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Purification and Partial Characterization of Two Soluble NAD(P)H Dehydrogenases from *Arum maculatum* Mitochondria[1]

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ABSTRACT

Two enzyme systems carrying out the oxidation of NAD(P)H in the presence of various electron acceptors have been isolated and partially characterized from the supernatant of frozen-thawed mitochondria from *Arum maculatum* spadices. The two systems contain flavoproteins and differ by their ability to oxidize NADH or NADPH, optimum pH and pI values, sensitivity to Ca^{2+} and EGTA, denaturation by 4 molar urea, molecular mass, and number of subunits. These properties, together with methodological considerations, are compatible with the location of these enzyme activities on the outer surface of the inner mitochondrial membrane, and support the hypothesis of the existence of two separate dehydrogenases responsible for the mitochondrial oxidation of cytosolic NADH and NADPH.

By comparison with animal mitochondria, the oxidation of NADH in plant mitochondria is far more complex. Only two oxidation systems have been reported for animal mitochondria: one, localized in the outer membrane, is probably of minor importance; the other one is the well known complex I of the inner membrane, facing the matrix compartment. The latter system is linked to energy transduction, and carries out the reoxidation of the reduced equivalents generated by the many NAD-linked dehydrogenases present in the matrix. It is strongly sensitive to rotenone and piericidin (14).

In addition to these two systems, plant mitochondria possess two—and possibly three—other systems carrying out the reoxidation of the NAD(P)H produced in the matrix or in the cytosol (11, 24, 29). These additional systems are all localized on the inner membrane. One of them is the external NADH dehydrogenase (10, 22) localized on the outer surface of the inner membrane. It is rotenone-insensitive, but antimycin- and cyanide-sensitive (2). It is also stimulated by low Ca^{2+} concentrations and inhibited by Ca^{2+} chelators (3, 25, 27). Two different dehydrogenases could correspond to this system, one specific for NADH and another one specific for NADPH (1, 12, 18, 25). Finally, localized on the inner surface of the inner membrane, another NADH dehydrogenase is also present which may act as a bypass to complex I. This dehydrogenase differs from complex I by its size, its insensitivity to rotenone and piericidin, and its lower affinity for NADH, and is not linked to energy transduction (2, 22, 26). Such a variety of NAD(P)H dehydrogenases confers to plant mitochondria, through the control of the NAD(P)H/NAD(P) ratio, a particular capacity to regulate the activities of the various metabolic pathways taking place in the cytosol or in the matrix.

These various NAD(P)H-oxidizing systems, however, have been mainly characterized on an operational basis: V_{max}, K_m, sensitivity to activators or inhibitors, localization, etc., and very little is known about their molecular properties. For instance, very little information is available on the isolation, structure, and polypeptide composition of complex I in plant mitochondria (8, 11, 24).

Only very recently have some studies been carried out on the isolation of plant mitochondria NADH dehydrogenases from various tissues: cauliflower buds (17), mung bean hypocotyls (6, 8), and *Arum maculatum* spadices (4, 5, 7, 8). The latter plant material is particularly suitable for studies on the external NADH dehydrogenase, since this system is exceptionally active in this tissue (21). Besides, these mitochondria oxidize both NADH and NADPH (at a rate about 40% the rate with NADH) (7). Sonication (5) and various detergents, such as Triton X-100 (6, 8, 17), deoxycholate (7), and lauryl dimethylamine *N*-oxide (4), were used to solubilize the NADH dehydrogenases. Due to its high activity in plant mitochondria and to the experimental procedures used, one generally assumes that the isolated enzyme is the rotenone-insensitive external NADH dehydrogenase, whose molecular properties have been partially characterized. Such a dehydrogenase was also characterized from the mitochondria of the yeast *Saccharomyces cerevisiae* (9). However, problems of contamination by the other NADH dehydrogenases do exist, that are generally not taken into consideration.

The aim of this paper is to contribute to a better knowledge of the various NADH dehydrogenases present in plant mitochondria. It reports on the characterization of two NADH dehydrogenases, which are easily solubilized by using a freeze/thaw technique. Their partial purification and characterization as well as their presumptive location within the mitochondrion are reported.

MATERIALS AND METHODS

Preparation of Mitochondria and Submitochondrial Fractions

Mitochondria were isolated from the sterile part (club) of *Arum maculatum* L. spadices. Plant material was collected in

[1] This paper is respectfully dedicated to the memory of Professor J. B. Biale whose soft and warm personality will be long remembered.

spring time in woods near Paris. The spadices were at the developmental stage γ (spathe fully developed but not yet open, preceding the respiratory crisis linked to thermogenesis) as defined by Lance (20). Mitochondria were isolated according to Lance and Chauveau (21) and purified on a Percoll gradient according to Moreau and Romani (28). Mitochondria were either used immediately for activity measurements or stored at −40°C for several weeks until use. *A. maculatum* spadices being available during only a short period of time (April 15–May 10) each year, the experiments reported in this paper were carried out over a period of 3 years. Sensible variations in mitochondrial activities were observed, due to variable weather conditions during plant growth and to the difficulty to accurately select a well defined developmental stage (γ), large variations in tissue respiration and mitochondrial activities taking place during spadix development (20, 21). The range of variation for the extreme values were in the order of ±35% with respect to the mean value. For this reason, no particular attempt was made to measure yields accurately, rather attention was focused on the highest purity available for the different preparations (which goes with low yields). In a typical experiment, mitochondria (*ca.* 60 mg protein, 30 mg mL^{-1}) were slowly thawed (5°C) and centrifuged at 27,000*g* for 20 min. After discarding a large amount of fluffy layer, the pellet was washed with a large volume (40 mL) of 10 mM phosphate buffer (pH 7.4), centrifuged at 27,000*g* for another 20 min, and the resulting pellet, after elimination of a residual fluffy layer, was finally suspended in a small volume of the same buffer. This constituted the membrane fraction (*ca.* 15 mg protein, 8 mg protein mL^{-1}, yield: *ca.* 25%). On the other hand, the first supernatant was centrifuged at 27,000*g* for 20 min and then two times at 100,000*g* for 60 min, the precipitate being discarded after each centrifugation. The final supernatant was filtered through a 0.22 μm Millipore filter to remove any residual contaminating membrane fragments. This filtrate, containing matrix and membrane solubilized proteins, constituted the soluble fraction (*ca.* 12 mg protein, 15 mg protein mL^{-1}, yield: *ca.* 20%).

NADH Dehydrogenase Purification

The NADH dehydrogenases of the soluble fraction were purified by anion exchange chromatography (FPLC[2]) (LKB). The samples were applied to a Mono Q column equilibrated with 20 mM Tris-HCl buffer (pH 7.5). Proteins were eluted with a linear (0–0.5 M) NaCl gradient in the same buffer.

Enzyme Activities

Cyt *c* oxidase activity was measured spectrophotometrically at 550 nm in 10 mM phosphate buffer (pH 7.4) in the presence of 50 μM reduced Cyt *c*. Cyt *c* was reduced by adding a few crystals of $Na_2S_2O_4$, any excess being eliminated by vigorous aeration. Mitochondrial integrity was measured by assaying cytochrome oxidase activity in a medium containing 0.3 M sucrose and 10 mM phosphate buffer (pH 7.4) by comparison with a medium devoid of sucrose. NAD(P)H dehydrogenase activity was determined spectrophotometrically in the same medium by measuring the rate of reduction of 50 μM Cyt *c* (NADH-Cyt *c* reductase) or 1 mM $K_3Fe(CN)_6$ (NADH-FeCN reductase), at 550 nm ($\Delta\epsilon$ = 21 mM^{-1} cm^{-1}) and 420 nm ($\Delta\epsilon$ = 1.0 mM^{-1} cm^{-1}) respectively, in the presence of 1 mM NADH (or NADPH) and 1 mM KCN (to abolish any Cyt *c* oxidase activity). Depending on the activity and the fraction studied (membrane, soluble), between 1 and 50 μg protein ml^{-1} was used to give a measurable rate of NAD(P)H reductase activity, in a final volume of 2.5 mL. As to the other electron acceptors, 300 μM juglone, 300 μM duroquinone, 40 μM coenzyme Q_6 (ubiquinone 30), the rate of NADH oxidation was monitored in the same medium by following the decrease of absorbance at 340 nm ($\Delta\epsilon$ = 6.22 mM^{-1} cm^{-1}). Since electron acceptors accept one (Cyt *c*, FeCN) or two (quinones) electrons, all NAD(P)H dehydrogenase activities were expressed in equivalents of NAD(P)H oxidized. All chemicals were from Sigma. Protein was determined by the Bradford method according to Fanger (13).

Electrophoresis

Nondenaturing gel electrophoresis was performed at 10°C in 10% polyacrylamide gels containing 0.1% Chaps and 0.3 M Tris-HCl buffer (pH 8.8). Gels were either stained with Coomassie blue for protein visualization or used for localization of NADH dehydrogenase activity by incubating the gels in 10 mM phosphate buffer (pH 7.4) in the presence of 0.5 mM NADH and 0.25 mg mL^{-1} (0.3 mM) NBT (30). Whole mitochondria or the membrane fraction were first solubilized in the presence of 10% Chaps, centrifuged at 11,000*g* for 10 min, and finally filtered on a 0.22 μm Millipore filter before being applied to the gel. Denaturing electrophoresis was performed on a discontinuous system according Laemli (19), pieces of nondenaturing gels containing NADH activity being placed in the wells. Then the gels were stained with Coomassie blue.

Electrofocusing

Electrofocusing of purified NADH dehydrogenases was performed with 4% polyacrylamide gel rods containing 1% ampholines (pH 5–8) (Servalyt 5–8, Serva). Then the gel rods were cut into 0.5 cm long pieces and incubated for 4 h in 0.1% NaCl for the determination of the pH gradient. NADH dehydrogenase activity was localized on the gel pieces by the NBT reaction as described above.

RESULTS

NADH Dehydrogenase Activities

Mitochondrial activities were measured on fresh and frozen-thawed mitochondria as well as on the soluble and membrane fractions isolated therefrom. The results are reported in Table I as specific activities (and do not represent quantitative distributions between the various mitochondrial fractions).

Cyt *c* oxidase, measured in a low tonicity medium (10 mM phosphate buffer) to rupture the outer membrane in order to release maximum activity, was slightly decreased (*ca.* 10%) in frozen-thawed mitochondria and increased (50%) in the membrane fraction. Very little activity (0.3%) was present in

[2] Abbreviations: FPLC, fast protein liquid chromatography; Chaps, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate; FeCN, ferricyanide; NBT, nitro blue tetrazolium.

Table 1. *Distribution of Specific Enzyme Activities in Mitochondria and Mitochondrial Fractions*

All activities were measured at pH 7.4 as indicated in "Materials and Methods" and are expressed with reference to reduced Cyt *c* (Cyt *c* oxidase) or NAD(P)H (reductase activities) oxidized. The rotenone-insensitive activity is calculated as the difference between total and rotenone-sensitive activity. Results are mean values of four to six experiments.

Fraction	Cyt *c* Oxidase	NADH-Cyt *c* Reductase			NADPH-Cyt *c* Reductase			NADH-FeCN Reductase	NADPH-FeCN Reductase
		Total	Rotenone-insensitive	Rotenone-sensitive	Total	Rotenone-insensitive	Rotenone-sensitive		
		μmol min⁻¹ mg⁻¹ protein							
Fresh mitochondria	0.93[a]	1.05	0.52	0.53	0.35	0.35	0	2.82	0.90
Frozen-thawed mitochondria	0.85	0.92	0.47	0.45	0.21	0.21	0	4.44	1.10
Membrane fraction	1.58	0.76	0.30	0.46	0.11	0.11	0	4.98	1.00
Soluble fraction	0.003	0.01	0	0	0.005	0	0	3.12	0.80

[a] The value of the activity measured in 0.3 M sucrose-10 mM phosphate buffer was about 0.10 to 0.05 μmol min^{-1} mg^{-1} protein, corresponding to an outer membrane integrity of 90 to 95%. By comparison, the integrity of frozen-thawed mitochondria was only 1 to 3%.

the soluble fraction, indicating that this fraction was not contaminated by inner mitochondrial membranes.

NADH dehydrogenase activity, measured as NADH-Cyt *c* reductase, decreased in frozen-thawed mitochondria (10%) but also in the membrane fraction (25%), this decrease being entirely due to the rotenone-insensitive activity, the rotenone-sensitive activity remaining at the same level. No activity could be found in the soluble fraction. NADH-Cyt *c* reductase activity (which is sensitive to antimycin) requires the integrity of complexes I and III as well as the presence of ubiquinone for full activity. This again points to the absence of contamination of the soluble fraction by the inner membranes.

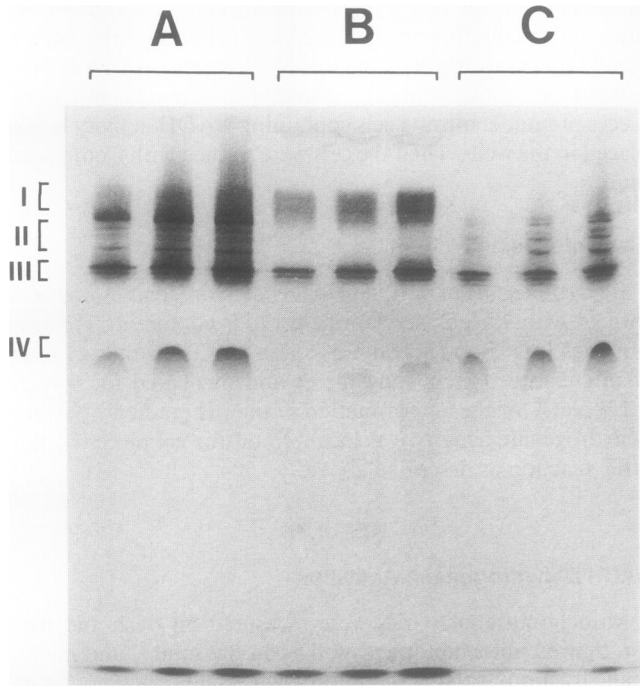

Figure 1. Nondissociating polyacrylamide gel analysis of *A. maculatum* mitochondria. A, whole mitochondria; B, membrane fraction; C, soluble fraction. Proteins from whole mitochondria and membrane fraction were solubilized by 10% Chaps. Protein (from left to right): A, 12, 24, and 48 μg; B, 5, 10, and 15 μg; C, 6, 12, and 24 μg. NADH dehydrogenase activities are visualized by coloration with NBT.

The same general remark could be made for the NADPH dehydrogenase activity. However, this activity was lower than the NADH dehydrogenase activity (between and 15 and 35%, depending on the fraction). This is in accordance with the NAD(P)H oxidase activities measured on whole, fresh mitochondria (7). This activity was antimycin-sensitive (not shown) and quite insensitive to rotenone, indicating that complex I does not participate in this oxidation.

Similar ratios were observed between NADH- and NADPH-FeCN reductase activities. However, these activities markedly increased in frozen-thawed mitochondria, due to the disappearance of the permeability barrier to FeCN in the inner membranes, and in the membrane fraction. Moreover, they were present in the soluble fraction at about the same specific activity as in the membrane fraction. In no case were these activities sensitive to antimycin (while succinate-FeCN reductase was 50% sensitive, not shown). Therefore, these activities appear to be equally distributed between the membrane and the soluble fraction, indicating that part of the activity is strongly membrane-bound, another part being easily solubilized through the freeze-thaw technique.

Nondenaturing Gel Electrophoresis

The NADH dehydrogenase activities of the Chaps-solubilized mitochondria and membrane fraction and of the soluble fraction were visualized on nondenaturing polyacrylamide gels stained with NBT (Fig. 1).

Whole, fresh mitochondria display at least nine bands of activity, distributed into four groups. The upper, slowly moving group (I), not very well defined, is present in whole mitochondria and in the membrane fraction. The second group (II) (3–4 bands) seems to be specific of the soluble fraction. The third group (III) includes three bands found both in the membrane and the soluble fractions. The last band (IV), with the greatest mobility, is distinctly apart from the others and belongs to the soluble fraction.

FPLC Analysis of the Soluble Fraction

FPLC analysis of the soluble fraction (Fig. 2) shows that the major part of the proteins were eluted by weak NaCl concentrations (below 150 mM). Two distinct peaks with high NADH-FeCN reductase activities were visible, corresponding to two protein peaks, which will be referred to as NADH dehydrogenases I (DH_1) and II (DH_2). The first one, DH_1, was eluted at 150 mM NaCl as a very sharp protein peak. The second one, DH_2, was eluted at 225 mM NaCl as a larger protein peak. Compared to the values of Table I (soluble fraction) the increase in specific activity for both DH_1 and DH_2 was about 20-fold, due to the elimination of a majority of matrix proteins from the soluble fraction.

Samples of eluates corresponding to DH_1 (samples 18–21) and to DH_2 (samples 31–40) were electrophoresed on non-denaturing gels. DH_1 exhibited only a single band of activity as visualized with the NBT technique (Fig. 3A). On the opposite, the electrophoresis of the DH_2 peak showed three very close major bands of activity (and several additional ones of slower mobilities) (Fig. 3B). This agrees well with the fact that the protein peak of DH_2 is not very sharp (Fig. 2). Staining with Coomassie blue (Fig. 3, C and D) showed that some other membrane and matrix proteins, besides the NADH dehydrogenases, were still present in the soluble fractions separated by FPLC.

Properties of the Soluble NADH Dehydrogenases

Both NADH dehydrogenases were able to oxidize NADH at similar rates, using FeCN as the electron acceptor (Table II). When artificial quinones (juglone, duroquinone) and coenzyme Q_6 were used as electron acceptors, the activity was rather reduced with DH_1 as compared to DH_2, for which juglone and duroquinone were very good electron acceptors. In both cases, coenzyme Q_6 (ubiquinone 30) was a very poor electron acceptor. On the other hand, DH_1 was able to oxidize NADPH at an appreciable rate, about half the rate of NADH oxidation, as do fresh *A. maculatum* mitochondria (7) (*cf.* Table I), while the ability of DH_2 to oxidize NADPH was very poor (20 times less as compared to NADH). This was observed with either FeCN or juglone as electron acceptors. From the data of Table II, it appears that DH_1 is more specific of the oxidation of NADPH and DH_2 of the oxidation of NADH.

Both dehydrogenases are (at least partly) flavoproteins as indicated by their typical fluorescence emission spectra (Fig. 4). Both dehydrogenases DH_1 and DH_2 exhibited two emission maxima around 470 and 515 nm.

The two dehydrogenases differed also by their optimum pH for maximal activity. The oxidation of NADH was maximum at pH 7.0 and that of NADPH at pH 6.5 for DH_1, while the oxidation of NADH had a sharp maximum at pH 5.5 for DH_2 (Fig. 5). Moreover, the oxidation of NADH by DH_1 was strongly resistant to alkaline pHs. From this figure it appears that the value of pH 7.4, which was used to assay the NAD(P)H reductase activities with the different fractions (Table I) or the different electron acceptors (Table II), was not optimal. However, despite variations in activities due to the heterogeneity of the plant material, the values of Fig. 5,

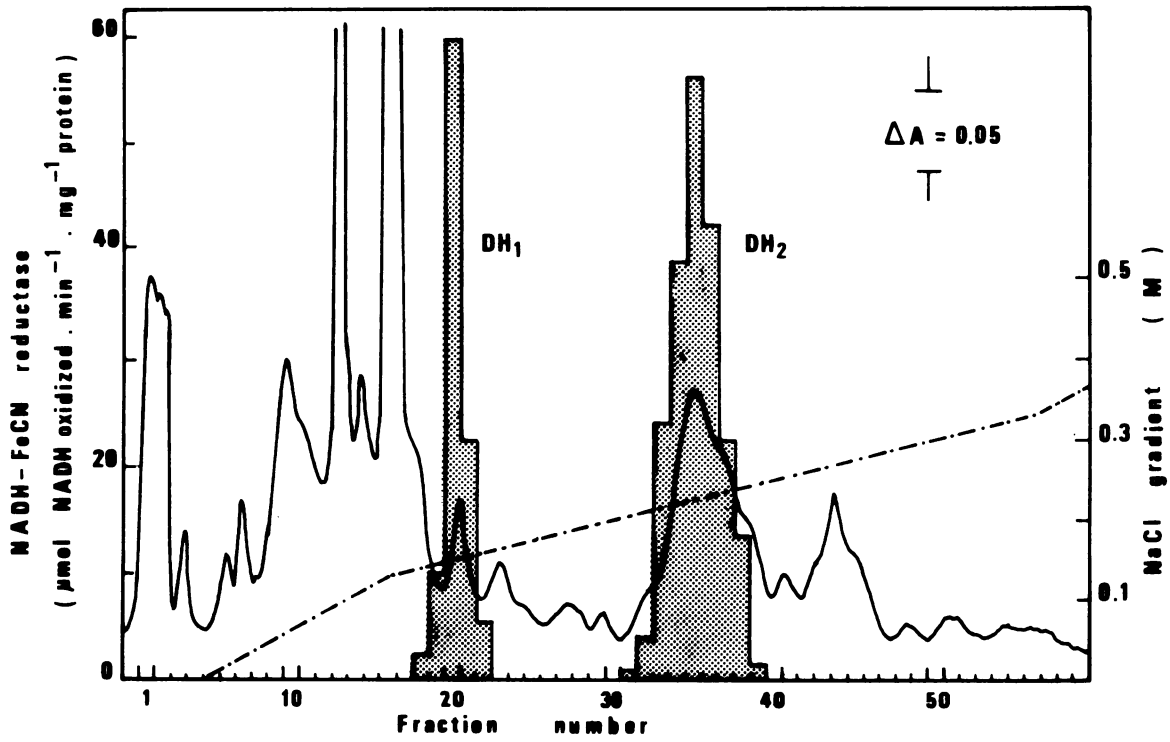

Figure 2. Separation by FPLC anion exchange chromatography of NADH dehydrogenases from the soluble fraction of *A. maculatum* mitochondria. Proteins were eluted by an NaCl gradient (0 to 500 mM) at pH 7.4 (dotted line). NADH-FeCN reductase activities (DH_1, DH_2), measured at pH 7.4, are represented by the hatched areas. The protein profile (solid line) is the absorbance measured at 280 nm.

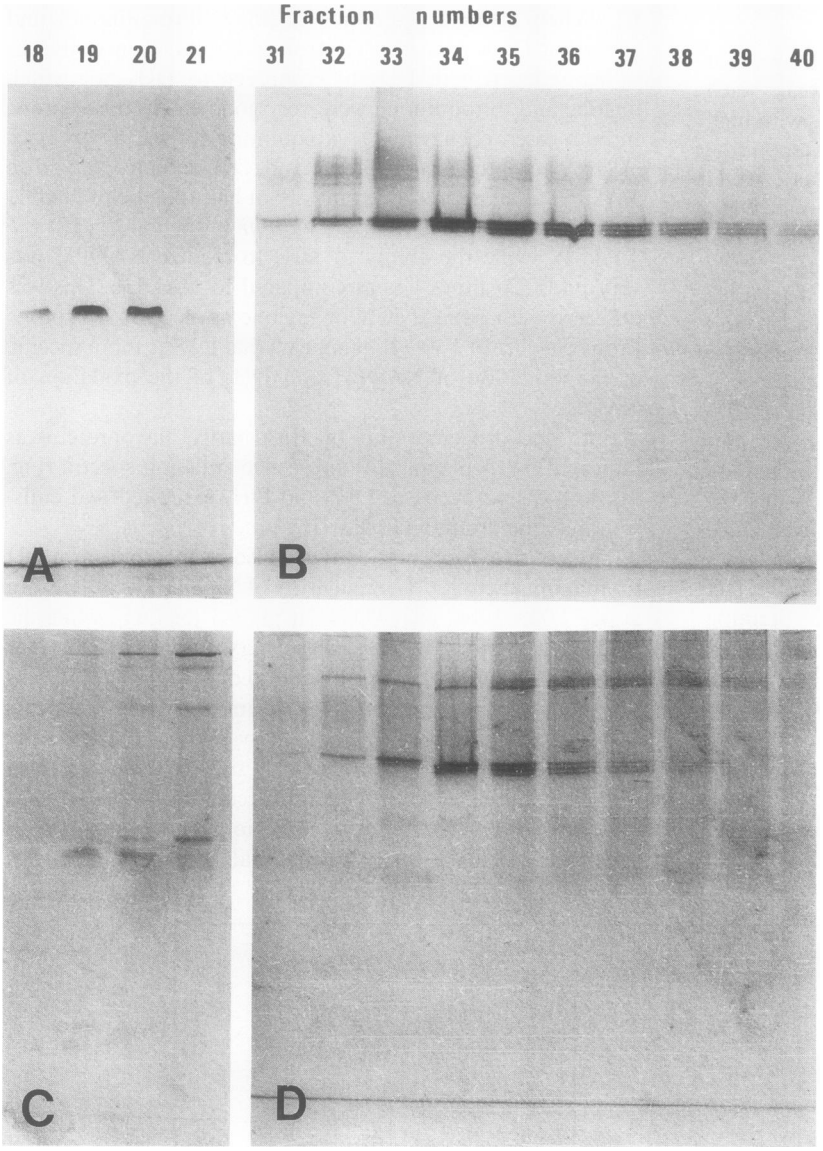

Figure 3. Nondissociating polyacrylamide gel electrophoresis of various fractions obtained by FPLC separation. A and C, DH_1 peak; B and D, DH_2 peak. A and B, NBT staining; C and D, Coomassie blue staining. Fraction numbers are those of Figure 2.

considered at pH 7.4, confirm the conclusions drawn from Table I, *i.e.* DH_1 specifically oxidizes NADPH, and as for DH_2 the ratio of the NADPH/NADH reductase activities is only a few percents.

The NADH-FeCN reductase activity of DH_1 was insensitive to EGTA and CA^{2+} ions, whereas the same activity with DH_2 was strongly inhibited (44%) by 5 mM EGTA and fully restored by a subsequent addition of 8 mM $CaCl_2$ (Fig. 6, A and C). Such an effect of Ca^{2+} and EGTA is not observed when using intact *A. maculatum* mitochondria because these mitochondria are quite heavily loaded with Ca^{2+}, depending on the developmental stage (between 100 and 500 nmol Ca^{2+} mg^{-1} protein, unpublished data). Figure 6 also clearly shows a differential effect of Ca^{2+} and EGTA on the two types of dehydrogenases. Moreover, the activity of DH_1 was reduced by only 30% in the presence of 4 M urea while that of DH_2 was completely abolished (Fig. 6, B and D).

The pI values for both isolated dehydrogenases were determined through electrofocusing. Several determinations led to an average value of 5.0 for DH_1. Values of 5.25, 5.20, and 5.10 were obtained for the three bands of DH_2 (results not shown).

Finally, the determination of the molecular masses was made from nondenaturing gel stained with NBT. The strips showing NADH dehydrogenase activity were cut off, equilibrated with 2.3% SDS in 0.25 M Tris-HCl (pH 6.8) and electrophoresed on SDS polyacrylamide gel (Fig. 7). The single band of DH_1 (Fig. 3A) was resolved into three polypeptides of 32, 33.5, and 34 kD (Fig. 7A). As to the three bands of DH_2 (Fig. 3B), they were resolved into five different bands of higher molecular masses with a major polypeptide of 54 kD, the other ones being polypeptides of 59, 62, 62.5, 72, and 98 kD (Fig. 7B).

DISCUSSION

The above results clearly indicate that two easily soluble NADH dehydrogenases can be isolated from *A. maculatum*

Table II. *Activities of Purified NADH Dehydrogenases (DH_1 and DH_2) with Various Electron Donors and Acceptors*

All activities were measured at pH 7.4 in 10 mM phosphate buffer, and are all expressed with reference to NADH and NADPH oxidized. Results are mean values of three to five experiments.

Reductase Activity	DH_1	DH_2
	μmol min⁻¹ mg⁻¹ protein	
NADH-FeCN	60	56
NADH-juglone	44	194
NADH-duroquinone	7.5	54
NADH-coenzyme Q_6	2	3
NADPH-FeCN	35	2.5
NADPH-juglone	11	3

spadix mitochondria. Both dehydrogenases are flavoproteins but clearly differ by a number of characteristics: substrate specificity, sensitivity to inhibitors, pIs, optimum pHs for activity, and number of subunits. The major problem, however, is the assignment of a specific location within the organelle.

First of all, storage for two or three weeks at −40°C does not impair mitochondrial oxidative properties since cytochrome oxidase activity is maintained at its original level as in freshly prepared mitochondria. On the other hand, NADH- and NADPH-FeCN reductase activities are enhanced due to alteration of the inner membrane integrity (Table I). Sensitivity or resistance to rotenone are also well preserved. Therefore frozen *A. maculatum* mitochondria constitute a good starting material for the isolation of NAD(P)H dehydrogenases from plant mitochondria.

Cottingham and Moore (7) have shown that the membrane fraction obtained from frozen and phosphate buffer washed *A. maculatum* mitochondria is nearly free of outer membranes and of matrix components. From the results of Table I, this fraction indeed appears to consist of highly purified inner membranes (large amounts of ill-defined fluffy layer being eliminated during the purification procedure). Therefore, most of the outer membranes should be concentrated in

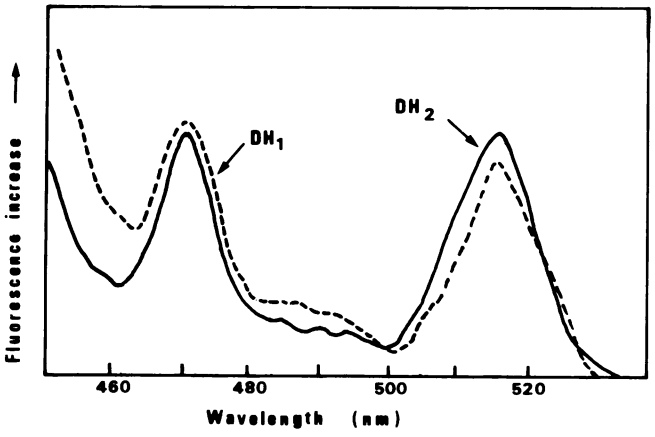

Figure 4. Fluorescence emission spectra of isolated NADH dehydrogenases, DH_1 and DH_2. Excitation light at 436 nm, aerobic conditions.

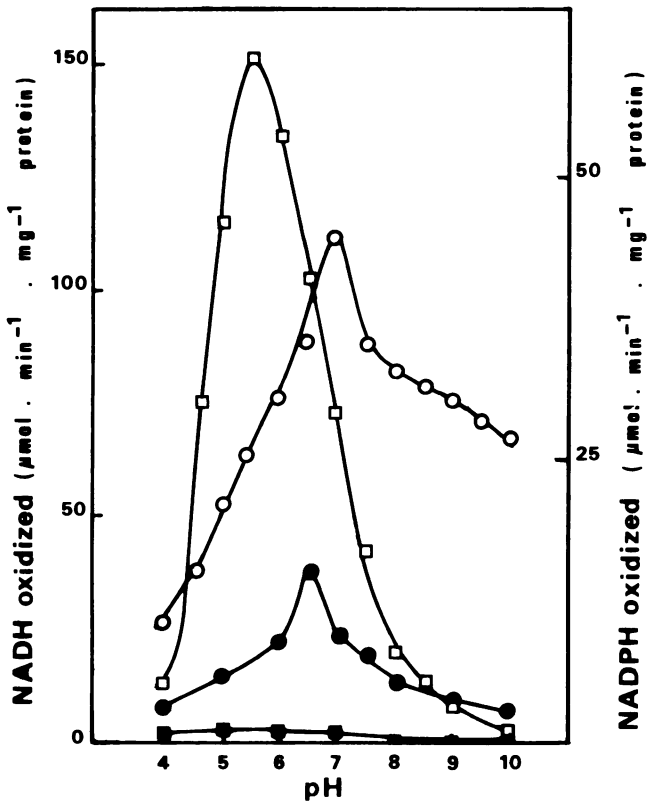

Figure 5. Effect of pH on NADH- and NADPH-FeCN reductase activities of the soluble NADH dehydrogenases isolated from *A. maculatum* mitochondria. Activities were measured in a 10 mM phosphate buffer adjusted at the desired pH. (○), NADH-DH_1; (●), NADPH-DH_1; (□), NADH-DH_2; (■), NADPH-DH_2.

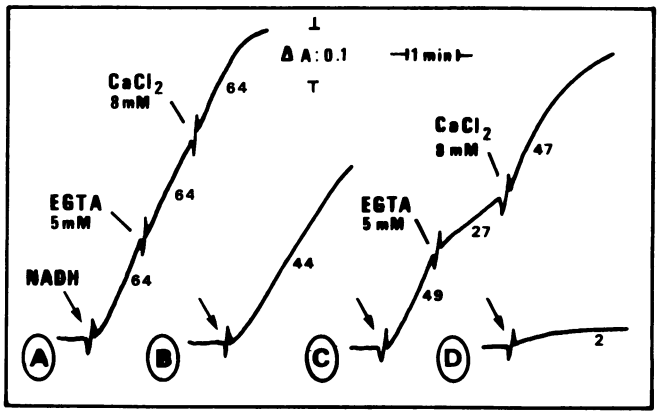

Figure 6. Effect of EGTA and $CaCl_2$ on the NADH-FeCN reductase activity of the soluble NADH dehydrogenases isolated from *A. maculatum* mitochondria. A and B, DH_1; C and D, DH_2 (fractions with the highest activities in Fig. 2). A and C, effect of EGTA and $CaCl_2$; B and D, effect of 4 M urea. Activities were measured in a 10 mM phosphate buffer at pH 7.4, and are expressed in μmol NADH oxidized min^{-1} mg^{-1} protein (figures along the traces).

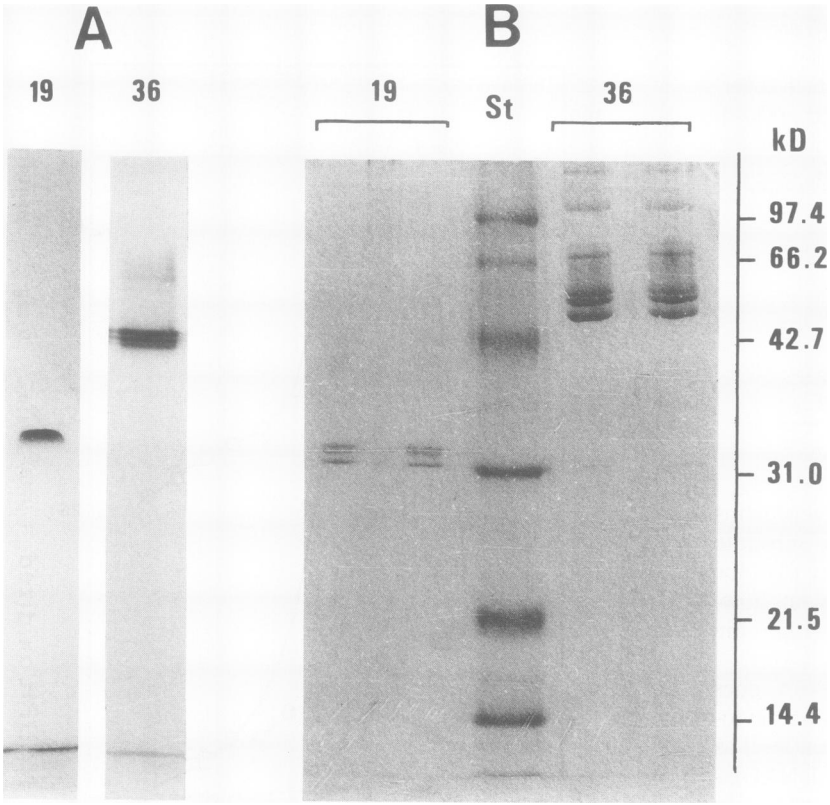

Figure 7. Determination of the polypeptide composition and molecular masses of DH_1 and DH_2. A, nondissociating gel electrophoresis of fractions with the highest activities in Figure 2. B, SDS gel electrophoresis of DH_1 and DH_2 gel fragments cut from A. St., standard molecular masses: phophorylase b (97.4 kD), BSA (66.2 kD), ovalbumin (42.7 kD), bovine carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), lysozyme (14.4 kD).

the first supernatant (see "Materials and Methods"). Further steps of high speed centrifugation and filtration through Millipore filters of this supernatant eliminate all outer membranes, and consequently the NADH dehydrogenase activity localized in this mitochondrial fraction. Moreover, it should be noted that this latter enzyme activity is rather weak, compared to others, and could not account for the levels of activity reported in Table I. Therefore, the starting material for the isolation of NADH dehydrogenase activities is a supernatant containing only enzyme systems loosely bound to the inner membranes, that are released by the freeze-thaw procedure, as well as the bulk of soluble matrix enzyme activities.

Nondenaturing gel electrophoresis of various mitochondrial fractions (Fig. 1) shows a number of bands displaying NADH dehydrogenase activity which is much higher than the three or four NADH dehydrogenase systems reported to be implicated in the oxidation of external and internal NADH in plant mitochondria (11). The nine or so bands of Figure 1 are close to the number of bands obtained by Cottingham and Moore (8) on gel electrophoresis of *A. maculatum* mitochondria carried out at pH 9.0. Some of these bands can be assigned specific locations on the inner membrane.

In particular the upper, ill-defined band (Fig. 1, group I) has been considered by Cottingham and Moore (8) as identical to complex I by reference to the NADH dehydrogenase of ox heart mitochondria. In agreement with that, it is to be noted that the membrane fraction from which this band is isolated has retained sensitivity to rotenone (Table I). The main band of group III (Fig. 1) is distributed between the membrane and the soluble fractions. It corresponds to the Y band of Cottingham and Moore (8). This band can be solubilized by physical means or Chaps (this paper) or treatment of the inner membranes by Triton X-100 (8). It was identified by Cottingham and Moore (8) as the external NADH dehydrogenase. It can account for a part of the high rotenone-insensitive NADH dehydrogenase activity of the membrane fraction (Table I). The band of higher mobility (group IV) and four or five bands of group II are exclusively associated with the soluble fraction.

FPLC analysis of the soluble fraction shows two distinct peaks of NADH dehydrogenase activity. They probably correspond to the high mobility band of group IV (DH_1) and to the three bands of group III (DH_2) (Fig. 1). The bands of group II are probably eluted after DH_2, but presumably they are too weak to yield a significant activity and allow further analysis. Both dehydrogenases (DH_1 and DH_2) have pIs close to 5, but they differ by all other properties.

DH_1 is not susceptible to denaturation by 4 mM urea (Fig. 6B). Since urea disrupts hydrophobic bonds (16), this indicates that DH_1 contains few hydrophobic bonding regions, which agrees with a weak integration in the inner mitochondrial membrane, an easy solubilization and the sharp peak shown in Figure 2. This could be an argument for the location of DH_1 on the outer surface of the inner mitochondrial membrane (10). A second argument for such a location is the ability of DH_1 to oxidize NADPH at an appreciable rate, in the same order of magnitude as for NADH (Table II, Fig. 5). The existence of such an NADPH dehydrogenase, distinct from the NADH dehydrogenase, on the outer surface of the

inner membrane has been postulated by Koeppe and Miller (18), Arron and Edwards (1), and Edmann *et al.* (12). However, this dehydrogenase in its solubilized form is not inhibited by EGTA nor reactivated by Ca^{2+} ions (Fig. 6), as is the NADPH dehydrogenase *in situ* (1, 18). Therefore DH_1 appears as a small protein, made of three polypeptides of very close molecular masses (32, 33.5, and 34 kD), that could possibly be identified with the external NADPH dehydrogenase. Such a protein could not be isolated by Cottingham and Moore (8) and Cook and Cammack (4, 5) from *A. maculatum* mitochondria, since in their work they considered only the membrane fraction, discarding the supernatant of frozen-thawed mitochondria. From cauliflower mitoplasts, Klein and Burke (17) have isolated two NADH dehydrogenases, one of them being able to oxidize NADPH (and showing no sensitivity to Ca^{2+}). However, it is difficult to equate this dehydrogenase with DH_1 due to its much more complex polypeptide composition (five polypeptides).

The activity of DH_2 is abolished in the presence of 4 M urea. Moreover, it is inhibited by 5 mM EGTA and reactivated by 8 mM Ca^{2+}. Though being in a solubilized form, it reacts like the external NADH dehydrogenase of the inner membrane of plant mitochondria (3, 23, 27). Under our experimental conditions, Ca^{2+} cannot exert its effect through the screening of charges at the surface of the inner membrane (15, 23, 25). Rather, this enzyme system should possess a Ca^{2+} binding site as postulated by some authors (27). DH_2 appears to be specific of the oxidation of NADH. This agrees with the conclusion of Arron and Edwards (1) and Møller and Palmer (25) indicating that external NADH and NADPH are oxidized by two different dehydrogenases. It also appears as an enzyme system with a rather complex polypeptide composition. Compared to other external NADH dehydrogenases that have been isolated, the activity of the solubilized enzyme (in this paper) is higher than those reported by Cook and Cammack (4, 5), Cottingham and Moore (7) and Cottingham *et al.* (6). Moreover, the polypeptide composition was different and no effect of Ca^{2+} or EGTA was observed by these authors.

It appears, therefore, that by using a very mild physical means (freeze/thaw) for membrane disruption of *A. maculatum* mitochondria, rather than sonication or detergents, two soluble NADH dehydrogenases can be isolated which possess quite different characteristics. The effect of Ca^{2+} and EGTA on the one hand, the specificity towards NADPH oxidation on the other hand, strongly suggest that these two dehydrogenases could be the external NADH and NADPH dehydrogenases located on the outer surface of the inner membrane of plant mitochondria. Therefore, the results reported in this paper lend a strong support to the existence of a specific dehydrogenase for external NADPH, as postulated by some authors. It is also the first report of the isolation and partial characterization of this system.

Many problems remain to be solved, however, while some new questions can be raised. The identification and the presumptive location of these dehydrogenases on the outer surface of the inner membrane are somewhat indirect. These activities cannot belong to the outer membrane, which is eliminated during the preparative procedure. It cannot be the complex I because of the relative simplicity of the polypeptide composition and insensitivity to rotenone, and it is difficult to identify these activities with the rotenone-insensitive internal NADH dehydrogenase because no capacity to oxidize NADPH (*cf.* DH_1) and no sensitivity to Ca^{2+} and EDTA (*cf.* DH_2) have been reported for this activity. Therefore, one is left with only the activities present on the outer surface of the inner membrane.

In addition new problems do appear. No consistent polypeptide composition has been reported for the various NAD(P)H dehydrogenases isolated so far (11, 24), and the present work does not contribute to the clarification of this situation. Besides, in this work, the NADH and NADPH dehydrogenases activities appear to be quite different in many aspects, while they are generally reported to display similar behaviors (effect of EDTA, of Ca^{2+}) when assayed *in situ* on intact mitochondria (11, 24). If for DH_1 the optimum pH for NADPH oxidation is lower than for NADH as generally observed (11), the optimum pH for NADH oxidation by DH_2 is much lower (5.5) than that (6.8) generally reported for the external NADH dehydrogenase (24).

These discrepancies, therefore, together with the results presented in this paper, raise many questions and show that much progress still remains to be done before a full understanding of the oxidation of endogenous and exogenous NAD(P)H by plant mitochondria is achieved. The main interest of this paper, however, is to present a simple method to isolate soluble NAD(P)H dehydrogenases from plant mitochondria and make further studies of these activities easier.

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