with the membrane fraction was observed. The membrane-impermeable protein cross-linking agent 3,3′-dithiobis-(sulfosuccinimidylpropionate) was used to further investigate the submitochondrial location of the 32-kD NADH dehydrogenase. The 32-kD protein was localized to the outer surface of the inner mitochondrial membrane or to the intermembrane space. The pH optimum for the enzyme was 7.0. The activity was found to be severely inhibited by *p*-chloromercuribenzoic acid, mersalyl, and dicumarol, and stimulated somewhat by flavin mononucleotide.

Plant mitochondria have several unique NAD(P)H DH activities including the exogenous NAD(P)H DH activities that are located on the outer surface of the inner mitochondrial membrane (Douce et al., 1973; Palmer and Ward, 1985; Møller and Lin, 1986). Another unique NAD(P)H DH activity faces the mitochondrial matrix and is rotenone insensitive and has low affinity for NAD(P)H (Møller and Palmer, 1982; Rasmusson et al., 1993). Oxidation of exogenous NADH and NADPH have often been observed to have different characteristics, supporting the view that two different enzymes are involved. There have also been a number of differences in exogenous NAD(P)H DH activities observed between species, thus making it difficult to derive a unifying model for the oxidation of exogenous NAD(P)H. However, exogenous NADH DH activity is characteristically observed to be stimulated by calcium in situ and is sensitive to flavones, particularly platanetin (Ravanel et al., 1986). Exogenous NAD(P)H DH activity is known to be only loosely associated with the outer surface of the inner mitochondrial membrane (Douce et al., 1973).

Several early attempts at purifying the proteins involved in the exogenous NAD(P)H DH did not result in a consensus about its protein composition (Cook and Cammack, 1984, 1985; Cottingham and Moore, 1984, 1988; Klein and Burke, 1984; Cottingham et al., 1986). We recently published our data about finding three NAD(P)H DH activities in red beet root mitochondria (Luethy et al., 1991). Two of these activities purified to single proteins of 42 and 31 kD. The 42-kD protein oxidized both NADH and NADPH, whereas the 31-kD protein was specific for NADH. The third activity was partially purified to a protein doublet near 55 kD and a 40-kD protein. This third activity oxidized only NADH and was found to be sensitive to platanetin, suggesting that this activity represents the traditional NADH DH. Since red beet root mitochondria also oxidize exogenous NADPH (Fredlund et al., 1991), we proposed that the 42-kD protein was responsible for this activity in red beet root mitochondria. Chauveau and Lance (1991) have recently published information on the purification of exogenous NAD(P)H DHs from *Arum maculatum* mitochondria. They found two activities, one associated with a cluster of proteins near 33 kD that oxidized both NADH and NADPH, and a second that was partially purified to a series of proteins with a 54-kD polypeptide being the most prevalent. They observed that the 54-kD DH was stimulated by calcium, suggesting that it was the traditional exogenous NADH DH. They further proposed that the 33-kD DH was the exogenous NADPH DH. Both DHs were found to be flavoproteins.

Comparison of these recent findings with the literature suggests that exogenous NAD(P)H DH activity could result

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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; 2D, two-dimensional.

from the combined activities of three different proteins. The 55 (54–55)-kD DH would appear to be the traditional exogenous NADH DH based on its sensitivity to platanetin (Luethy et al., 1991) and stimulation by calcium (Chauveau and Lance, 1991). The roles of the 42- and 32 (31–33)-kD DHs are less clear. The three DHs have different cofactor specificities depending on the plant species.

In this paper we describe purification and characterization of a 32-kD NADH DH activity from corn (*Zea mays* L.) mitochondria. This activity was localized to either the outer surface of the inner mitochondrial membrane or to the intermembrane space. The properties of this DH are compared to the 32-kD activities observed in *Arum maculatum* and in red beet root. The data to date support the view that the 32-kD NAD(P)H DH activity may be an exogenous NAD(P)H DH. A preliminary report of some of these results has been presented elsewhere (Luethy et al., 1992).

MATERIALS AND METHODS

Corn (*Zea mays* L. B73) seed was obtained from the Nebraska Seed Foundation (Lincoln, NE). Corn seedlings were grown in the dark at 29°C for 4 to 5 d. Mitochondria were isolated from etiolated shoots and fractionated as previously described (Hayes et al., 1991). Fractions containing mitochondrial membranes, soluble proteins, and high mol wt soluble protein complexes were obtained. Proteins were quantitated by the Lowry method as modified by Larson et al. (1977). For Mono Q column fractions, the Integrated Separation Systems (Hyde Park, MA) Protein Gold system was used.

Enzyme Assays

NAD(P)H DH activities were measured as described by Luethy et al. (1991) using 1 mM NADH or 1 mM NADPH and 60 μM DCPIP as an artificial electron acceptor. Assays were performed at 25°C in 30 mM Mops, pH 7.0. The reduction of DCPIP was monitored at 600 nm (ϵ_{600nm} = 21.0 mM^{-1} cm^{-1}). Nonenzymatic reduction of DCPIP by NAD(P)H varied with pH and was subtracted from these data. When 1 mM Q_0 was used as the artificial electron acceptor, the utilization of NAD(P)H was followed at 340 nm (ϵ_{340nm} = 6.22 mM^{-1} cm^{-1}). All results presented are means of at least three separate experiments.

SDS-PAGE, Antibody Production, Immunoblotting, and Immunoprecipitation

Separation of mitochondrial proteins was performed using a 13 to 16% (w/v) acrylamide resolving gel and a 10% (w/v) stacking gel as reported by Elthon and McIntosh (1986). Proteins resolved by SDS-PAGE were visualized using Coomassie brilliant blue stain or by silver staining with the protocol of Merril et al. (1984). Protein standards used were Bio-Rad low mol wt standards. 2D IEF was performed as described by Barent and Elthon (1992). Polyclonal antibodies were generated as previously described (Elthon et al., 1989) using 1 to 1.5 μg of protein per injection. Immunoblots of SDS-PAGE were carried out as described by Elthon and McIntosh (1987). Each protein gel or immunoblot is representative of at least three similar experiments. Immunoprecipitation experiments were conducted with 50 μL of serum (either preimmune or anti-32-kD NADH DH) and approximately 20 μmol min^{-1} of NADH DH activity. The protein was incubated with the serum for 5 h at 4°C, 50 μL of Protein A Sepharose 4B Fast Flow beads were added, and the mixture was incubated with agitation at 4°C for an additional 2 h. The mixture was then centrifuged at 16,000*g* for 10 min in an Eppendorf microfuge, and the supernatant was assayed for NADH DH activity.

Cross-Linking of Mitochondrial Proteins

Mitochondrial proteins were cross-linked with the membrane-impermeable cross-linking agent DTSSP from Pierce (Rockford, IL). Freshly isolated intact mitochondria (13.2 mg) were incubated at 4°C for 2 h in 2.2 mL of PBS (10 mM KH_2PO_4, 150 mM NaCl, pH 7.2) containing 250 mM Suc and 2 mM DTSSP. The reaction was stopped by addition of Tris to a final concentration of 40 mM, followed by incubation at 4°C for 15 min.

Isolation of Mitochondrial Outer Membranes

Outer membranes were isolated using a modification of the method of Mannella and Bonner (1975). Freshly isolated mitochondria (about 70 mg) were resuspended in a minimum volume (about 200 μL) of 250 mM Suc, 30 mM Mops (pH 8.0). The resuspended mitochondria were added to 50 volumes of vigorously stirred 10 mM Mops (pH 8.0) and stirred for 5 min. The resulting suspension was layered onto a step gradient consisting of 5 mL of 0.3, 0.6, 0.9, and 1.2 M Suc and centrifuged at 40,000*g* for 1 h in a Beckman SW28 rotor. Outer membranes were collected from the 0.6 to 0.9 M Suc interface. The outer membrane fraction was diluted with 3 volumes of 30 mM Mops (pH 8.0) and centrifuged at 60,000*g* for 90 min in a Sorvall T865.1 rotor. The outer membrane pellet was resuspended in 30 mM Mops (pH 8.0).

RESULTS

Purification of the 32-kD NADH DH

Several reports in the literature have indicated that the exogenous NAD(P)H DH activities are readily released from plant mitochondrial membranes by relatively gentle treatments (Douce et al., 1973; Cook and Cammack, 1985; Luethy et al., 1991). When mitochondria are subfractionated into membrane proteins, soluble proteins, and soluble proteins that exist as large protein complexes (Hayes et al., 1991), the soluble protein fraction contains most of the NAD(P)H DH activity. The specific activity of NADH DH in the subfractions was whole (0.474), membrane (0.246), complex (0.470), and soluble (1.67 μmol DCPIP min^{-1} mg^{-1} protein). Up to three NAD(P)H DH activities have been reported in the soluble fraction after mitochondrial disruption (Chauveau and Lance, 1991; Luethy et al., 1991). Thus, the specific activities measured are potentially the combination of several activities. The soluble fraction of mitochondria served as a convenient starting material for purification of exogenous NAD(P)H DH

activities because a large amount of these activities were released from the membrane without the use of detergent.

The solubilized NAD(P)H DH activity was initially applied to an anion-exchange column (Mono Q) and eluted with a linear gradient from 0 to 350 mM NaCl. A first peak of NADH DH activity eluted at about 100 mM NaCl and a second peak eluted near 200 mM NaCl. SDS-PAGE analysis of the peak fractions revealed that the protein profiles were still fairly complex at this stage (data not shown). Peak 1 on average had a specific activity of 4.86 μmol min^{-1} mg^{-1} protein. Peak 2 had an average specific NADH DH activity of 22 μmol min^{-1} mg^{-1} protein and also exhibited a NADPH DH activity of 33 μmol min^{-1} mg^{-1} protein.

The four fractions that contained the greatest Mono Q peak 1 NADH DH activity were pooled and concentrated. The protein sample (about 300 μg) was then made to 4 M NaCl and applied to a Phenyl-Superose (hydrophobic interaction) column. The activity was eluted with a 4 to 0 M linear NaCl gradient. A single peak of NADH DH activity eluted in the range of 1 to 0.5 M NaCl. At this point, the activity was purified to a single protein of 32 kD (Fig. 1). Table I shows the yield and specific activities at different stages of purification of the 32-kD NADH DH. A purification factor or fold purification is not relevant in this purification due to the multiple NADH DH activities in both the whole and soluble fractions. Considerable enzyme inactivation occurred as evidenced by a final yield of 0.4% of the initial total mitochondrial activity. The yield of purified protein was 0.006% of total mitochondrial protein.

For further characterization of the 32-kD NADH DH, a polyclonal antibody against this protein was generated. Mice were injected with the 32-kD antigen as described in "Materials and Methods." A specific polyclonal antibody was obtained within 4 weeks of the initial injection. The anti-32-kD polyclonal antibody was compared to preimmune serum in immunoprecipitation experiments (described in "Materials and Methods") to further establish that the 32-kD protein was responsible for the NADH DH activity. The anti-32-kD antibody was found to immunoprecipitate 26% of the NADH DH activity as compared to preimmune serum. Figure 2 shows an immunoblot of the mitochondrial subfractions probed with the anti-32-kD antibody. Lane 1 (Whole Mitos) shows the reaction of the antibody in whole mitochondria. Some association was detected with the membrane fraction in lane 2 (Membranes), as was the case with the complex fraction. The data indicated that the 32-kD NADH DH protein was enriched in the soluble protein fraction after mitochondrial subfractionation. These results indicate that if the 32-kD DH is a membrane protein in situ, then it is loosely associated with the membrane.

The antibody to the 32-kD NADH DH was used to analyze cross-reactivity with mitochondria from different plant species (Fig. 3). The antibodies were found to react with a similar 32-kD protein in all of the plant species tested. The response in red beet root mitochondria was weak, which correlates with the low levels of this protein found in red beet root mitochondria (Luethy et al., 1991). The antibodies also rec-

Table I. *Specific activities observed during purification of the 32-kD NADH DH*

Activities were measured as NADH-dependent DCPIP reduction.

Purification Step	Total Activity	Total Protein	Specific Activity
	μmol min^{-1}	mg	μmol min^{-1} mg^{-1}
Whole mitochondria	30.0	65.1	0.474
Soluble proteins	13.2	8.41	1.67
Mono-Q peak 1	0.810	0.282	4.86
Phenyl-Superose	0.122	0.004	29.0

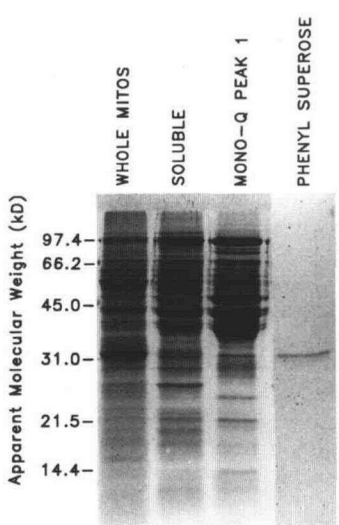

Figure 1. SDS-PAGE of purification steps for the 32-kD NADH DH. Twenty micrograms of protein was loaded in the first three lanes, which consisted of whole mitochondria, the soluble fraction of mitochondria, and the first NADH DH activity peak from Mono Q. The entire Phenyl-Superose peak was loaded in the far-right lane. Proteins were visualized by Coomassie brilliant blue staining.

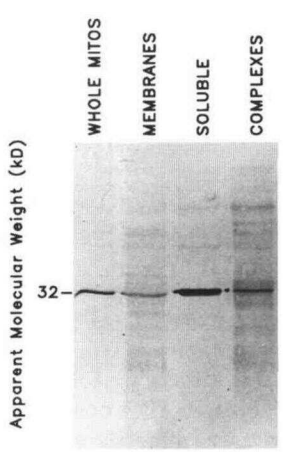

Figure 2. Immunoblot of mitochondrial subfractions probed with anti-32-kD DH NADH DH polyclonal antibodies. Mitochondrial fractionation was performed as described in "Materials and Methods." Protein (20 μg) from each fraction was separated by SDS-PAGE and transferred to nitrocellulose.

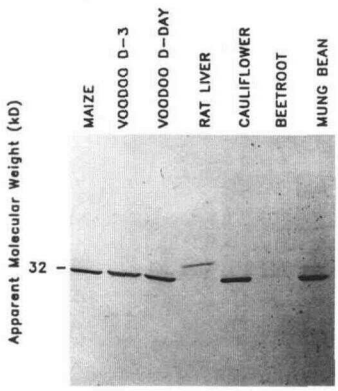

Figure 3. Immunoblot of mitochondria from several different species probed with anti-32-kD NADH DH antibody. Thirty micrograms of protein was loaded into each lane. Plant mitochondria tested were maize (*Z. mays* L.) etiolated shoots, voodoo lily (*Sauromatum guttatum* Schott) appendices from 3 d prior to flowering (D-3) and the day of flowering (D-day), cauliflower (*Brassica oleracea* L.) inflorescences, red beet (*Beta vulgaris* L.) roots, and mung bean (*Vigna radiata* L.) etiolated shoots. Rat liver represents the only species of animal mitochondria tested.

ognized a 40-kD protein in rat liver mitochondria, the significance of which is not clear because of the difference in molecular mass.

2D Analyses of the 32-kD NADH DH

The polyclonal antibodies were used to identify the 32-kD NADH DH in the mitochondrial 2D profile using 2D immunoblots (Fig. 4). The results show that the 32-kD NADH DH consists of two major isoelectric forms and one minor form. These results are consistent with 2D gels of the purified 32-kD NADH DH (results not shown). Based on quantitation of proteins in maize mitochondria from 2D gels (Lund et al., 1992), the 32-kD NADH DH constitutes 0.74% of total mitochondrial protein.

Characterization of the 32-kD NADH DH Activity

The 32-kD NADH DH was further characterized to determine its pH optimum, inhibition characteristics, and substrate specificity. Figure 5 shows the pH profile of the 32-kD NADH DH. The apparent optimal activity is around pH 7.0. Table II shows the effect of various inhibitors, flavins, and sulfhydryl modifying reagents on the activity of the 32-kD NADH DH. The activity was found to be insensitive to EGTA or Ca^{2+} and was unaffected by high concentrations of NaCl or KCl. The activity was stimulated somewhat (11%) by flavin mononucleotide but not by flavin adenine dinucleotide, suggesting that it may be linked to flavin mononucleotide. The presence of NAD, NADP, ADP, or ATP had no effect on the activity. The activity was inhibited severely by the sulfhydryl agents pCMB and mersalyl, but not by NEM. The outer membrane NADH DH inhibitor 2,4-D (Mannella and Bonner, 1978) and the complex I inhibitor rotenone had no effect on the activity. Antimycin A did not inhibit the 32-kD NADH DH. Several flavins and flavin-like compounds were tested

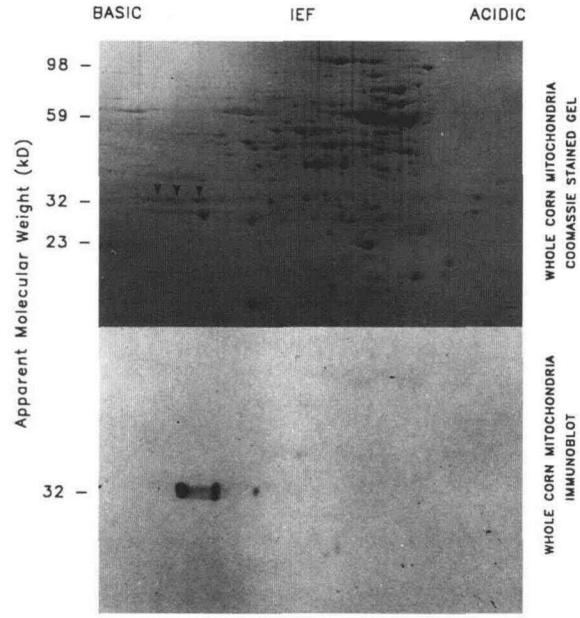

Figure 4. 2D mapping of the 32-kD NADH DH using polyclonal antibodies. In the upper panel the proteins were stained with Coomassie brilliant blue. The lower panel is an immunoblot of a similar gel probed with the anti-32-kD NADH DH polyclonal antibody. Approximately 300 μg of whole mitochondrial protein was loaded in the first dimension for both the gel and the immunoblot.

for their effect on the activity. Dicumarol was the only one that resulted in a high degree of inhibition. The bud extract that contained platanetin, an inhibitor of the exogenous NADH DH in situ, inhibited the 32-kD NADH DH somewhat (20%).

Utilization of Different Cofactors and Electron Acceptors by the 32-kD NADH DH

The ability of the 32-kD NADH DH to use NADH, NADPH, deamino-NADH, and deamino-NADPH was eval-

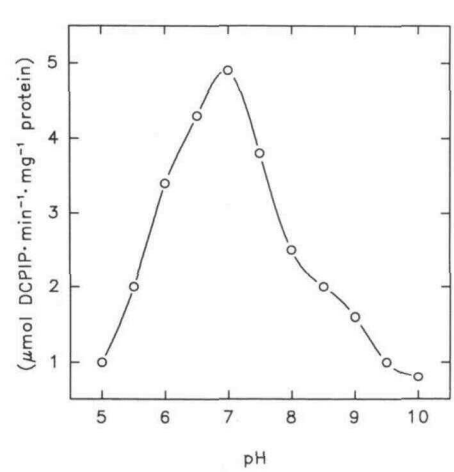

Figure 5. Effect of pH on activity of the 32-kD NADH DH. The pH optimum was measured using a buffer consisting of 30 mM bis-Tris-propane.

Table II. *Effect of various compounds on activity of the 32-kD NADH DH*

The following compounds were solubilized in 100% DMSO: pCMB, 2,4-D, rotenone, flavone, phloretin, phloridzin, kaempherol, and apigenin. Antimycin A was dissolved in 100% ethanol. The bud extract was prepared as described by Luethy et al. (1991). NADH (1 mM) was used as the substrate except for the testing of 2,4-D, in which 100 μM was used. The average control rate was 2.52 μmol min^{-1} mg^{-1} protein.

Treatment	Percent of Control
5 mM EGTA	97
1 mM $CaCl_2$	107
1 M NaCl	81
1 M KCl	89
1 mM flavin adenine dinucleotide	99
1 mM flavin mononucleotide	111
200 μM NAD	100
200 μM NADP	99
200 μM ADP	100
200 μM ATP	96
150 μM NEM	103
150 μM pCMB	2
150 μM mersalyl	0
150 μM 2,4-D	96
8 μM rotenone	100
1 μM antimycin A	101
150 μM dicumarol	22
150 μM flavone	108
150 μM phloretin	107
150 μM phloridzin	83
150 μM kaempherol	79
150 μM apigenin	82
Bud extract	80

uated. The concentration of each cofactor was adjusted to yield a final concentration of 1 mM based on the A_{340}. The degree of utilization of each cofactor was 2.52 μmol min^{-1} mg^{-1} protein with NADH (100%), NADPH (1%), deamino-NADH (105%), and deamino-NADPH (11%). The 32-kD DH was found to reduce the artificial electron acceptor Q_0 (1 mM) at 41% of its rate with DCPIP (60 μM). The 32-kD DH was found to be incapable of reducing oxygen.

Submitochondrial Localization of the 32-kD NADH DH

The water-soluble membrane-impermeable protein cross-linking agent DTSSP (Pierce) was used to investigate the submitochondrial location of the 32-kD NADH DH. This cross-linking agent reacts with free amino groups, is homobifunctional, and is cleavable with βME. Freshly isolated intact mitochondria were incubated in the presence of DTSSP for 2 h at 4°C. The cross-linking reaction was then quenched with Tris. DTSSP was expected to pass through porin in the outer membrane and thus cross-link proteins on either surface of the outer membrane, proteins soluble in the intermembrane space, and proteins on the outer surface of the inner membrane. Proteins inside of the inner membrane barrier would not be expected to be cross-linked. Cross-linking was evaluated by following the migration of the cross-linked proteins to higher mol wt in SDS-PAGE in the absence of βME. In the presence of βME, the cross-links were broken and the proteins migrated to normal mol wt (Fig. 6). The two left lanes of Figure 6 show controls in which mitochondria that were not cross-linked were subjected to SDS-PAGE without and with βME.

The cross-linking of individual proteins can be followed in these experiments with antibodies, as shown in Figure 7. Gels similar to that shown in Figure 6 were transferred to nitrocellulose for probing. The upper panel shows an immunoblot that was probed with polyclonal antibodies to the soluble matrix enzyme malate DH. The results indicate that the vast majority of the malate DH was not cross-linked. The middle panel shows a similar blot probed with monoclonal antibodies to the β subunit of the F_1-ATPase (Luethy et al., 1993), which is an enzyme that is membrane bound and facing the matrix. No cross-linking of the ATPase was observed. These results show that DTSSP did not penetrate the inner membrane barrier. The lower panel of Figure 7 presents the results with polyclonal antibodies to the 32-kD NADH DH. The results show clearly that the 32-kD NADH DH is located external to the inner membrane barrier. In another experiment, a blot similar to that shown in Figure 7 was probed with monoclonal antibodies to the alternative oxidase. Regions of the alternative oxidase are believed to be exposed to the intermembrane space (Rhoads and McIntosh, 1991). The results showed that the alternative oxidase became cross-linked, indicating that the DTSSP was accessible to the outer surface of the inner mitochondrial membrane (data not shown).

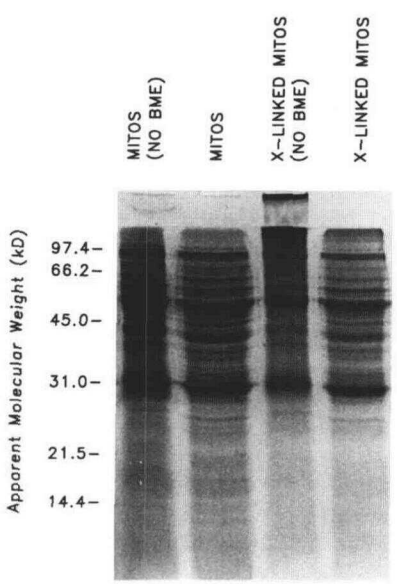

Figure 6. SDS-PAGE analysis of the cross-linking of proteins in intact mitochondria with DTSSP. Freshly isolated mitochondria were incubated either without (Mitos) or with DTSSP (X-Linked Mitos) as described in "Materials and Methods." Mitochondrial proteins were then separated by SDS-PAGE in the presence or absence of β-ME (No BME). Approximately 20 μg of mitochondrial protein was loaded per lane.

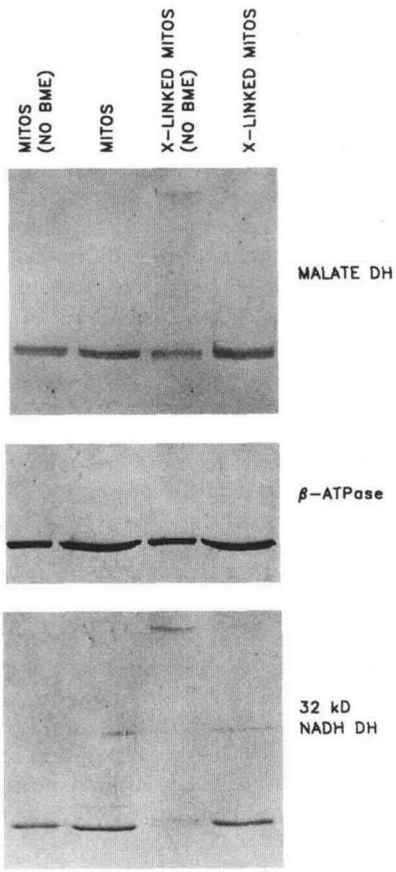

Figure 7. Localization of various mitochondrial proteins through immunoblot analyses of cross-linked mitochondria. Protein gels similar to those shown in Figure 6 were transferred to nitrocellulose for immunoblotting. The upper panel was probed with polyclonal antibodies to malate DH, the middle panel with monoclonals to the β subunit of the F_1-ATPase, and the lower panel with polyclonals to the 32-kD NADH DH.

Control experiments were performed to ensure that DTSSP was capable of cross-linking malate DH and the β subunit of the ATPase. The cross-linking was performed as described above, except that the mitochondria were briefly sonicated in the presence of DTSSP. Under these conditions, which circumvented the inner membrane barrier, malate DH and the β subunit of the ATPase became cross-linked (Fig. 8).

The above experiments show clearly that the 32-kD NADH DH is located external to the inner membrane. Experiments were then performed to determine if the 32-kD NADH DH was associated with the outer membrane. The outer membrane has previously been shown to contain a NADH DH activity, and this activity (measured as NADH-dependent Cyt *c* reduction) has been shown to be insensitive to antimycin A (Douce et al., 1973). NADH-dependent Cyt *c* reductase activity associated with the inner membrane is inhibited by antimycin, since the electrons pass through the Cyt bc_1 complex. Outer membranes were isolated using a modification of the procedure of Mannella and Bonner (1975) as described in "Materials and Methods," and were found to have NADH-dependent Cyt *c* reductase activity that was insensitive to antimycin. A comparison of the protein profile of the isolated outer membranes with whole mitochondria is presented in the upper panel of Figure 9. The outer membranes contain few proteins, but the profile is distinct. Gels similar to these were transferred to nitrocellulose for immunoblotting. Monoclonal antibodies to the alternative oxidase were used as a marker for the presence of inner membranes because the alternative oxidase is an integral membrane protein (Rhoads and McIntosh, 1991). No alternative oxidase protein was found in the outer membrane preparation (middle panel). When similar blots were probed with polyclonal antibodies to the 32-kD NADH DH, no association with the outer membrane was observed.

These experiments localize the 32-kD NADH DH to either the intermembrane space or to the outer surface of the inner mitochondrial membrane. It may not be possible to differentiate between these two localizations because the 32-kD NADH DH may have only a loose association with the inner membrane in situ.

DISCUSSION

Previous work on purification of the exogenous NAD(P)H DHs from plant mitochondria has indicated that these DHs are readily released from the inner membrane during mitochondrial disruption (Douce et al., 1973; Cook and Cammack, 1985). The resulting appearance of NAD(P)H DH activities in the soluble fraction during mitochondrial disruption has been shown to correlate with decreased activities associated with the membranes (Chauveau and Lance, 1991; Luethy et al., 1991). A 32-kD protein has often been observed in the resulting soluble fraction (Klein and Burke, 1984; Cook and Cammack, 1985; Chauveau and Lance, 1991; Luethy et al., 1991). We have purified a NADH DH activity from maize mitochondria to a 32-kD protein and have raised polyclonal antibodies to the protein. In immunoprecipitation experiments, these antibodies were found to precipitate the activity to a reasonable degree (26%). The antibodies were used to evaluate the distribution of the 32-kD protein following

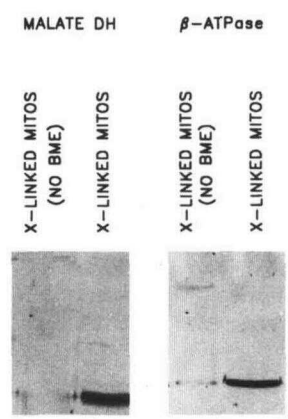

Figure 8. Cross-linking of proteins in mitochondria sonicated in the presence of DTSSP. Mitochondria were sonicated in the presence of DTSSP to ensure that malate DH and the β subunit of the F_1-ATPase could be cross-linked. Other conditions are the same as in Figure 7.

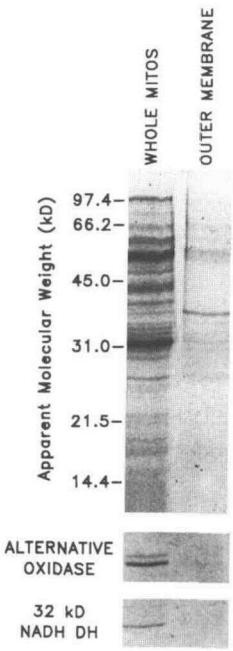

Figure 9. Immunoblot comparison of whole mitochondria and isolated mitochondrial outer membranes. The upper panel is a Coomassie-stained gel of whole mitochondria and outer membranes. The two lower panels are immunoblots of similar gels probed with monoclonal antibodies to the alternative oxidase and polyclonal antibodies to the 32-kD NADH DH. Approximately 5 μg of protein was loaded per lane.

submitochondrial fractionation. Consistent with the literature, the protein was enriched in the soluble protein fraction; however, some was associated with the membrane fraction. Immunoblots of mitochondria from different plant species indicated that a similar mol wt protein was immunoreactive in all species tested. The antibodies also reacted with a 40-kD protein in rat liver mitochondria. This could suggest either that a change in molecular mass of the DH has occurred or that the antibody is cross-reacting with a different protein. The submitochondrial location of the 32-kD NADH DH was further evaluated in maize using the membrane-impermeable cross-linking agent DTSSP. These results clearly indicated that the 32-kD NADH DH was located on the outer surface of the inner membrane or in the intermembrane space. These findings are consistent with the literature, which indicates a loose association of exogenous NAD(P)H DH activities with the outer surface of the inner membrane.

Our work on the red beet root and maize 32-kD NADH DHs has indicated that they both are specific for NADH. However, the *Arum* 32-kD NADH DH was shown to oxidize both NADH and NADPH (Chauveau and Lance, 1991), and thus the cofactor specificity is likely dependent on the species. It has been shown with other enzymes that single amino acid changes can change the cofactor specificity (Feeney et al., 1990; Haeffner-Gormley et al., 1992); therefore, species-dependent cofactor specificity is not surprising. The 32-kD NADH DH was found to effectively utilize deamino-NADH as a substrate. This is in contrast to the suggestion that only complex I-type enzymes utilize deamino-NADH (Matsushita et al., 1987).

The results from our laboratory with maize and those of Chauveau and Lance (1991) with *Arum* are similar in many regards. We observed a similar elution profile from Mono Q, similar mol wts, and a similar pH optimum for NADH oxidation, both proteins may be flavoproteins, rotenone did not affect the activity, and no effect of Ca^{2+} or EGTA was observed. We found that DCPIP and Q_0 were effective electron acceptors. Chauveau and Lance observed that ferricyanide and quinones were effective electron acceptors. When the data from all of these experiments are considered, the 32-kD NAD(P)H DH remains a strong candidate for an exogenous DH.

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