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Identification and Characterization of an Inducible NAD(P)H Dehydrogenase from Red Beetroot Mitochondria[1]

R. Ian Menz and David A. Day*

Division of Biochemistry and Molecular Biology and the Cooperative Research Centre for Plant Science, The Australian National University, Canberra, Australian Capital Territory 0200, Australia

Exogenous NADH oxidation of mitochondria isolated from red beetroots (*Beta vulgaris* L.) increased dramatically upon slicing and aging the tissue. Anion-exchange chromatography of soluble fractions derived by sonication from fresh and aged beetroot mitochondria yielded three NADH dehydrogenase activity peaks. The third peak from aged beetroot mitochondria was separated into two activities by blue-affinity chromatography. One of these (the unbound peak) readily oxidized dihydrolipoamide, whereas the other (the bound peak) did not. The latter was an NAD(P)H dehydrogenase with high quinone and ferricyanide reductase activity and was absent from fresh beet mitochondria. Further affinity chromatography of the NAD(P)H dehydrogenase indicated enrichment of a 58-kD polypeptide on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We propose that this 58-kD protein is the inducible, external NADH dehydrogenase.

Plant and fungal mitochondria contain several unique NAD(P)H dehydrogenases that are linked to the respiratory chain (Møller and Lin, 1986; Douce and Neuberger, 1989). One of these enzymes is located on the outside of the inner membrane and is involved in the oxidation of cytosolic NADH (Møller and Lin, 1986; Douce and Neuberger, 1989; Roberts et al., 1995). However, there is at least one internally facing dehydrogenase that provides a rotenone-insensitive bypass of complex I for the oxidation of matrix NAD(P)H (Rasmusson and Møller, 1991). Several attempts have been made to purify these unique enzymes (Cook and Cammack, 1984, 1985; Cottingham and Moore, 1984; Klein and Burke, 1984; Chauveau and Lance, 1990; Luethy et al., 1991, 1995; Rasmusson et al., 1993; Knudten et al., 1994). These preparations were often found to contain polypeptides of 30 to 40 or 50 to 60 kD, although direct comparisons are difficult because different species and methodologies were used in the different studies. Although a precise identification of these enzymes has not been achieved, a strong case for a 58-kD protein to be the external NADH dehydrogenase has been made (Luethy et al., 1991, 1995).

Mitochondria isolated from fresh red beetroots (*Beta vulgaris* L.) oxidize exogenous NADH poorly, but this activity can be induced by "aging" the tissue in a dilute $CaSO_4$ solution (Day et al., 1976; Rayner and Wiskich, 1983). This property makes aged red beetroot an ideal tissue for the isolation of the exogenous NADH dehydrogenase, because it can be identified by its low levels in mitochondria from fresh tissue. Recently, several groups have concentrated on isolating NAD(P)H dehydrogenases from the soluble fraction of red beetroot mitochondria (Luethy et al., 1991; Rasmusson et al., 1993; Soole and Menz, 1995). This fraction is generated by the sonication of the mitochondria followed by centrifugation to remove membrane fragments and vesicles. The enzyme(s) responsible for cytosolic NAD(P)H oxidation is likely to be present in the soluble fraction, because it is believed to be loosely bound to the outer surface of the inner membrane (Douce et al., 1973). Use of the soluble fraction has the added advantage that it avoids the large-molecular-mass complex I, which remains in the membrane.

Anion-exchange chromatography of both the fresh (Rasmusson et al., 1993; Soole and Menz, 1995) and aged (Luethy et al., 1991) beetroot soluble fractions results in the separation of three NADH dehydrogenase activity peaks. The first activity peak has been purified to a polypeptide with a molecular mass of approximately 42 kD by Luethy et al. (1991) and Soole and Menz (1995), whereas Rasmusson et al. (1993) purified their first activity peak to a polypeptide with a molecular mass of 26 kD. It has been suggested that the rotenone-insensitive endogenous NAD(P)H dehydrogenase is responsible for the first activity peaks (Rasmusson et al., 1993; Soole and Menz, 1995). All three groups found that their second activity peaks were due to an enzyme with a molecular mass of 32 to 35 kD (Luethy et al., 1991; Rasmusson et al., 1993; Soole and Menz, 1995). Luethy et al. (1991) purified this peak to a single 32-kD protein.

The third activity peak isolated from mitochondria from aged beetroot (Luethy et al., 1991) was inhibited by plane tree bud extract, which contained platanetin, a potent inhibitor of exogenous NADH oxidation (Ravanel et al., 1986). Recently, it has been shown not to be a specific inhibitor (Roberts et al., 1996). Therefore, Luethy et al. (1991) proposed that exogenous NADH oxidation was me-

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* Corresponding author; e-mail david.day@anu.edu.au; fax 61–6–2490313.

Abbreviations: dicumarol, 3–3′-methylene-bis-(4-hydroxycoumarin); FeCN, potassium ferricyanide; flavone, 2-phenyl-4H-1-benzopryan-4-one; lipoamide, DL-6,8 thiotic acid amide; p-CMB, *p*-chloromecuribenzoic acid; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone.

diated by the enzyme that gave rise to this activity peak, and it was subsequently found that the activity was associated with a 58-kD protein (Luethy et al., 1995). In contrast, Rasmusson et al. (1993) found that their third activity peak, also containing a 58-kD protein, possessed dihydrolipoamide dehydrogenase activity and was 98% inhibited by arsenite, a potent inhibitor of this enzyme. Rasmusson et al. (1993) proposed that their third activity peak and that of Luethy et al. (1991) was due to a lipoamide dehydrogenase that was the L-protein component of the pyruvate and 2-oxoglutarate dehydrogenase complexes.

More recently, enzymes analogous to the 32-kD (second activity peak) and 58-kD (third activity peak) enzymes of aged beetroot mitochondria have been purified from maize mitochondria (Knudten et al., 1994, Luethy et al., 1995). Both of these maize enzymes have been localized to the outer surface of the inner membrane or the intermembrane space. This location implies that one or both of these enzymes could be involved in exogenous NAD(P)H oxidation.

We have attempted to resolve the discrepancy between Luethy et al. (1995) and Rasmusson et al. (1993) by comparing the anion-exchange profiles of the soluble fractions of mitochondria from fresh and aged beetroot tissue. We have found that the third activity peak of aged beetroot mitochondria can be separated into two components. One of these is a dihydrolipoamide dehydrogenase, whereas the other is an NAD(P)H dehydrogenase that has been purified to a single 58-kD polypeptide. We propose that the 58-kD protein is the inducible, external NADH dehydrogenase.

MATERIALS AND METHODS

Red beetroots (*Beta vulgaris* L.) were purchased at local markets. Beetroot tissue was aged as previously described by Rayner et al. (1983). Mitochondria were prepared as previously described by Soole et al. (1990), except that the aged tissue was homogenized (200-g batches) in a medium (250 mL) containing 0.4 M Suc, 5 mM EDTA, and 30 mM Tes, all at pH 7.4, using several 1-s bursts (setting 7) of a Polytron PT 10–35 fitted with a PTG 35/2M aggregate (Kinematica, Kriens, Switzerland). Mitochondria (approximately 50 mg) were resuspended in 20 mM Tris/HCl (pH 8.0) containing 1 mM PMSF and 5 μM *trans*-epoxysuccinyl-L-luecylamido-(4-guanidino)butane protease inhibitor and sonicated in five 5-s bursts using an ultrasonic disintegrator (MSE, Sussex, UK) at maximum power. The sonicated mitochondria were then centrifuged at 300,000*g* for 45 min, and the resulting supernatant represented the soluble fraction.

All chromatographic procedures were carried out using a fast protein liquid chromatography system (Pharmacia). Anion-exchange chromatography was performed on a 1-mL column (resource Q, Pharmacia), whereas blue-affinity chromatography was performed on a Pharmacia Hi-Trap blue column. The buffer used for both of these chromatographic procedures was 20 mM Tris/HCl (pH 8.0), and proteins were eluted with gradients of NaCl (0–350 mM) or NADPH (0–10 μM) as indicated. Gel-filtration chromatography was carried out on a calibrated column (Superdex 75, Pharmacia) using a buffer containing 50 mM KH_2PO_4 (pH 7.0) and 0.15 M NaCl.

Protein concentrations were estimated using the method of Lowry et al. (1951). Electrophoresis was carried out using the method of Laemmli (1970) with 12% (w/v) polyacrylamide resolving gels, and proteins were stained using Coomassie brilliant blue. Noncovalently bound flavin was removed from the protein by boiling for 3 min, and the flavin was determined fluorometrically by the method of Seigel (1978).

Oxygen-consumption assays were carried out polarographically at 25°C using an oxygen electrode (Rank Brothers, Cambridge, UK) with 2 mL of a standard reaction medium that consisted of 0.25 M Suc, 10 mM Tes, 10 mM KH_2PO_4, and 5 mM $MgCl_2$, all at pH 7.2. External NAD(P)H:Q_0 reductase and NADH:lipoamide reductase activities were measured in 10 mM 1,3-bis(Tris[hydroxymethyl]-methylamino)propane buffer (pH 7.2) containing 200 μM NAD(P)H and either 200 μM Q_0 or 1 mM lipoamide. The reduction of NAD(P)H was monitored at 340 nm (extinction coefficient at 340 nm = 6.22 mM^{-1} cm^{-1}).

RESULTS

Comparison of NAD(P)H Oxidizing Pathways of Fresh and Aged Beetroot Mitochondria

The specific activities of several NAD(P)H oxidizing activities were measured in mitochondria that were prepared from fresh and aged beetroot tissue and are compared in Table I. State-3 malate oxidation in the presence and absence of rotenone was used to estimate complex I and rotenone-insensitive endogenous NADH oxidation, respectively. Exogenous NAD(P)H oxidation was measured in the presence of Ca^{2+} to ensure maximal rates.

Table I. *Comparison of NADH oxidizing pathways in fresh and aged beetroot mitochondria*

Oxygen uptake and lipoamide dehydrogenase activity were assayed as detailed in "Materials and Methods." The concentrations for substrates and inhibitors for the oxygen consumption assays were: 10 mM malate, 10 mM glutamate, 25 μM rotenone, 1 mM NADH, and 1 mM NADPH. To ensure maximal rates 1.25 mM $CaCl_2$ was added for measurement of NAD(P)H oxidation. Rates are means of three experiments with one preparation, although similar trends were observed with other preparations.

Substrate	Specific Activity	
	Fresh mitochondria	Aged mitochondria
	nmol O_2 (NADH) min^{-1} mg^{-1} protein	
Malate/glutamate	76	85
Malate/glutamate + rotenone	12	23
NADH	13	129
NADPH	3	12
Lipoamide dehydrogenase	133[a]	96[a]

[a] nmol NADH min^{-1} mg^{-1} protein.

The specific activities for the majority of the NAD(P)H oxidizing pathways measured increased to some degree upon slicing and aging of the tissue. However, exogenous NADH oxidation increased to the greatest extent, by 116 nmol min^{-1} mg^{-1}, a 10-fold relative increase (Table I). The presence of low levels of exogenous NADH oxidation in fresh beet, which increase with aging, is consistent with previous reports (Fredlund et al., 1991; Luethy et al., 1991). We found that this activity varies substantially with both cultivar and geographic location, which presumably explains the variability reported in the literature (R.I. Menz, unpublished observation).

In contrast to external NADH oxidation, the specific activities of the other pathways were increased to a much lesser extent. External NADPH oxidation was increased 4-fold, but this amounted to a total increase in specific activity of only 9 nmol O_2 min^{-1} mg^{-1}. This result is consistent with previous work (Fredlund et al., 1991) as well as with the observation that exogenous NADH oxidation is mediated by an enzyme that is distinct from that which catalyzes exogenous NADPH oxidation (Roberts et al., 1995). State-3 malate oxidation in the absence of rotenone (i.e. complex-I activity) was also only slightly increased (9 nmol O_2 min^{-1} mg^{-1}). Likewise, although malate oxidation in the presence of rotenone (internal rotenone-insensitive activity) was doubled, its specific activity increased by only 11 nmol O_2 min^{-1} mg^{-1}. In contrast to the other NAD(P)H oxidizing activities, lipoamide dehydrogenase activity decreased (Table I).

From these comparative data it is evident that the aged mitochondria are dramatically enriched in the enzyme involved in exogenous NADH oxidation when compared with the other NAD(P)H-utilizing pathways. The data also suggest that the enzyme responsible for exogenous NADH oxidation is unlikely to possess lipoamide dehydrogenase activity, since exogenous NADH oxidation was increased 10-fold by aging and lipoamide dehydrogenase activity was reduced.

Identification of an Age-Induced NAD(P)H Dehydrogenase in Beetroot Mitochondria

The soluble fractions generated from the mitochondria of both fresh and aged beetroot tissues were subjected to anion-exchange chromatography, and the resulting fractions were assayed for NADH:Q_0 reductase activity. Three peaks of activity were resolved from both the fresh and aged material (Fig. 1), which is consistent with previous reports that used the soluble fraction of both fresh (Rasmusson et al., 1993) and aged (Luethy et al., 1991) beetroot mitochondria. However, when the NADH:Q_0 reductase elution profiles were normalized relative to activity peak 1, it was evident that the profiles were not identical (Fig. 1). Subtraction of the "fresh" profile from the "aged" revealed the presence of additional NADH:Q_0 reductase activity in the aged profile. This extra activity peak could be resolved only by subtraction and was not evident in the aged profile (Fig. 1). This extra or age-induced NADH:Q_0 reductase activity eluted from the anion-exchange column at NaCl concentrations of 200 to 300 mM and, therefore, overlapped

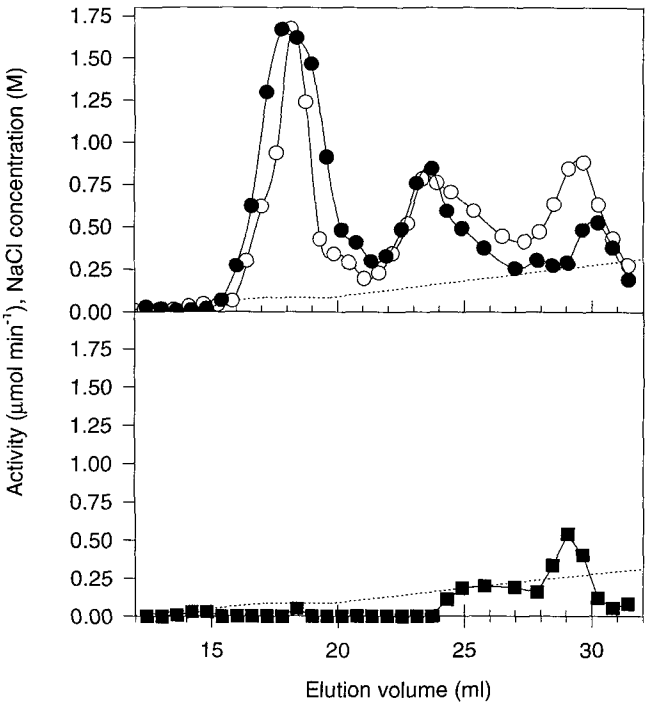

Figure 1. Anion-exchange elution profiles of fresh and aged beetroot mitochondrial soluble proteins. The top panel is a typical NADH:Q_0 reductase activity profile for fresh (●) and aged (○) mitochondrial soluble fraction when the resource Q column was eluted with a 0 to 350 mM NaCl gradient (- - - -). The profiles have been normalized with respect to peak 1. The bottom panel is the profile generated by electronically subtracting the fresh from the aged profile (■).

with both activity peak 3 and to some degree activity peak 2. Because exogenous NADH dehydrogenase activity was substantially increased by aging (Table I), it is likely that the extra peak of NADH:Q_0 reductase activity in the aged profile is attributable to the exogenous NADH dehydrogenase.

Purification of the Age-Induced NADH Dehydrogenase

The fractions from the aged profile that corresponded to the age-induced peak (27.5–30 mL; Fig. 1) were diluted 2-fold to reduce the NaCl concentration and then applied to a blue-affinity column; for a comparison, the same region from the fresh profile was treated in the same manner. The column was eluted with a NaCl gradient, and the resulting fractions were assayed for NADH:FeCN reductase activity (Fig. 2). Two peaks of NADH:FeCN reductase activity were detected in the material prepared from mitochondria from aged tissue. Only one activity peak was detected in the material prepared from mitochondria from fresh tissue, and this corresponded to the first and largest peak observed with the aged material (Fig. 2). This peak (the unbound fraction) eluted during the load and wash steps prior to elution with NaCl (Fig. 2). The second peak, resolved from the aged material (the bound fraction), started to elute from the column at a NaCl concentration of approximately 0.2 M (Fig. 2). Examination of the ratios of activities with different electron acceptors revealed that the

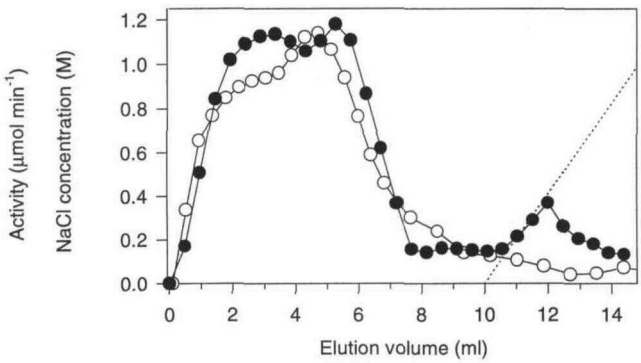

Figure 2. Blue-affinity chromatography of the age-induced peak resolved by anion exchange. The pooled sample corresponding to the age-induced peak resolved by anion exchange of soluble mitochondrial proteins from aged tissue (●) was subject to blue-affinity chromatography. This is compared with a sample from the same region of the anion-exchange profile in soluble mitochondrial proteins from fresh tissue (○). The samples were loaded and washed prior to elution with a 0 to 1 M NaCl gradient. The NADH:FeCN reductase activity profiles are shown.

unbound fractions from both the fresh and aged material were similar (not shown), whereas the activity of the bound fraction was most likely due to a different enzyme (Table II). The unbound fraction had its greatest activity with FeCN as an acceptor, but it also had high activity with lipoamide as an acceptor; its activity with Q_0 as an acceptor was much less (Table II). In contrast the bound fraction exhibited similar activities with both FeCN and Q_0 as acceptors and had an extremely low rate with lipoamide as an acceptor (Table II). Therefore, it seems that the soluble fraction derived from the aged tissue contained an additional activity that could be resolved by its tight binding to blue-affinity columns.

The age-induced activity was purified by applying it again to a blue-affinity column, as in Figure 2, followed by washing with 5 mL of 150 mM NaCl prior to elution with a gradient of 0 to 10 μM NADPH (Fig. 3). The majority of the NADH:Q_0 reductase activity eluted from the column at an NADPH concentration of approximately 2.5 μM (Fig. 3). SDS-PAGE analysis of this fraction showed only one major polypeptide with an apparent molecular mass of 58 kD (Fig. 4). The native molecular mass determined by gel filtration also was found to be approximately 58 kD (Table III). These two results suggest that the age-induced activity

Table II. *Comparison of bound and unbound fractions from the blue-affinity column*

Assays were performed as described in "Materials and Methods." Rates are means of three experiments.

Assay	Total Activity	
	Unbound fraction	Bound fraction
	μmol NADH min⁻¹	
NADH:Q_0	0.520	0.114
NADH:FeCN	3.450	0.105
NADH:lipoamide	2.150	0.006

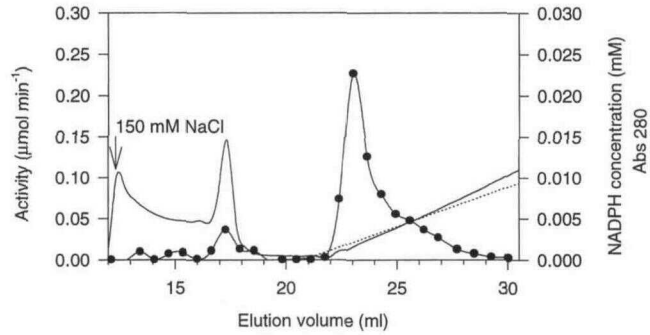

Figure 3. Purification of aged-induced dehydrogenase by NADPH elution from a blue-affinity column. The pooled sample from the anion-exchange column was loaded onto the blue-affinity column. Prior to elution with a 0 to 10 μM NADPH gradient (- - - -), the column was washed with 5 mL of 150 mM NaCl. The NADH:Q_0 reductase activity (●) and the A_{280} (—) are shown.

may be mediated by a single-subunit enzyme with a molecular mass of 58 kD.

The purified preparation had its greatest activity with NADH as a substrate and Q_0 as an acceptor (Table III). It oxidized NADPH at approximately 70% and deamino-NADH at approximately 45% of the rate with NADH (Table III). The rate of NADH oxidation with FeCN as an acceptor was 0.6 of that with Q_0 as an acceptor (Table III). No lipoamide dehydrogenase activity could be detected in the purified sample, showing that the purified enzyme was not a lipoamide dehydrogenase.

Characterization of the Age-Induced NADH Dehydrogenase

The kinetic data were found to best fit a Michaelis-Menten equation for a bi-substrate reaction with a ping-pong mechanism (Fig. 5). The estimated apparent K_m for NADH was 115 ± 25 μM and the apparent K_m for Q_0 was 53 ± 12 μM (Fig. 5). The enzyme had an estimated V_{max} of 405 ± 54 μmol min^{-1} mg^{-1} (Fig. 5), and the pH optimum for the enzyme was found to be pH 7.0 (Fig. 6), although

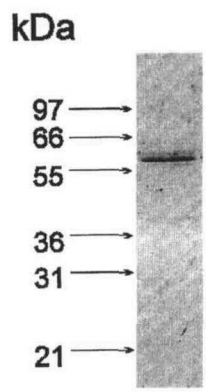

Figure 4. SDS-PAGE analysis of the purified age-induced NADH dehydrogenase. Coomassie blue-stained gel of the affinity-purified enzyme is shown; the migration of molecular-mass standards is indicated in kD.

Table III. *Some characteristics of the newly identified NAD(P)H dehydrogenase*

All rates are expressed relative to the NADH:Q_0 reductase activity (100% = 250 μmol min^{-1} mg^{-1} protein). The native molecular mass was estimated by its elution from a calibrated Superdex 75 column. SDS-PAGE molecular mass was estimated by comparison with the migration of molecular mass standards.

Assay	Relative Activity or Molecular Mass
NADH:Q_0	100
NADPH:Q_0	70
DANADH[a]:Q_0	45
NADH:lipoamide	Not detected
NADH:FeCN/NADH:Q_0	0.6
Native molecular mass (kD)	58
SDS-PAGE molecular mass (kD)	58

[a] Nicotinamide hypoxanthine dinucleotide, reduced form.

the enzyme maintained greater than 80% of its maximal activity between pH 5.5 and 8.0 (Fig. 6).

The degree of purification and percentage yield of the purified protein could not be determined accurately because there are several enzymes capable of reducing NADH in the presence of artificial electron acceptors in the starting mitochondria. Without a specific assay that can be performed on both intact mitochondria and the purified enzyme, these factors cannot be determined. However, the amount of purified protein recovered was quite low, typically 1 to 3 μg from approximately 40 mg of mitochondrial protein. This low recovery could reflect either a poor yield for the purification or a low abundance of the protein in

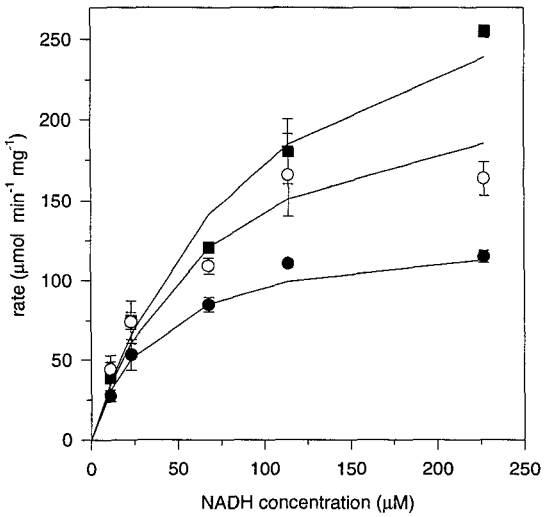

Figure 5. Kinetic analysis of the purified, age-induced NADH dehydrogenase. The NADH:Q_0 reductase activities were measured using three different concentrations of Q_0: 20 (●) 66 (○), and 200 μM (■). The error bars indicate the SE. The data were found to best fit a Michaelis-Menten equation for a bi-substrate reaction with a ping-pong mechanism. The estimated apparent K_m for NADH was 115 ± 25 μM, and the apparent K_m for Q_0 was 53 ± 12 μM. The enzyme had an estimated V_{max} of 405 ± 54 μmol min^{-1} mg^{-1}. The predicted lines (—) are shown.

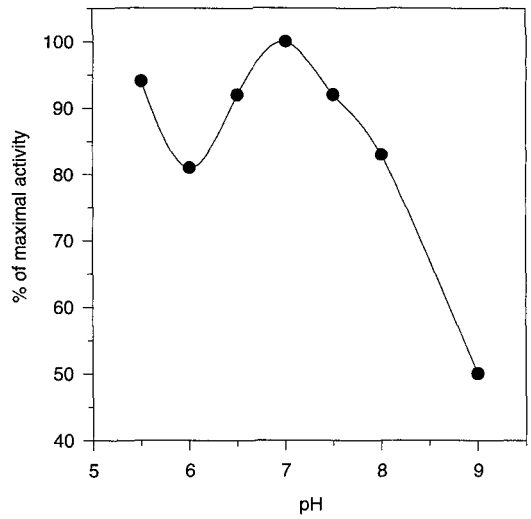

Figure 6. pH activity profile of purified age-induced NADH dehydrogenase. NADH:Q_0 reductase activity (●) was measured in a buffer containing 10 mM 1,3-bis(Tris[hydroxymethyl]-methylamino)propane and 10 mM Mes titrated to the pH indicated. The data are means of three experiments with one preparation, although a similar trend was observed with other preparations.

mitochondria. The determination of accurate specific activities was hampered by the low protein concentrations, which made detection variable with a number of methods, and the added complication that activity was lost with storage of the samples. Therefore, the specific activities expressed throughout this paper reflect the highest rates observed.

Fluorometric flavin analysis (Siegel, 1978) revealed that the purified protein contained noncovalently bound FAD, although the total flavin-to-protein ratio was determined to be only 1:2.5. This substoichiometric amount of flavin reflects the difficulty in determining the true protein concentration, although the presence of some degraded protein in the preparation cannot be excluded.

The NADH:Q_0 reductase activity of the purified preparation was not dependent on added Ca, since EGTA and $CaCl_2$ had no effect (Table IV). The cofactors NAD and ADP had a minimal effect on the age-induced activity, inhibiting only 5 and 8%, respectively (Table IV). NADP and ATP had a greater effect, inhibiting NADH:Q_0 reductase activity 22 and 32%, respectively (Table IV). The inhibitors p-CMB and dicumarol were the most effective of those tested, inhibiting the NADH:Q_0 reductase rate by 48 and 86%, respectively (Table IV). Mersalyl and platanetin also inhibited the activity 26 and 16%, respectively (Table IV), whereas flavone had a minimal effect (Table IV).

DISCUSSION

The additional NAD(P)H dehydrogenase purified in this paper appears to be similar to the 58-kD protein previously purified from beetroot mitochondria by Luethy et al. (1991, 1995). Both activities are due to proteins with similar molecular masses and have similar activities in the presence of different activators and inhibitors. However, there are sev-

Table IV. *The effects of several activators and inhibitors of NADH dehydrogenases on the NADH:Q_0 reductase activity of the purified age-induced dehydrogenase*

All activities are expressed relative to the control rate, which was 250 μmol min^{-1} mg^{-1}. The results are means of three experiments.

Addition	Relative Activity
	% of control
None	100
5 mM EGTA	98
1 mM $CaCl_2$	100
200 μM NAD	95
200 μM NADP	78
200 μM ADP	92
200 μM ATP	68
150 μM p-CMB	52
150 μM Mersalyl	74
150 μM Dicumarol	14
150 μM Platanetin	84
150 μM Flavone	95

eral distinct differences between these enzymes. The 58-kD protein purified by Luethy et al. (1991) was inhibited 76% by plane tree bud extract and 80% by NAD; in contrast, the age-induced protein isolated here was inhibited only 16% by platanetin and 5% by NAD. Since the bud extract used by Luethy et al. (1991) would have contained several flavanoids (Ravanel et al., 1990), it is possible that a combination of these provided a more potent inhibitor than platanetin alone. The difference in the effect of NAD is not clear. Another difference is that the protein purified by Luethy et al. (1991) could not oxidize NADPH. The ability of the age-induced dehydrogenase to oxidize both NADH and NADPH is likely to be attributed to a change that occurs when the enzyme is released from its membrane environment, since intact mitochondria did not oxidize NADPH (Table I). Changes in substrate specificity are often observed when membrane-bound dehydrogenases are released. The specificities of the enzyme can also change depending on the assay conditions (Menz, 1995). In particular, the use of artificial quinones as acceptors may change specificity and affinity (Møller et al., 1996), which may also account for the different sensitivities to inhibitors observed by Luethy et al. (1991) and us.

Our results might explain the differences in the activities of the 58-kD proteins isolated by Luethy et al. (1991, 1995) and Rasmusson et al. (1993). Rasmusson et al. (1993) used beetroot mitochondria that had low levels of exogenous NADH dehydrogenase activity, and consequently, as shown in this paper (with mitochondria from fresh beets), only the lipoamide dehydrogenase activity was detected. In contrast, Luethy et al. (1991, 1995) used beetroot mitochondria that had substantial levels of exogenous NADH dehydrogenase activity and, therefore, they detected and purified a dehydrogenase similar to the age-induced activity purified here. Whether the preparation of Luethy et al. (1991, 1995) contained any lipoamide dehydrogenase activity is not clear. However, since lipoamide dehydrogenase is present in the matrix compartment and the majority of the antibody they prepared against this preparation recognized proteins outside of the matrix (Luethy et al., 1995), lipoamide dehydrogenase may have been a minor component. Beetroot lipoamide dehydrogenase has a NADH:2,6-dichlorophenol-indophenol reductase activity 10- to 15-fold lower than its NADH:FeCN reductase activity (Soole and Menz, 1995). Since Luethy et al. (1991, 1995) assayed only for NADH:2,6-dichlorophenol-indophenol activity, they may not have detected contaminating lipoamide dehydrogenase activity.

Several characteristics of the purified enzyme are similar to those observed for external NADH oxidation by intact mitochondria. A wide range of K_m values have been reported for external NADH oxidation, ranging from 10 to 100 μM (Møller et al., 1993), values that are similar to the 115 ± 25 μM determined here for the purified enzyme. Similarly, the reported pH optimum between 6.8 and 7.2 (Møller et al., 1993) for external NADH oxidation is similar to the maximum at pH 7.0, which was observed for the purified enzyme. The purified enzyme also showed sensitivity to dicumarol, p-CMB, mersalyl, dicumarol, and platanetin, all of which have been shown to inhibit external NADH oxidation by intact plant mitochondria (Møller and Lin, 1986). The most dramatic difference between the purified enzyme and exogenous NADH oxidation by intact mitochondria is the lack of stimulation by Ca. However, the loss of Ca sensitivity upon release from the membrane has been previously observed (Cook and Cammack, 1984; Cottingham and Moore, 1984) and also may be influenced by the use of artificial quinones (Møller et al., 1996.). Therefore, the characteristics of the purified enzyme supports the conclusion that it is responsible for external NADH oxidation by beet mitochondria.

CONCLUSION

We have identified an NAD(P)H dehydrogenase from beetroot that is induced by slicing and aging. This enzyme is most likely the exogenous NADH dehydrogenase, since no other activity was so markedly induced by aging. The enzyme appears analogous to the 58-kD proteins previously isolated from beetroot and maize mitochondria (Luethy et al., 1991, 1995), which have been localized to the outer side of the inner membrane or intermembrane space, an observation consistent with this enzyme being the exogenous NADH dehydrogenase. Although the 58-kD NADH dehydrogenase has a molecular mass similar to that of lipoamide dehydrogenase (Barrera et al., 1972), it is distinct from the latter and cannot reduce lipoamide. However, care must be taken to separate the two enzymes when investigating the external NADH dehydrogenase.

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LITERATURE CITED

Barrera CR, Namihira G, Hamilton C, Munk P, Eley MH, Linn TC, Reed LJ (1972) α-Keto acid dehydrogenase complexes. XVI. Studies on the subunit structure of the pyruvate dehydrogenase complexes from bovine kidney and heart. Arch Biochem Biophys **148:** 343–358

Chauveau M, Lance C (1990) Purification and partial characterization of two soluble NAD(P)H dehydrogenases from *Arum maculatum* mitochondria. Plant Physiol **95:** 934–942

Cook ND, Cammack R (1984) Purification and characterisation of the rotenone-insensitive NADH dehydrogenase of mitochondria from *Arum maculatum*. Eur J Biochem **141:** 573–577

Cook ND, Cammack R (1985) Properties of a soluble rotenone-insensitive NADH dehydrogenase released from *Arum maculatum* mitochondrial membranes by sonication. Biochim Biophys Acta **827:** 30–35

Cottingham IR, Moore AL (1984) Partial purification and properties of the external NADH dehydrogenase from cuckoo-pint (*Arum maculatum*) mitochondria. Biochem J **224:** 171–179

Day DA, Rayner JR, Wiskich JT (1976) Characteristics of NADH oxidation by beetroot mitochondria. Plant Physiol **58:** 38–42

Douce R, Mannella CA, Bonner WD (1973) The external NADH dehydrogenases of intact plant mitochondria. Biochim Biophys Acta **292:** 105–116

Douce R, Neuberger M (1989) The uniqueness of plant mitochondria. Annu Rev Plant Physiol Plant Mol Biol **40:** 371–414

Fredlund KM, Rasmusson AG, Møller IM (1991) Oxidation of external NAD(P)H by purified mitochondria from fresh and aged red beetroots (*Beta vulgaris* L.). Plant Physiol **97:** 99–103

Klein RR, Burke JJ (1984) Separation procedure and partial characterization of two NAD(P)H dehydrogenases from cauliflower mitochondria. Plant Physiol **76:** 436–441

Knudten AF, Thelen JJ, Luethy MH, Elthon TE (1994) Purification, characterisation and submitochondrial localization of the 32-kilodalton NADH dehydrogenase from maize. Plant Physiol **106:** 1115–1122

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Luethy MH, Hayes MK, Elthon TE (1991) Partial purification and characterization of three NAD(P)H dehydrogenases from *Beta vulgaris* mitochondria. Plant Physiol **97:** 1317–1322

Luethy MH, Thelen JJ, Knudten AF, Elthon TE (1995) Purification, characterization, and submitochondrial localization of a 58-kilodalton NAD(P)H dehydrogenase. Plant Physiol **107:** 443–450

Menz RI (1995) Purification and characterisation of plant mitochondrial NAD(P)H dehydrogenases. PhD thesis, The Australian National University, Canberra

Møller IM, Lin W (1986) Membrane-bound NAD(P)H dehydrogenases in higher plant cells. Annu Rev Plant Physiol **37:** 309–334

Møller IM, Rasmusson AG, Fredlund KM (1993) NAD(P)H-ubiquinone oxidoreductases in plant mitochondria. J Bioenerg Biomembr **25:** 377–384

Møller IM, Roberts TH, Rasmusson AG (1996) UQ-1 induces external deamino-NADH oxidation in potato tuber mitochondria. Plant Physiol **112:** 75–78

Rasmusson AG, Fredlund KM, Møller IM (1993) Purification of a rotenone-insensitive NAD(P)H dehydrogenase from the inner surface of the inner membrane of red beetroot mitochondria. Biochim Biophys Acta **1141:** 107–110

Rasmusson AG, Møller IM (1991) NAD(P)H dehydrogenases on the inner surface of the inner mitochondrial membrane studied using inside out submitochondrial particles. Physiol Plant **83:** 357–365

Ravanel P, Creuzet S, Tissut M (1990) Inhibitory effect of hydroxyflavones on the exogenous NADH dehydrogenase of plant mitochondrial inner membranes. Phytochemistry **29:** 441–445

Ravanel P, Tissut M, Douce R (1986) Platanetin. A potent natural uncoupler and inhibitor of the exogenous NADH dehydrogenase in intact plant mitochondria. Plant Physiol **80:** 500–504

Rayner JR, Wiskich JT (1983) Development of NADH oxidation by red beet mitochondria on slicing and ageing of the tissues. Aust J Plant Physiol **10:** 55–63

Roberts TH, Fredlund KM, Møller IM (1995) Direct evidence for the presence of two external NAD(P)H dehydrogenases coupled to the electron transport chain in plant mitochondria. FEBS Lett **373:** 307–309

Roberts TH, Rasmusson AG, Møller IM (1996) Platanetin and 7-iodo-acridone-4-carboxylic acid are not specific inhibitors of respiratory NAD(P)H dehydrogenases in potato tuber mitochondria. Physiol Plant **96:** 263–267

Siegel LM (1978) Quantitative determination of noncovalently bound flavins: types and methods of analysis. Methods Enzymol **50:** 419–429

Soole KL, Dry IB, James AT, Wiskich JT (1990) The kinetics of NADH oxidation by complex I of isolated plant mitochondria. Physiol Plant **80:** 75–82

Soole KL, Menz RI (1995) Functional and molecular aspects of the NADH dehydrogenases of plant mitochondria. J Bioenerg Biomemb **27:** 397–406