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Rapid Report

Purification of a rotenone-insensitive NAD(P)H dehydrogenase from the inner surface of the inner membrane of red beetroot mitochondria

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The soluble fraction of disrupted red beetroot mitochondria was resolved by anion-exchange chromatography. Three NADH-oxidising activities were found, including one duroquinone reductase oxidising both NADH and NADPH. This NAD(P)H-duroquinone reductase, which we assign as the internal rotenone-insensitive NAD(P)H dehydrogenase, was further purified by affinity chromatography into a 26 kDa polypeptide.

Plant mitochondria contain at least three different NAD(P)H dehydrogenases in the inner membrane, all three of which are coupled to the electron transport chain via ubiquinone [1]. Whether exogenous NADH and NADPH are oxidized by one or two enzymes on the outer surface of the inner membrane is still unclear. On the inner surface, plant mitochondria have, in addition to the rotenone-sensitive Complex I, a rotenone-insensitive, Ca^{2+}-dependent NADPH dehydrogenase, which may also oxidize NADH [2–4]. Complex I has recently been isolated from plant mitochondria [5] and several groups have reported a partial purification of an external NAD(P)H dehydrogenase but with a marked lack of consistency in size and properties [6–9].

In the present communication we report the first purification of the internal rotenone-insensitive NAD(P)H dehydrogenase from plant mitochondria. This work has been presented at two meetings [10,11].

Red beetroot (*Beta vulgaris* L.) mitochondria were prepared essentially as in Ref. 5 and stored at −80°C until used. The mitochondria were disrupted either by sonication [3] or by four cycles of freeze-thawing, both procedures carried out in 5 mM triethanolamine-HCl/0.5 mM EDTA (pH 7.6). Soluble proteins were separated from the membranes by centrifugation at 300 000 × *g* for 1 h.

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Abbreviations: DQ, duroquinone; UQ, ubiquinone.

The 300 000 × *g* supernatant was precipitated between 35 and 60% $(NH_4)_2SO_4$, and desalted on a PD-10 column (Pharmacia) preequilibrated in buffer A (20 mM triethanolamine-HCl (pH 7.6)). The enzyme fraction was loaded on a 2.6 × 9 cm DEAE Sepharose FF column, and after washing with one column volume of buffer A, eluted with a 0–0.25 M NaCl gradient in buffer A. Fractions containing > 50% of the NADPH-DQ activity in the peak fraction were pooled and dialyzed for 5 h against 100 volumes of buffer B (20 mN Mops-KOH (pH 7.0)). The dialyzate was loaded at 1 ml/min on an Blue Affi-Gel cartridge (Bio-Rad), which thereafter was washed with first 5 ml 100 mM KCl in buffer B, then with 30 ml buffer B. The enzyme was eluted with an NADPH gradient (0–2 μM in Fig. 2, but later 0–300 nM) in buffer B. All procedures were carried out at 4°C and, apart from the dialysis, in the presence of 0.5 mM phenylmethylsulphonyl fluoride and 2 μM E-64 (Sigma E 3132).

NAD(P)H oxidation with UQ analogues as acceptors was measured at 340 nm in 20 mM Mops-KOH (pH 7.2) and 200 ng/ml antimycin A (except in Table II). NAD(P)H was used at 100 μM. Ferricyanide and cytochrome *c* reduction were measured at 420 and 550 nm, respectively.

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) was measured as NADH consumption in a medium containing 150 mM K-P_i, 1 mM EDTA (pH 6.5), 100 μM NAD^+, 100 μM NADH and 0.6 mM DL-lipoamide.

For the determination of α- and β-specificity, [4-^{3}H]NAD (Amersham) was reduced with either alcohol dehydrogenase (EC 1.1.1.1) or glucose-6-phosphate de-

hydrogenase (EC 1.1.1.49) to produce [β- and α-^{3}H]NADH, respectively, and filtered through a 10 kDa membrane. Enzyme (2.5 nmol NADH/min) was incubated for 10 min with 0.18 μM [α- or β-^{3}H]NADH and 50 μM DQ in 50 mM Mops-KOH (pH 7.2). NAD^+ and H_2O were thereafter separated on a 9 ml Sephadex G-10 column and fractions were counted in a scintillation counter.

Native molecular weight was determined using a 1.6 × 65 cm Sephacryl 200 HR column and gel-filtration calibration kit (Pharmacia). The column was pre-equilibrated and eluted in buffer B + 100 mM NaCl at 0.15 ml/min.

SDS-PAGE were run on 12% gels according to Ref. 12 and stained with silver according to Ref. 13. Dilute samples were first concentrated using Centricon 10 microconcentrators. Protein was determined by the method of Bradford [14].

Mitochondria isolated from fresh beetroots were used as the starting material since these contain little or no external NAD(P)H dehydrogenase activity [15,16] which might otherwise make it difficult to identify the internal NAD(P)H dehydrogenase after release of the enzymes from the inner mitochondrial membrane. NADPH-DQ reductase activity was used to detect the internal NAD(P)H dehydrogenase [3,4].

The mitochondria were disrupted, either by freezing/thawing or by sonication, causing the release of the matrix proteins as well as practically all NADPH-DQ reductase activity not attributable to Complex I (results not shown). The amount of NAD(P)H-DQ reductase activity released is sufficient to account for rotenone-intensitive malate oxidation in intact red beetroot mitochondria [17].

This soluble fraction was fractionated by anion-exchange chromatography (Fig. 1). Peaks of NAD(P)H dehydrogenase activity were detected at 90, 120 and 190 mM NaCl (Peaks I, II and III, respectively).

Peak I was the only peak with NADPH-DQ reductase activity, making it the sole candidate for the internal, rotenone-insensitive NAD(P)H dehydrogenase. The NAD(P)H-DQ reductase activity in Peak I was rotenone-insensitive and specific for the β-hydrogen on the nicotinamide ring (results not shown). The latter observation shows that it is not due to outer membrane activity which is α-specific [18].

Peak II is an, as yet unidentified, soluble NADH-ferricyanide oxidoreductase. The NAD(P)H-DQ reductase activity observed under Peak II was due to tailing from Peak I. This was confirmed by passing Peak II through a gel-filtration column where the NADH-DQ and NADH-ferricyanide reductase activities did not coelute (results not shown). The native molecular mass of the Peak II enzyme was 35–40 kDa as determined by gel filtration.

Peak III showed high NADH-DQ and -ferricyanide reductase activities, but also very high dihydrolipoamide dehydrogenase activity. This activity was 99% inhibited by 2 mM arsenite. Thus, Peak III is dihydrolipoamide dehydrogenase; it is not the external NADH dehydrogenase as suggested by Luethy et al. [8] and Chauveau and Lance [9].

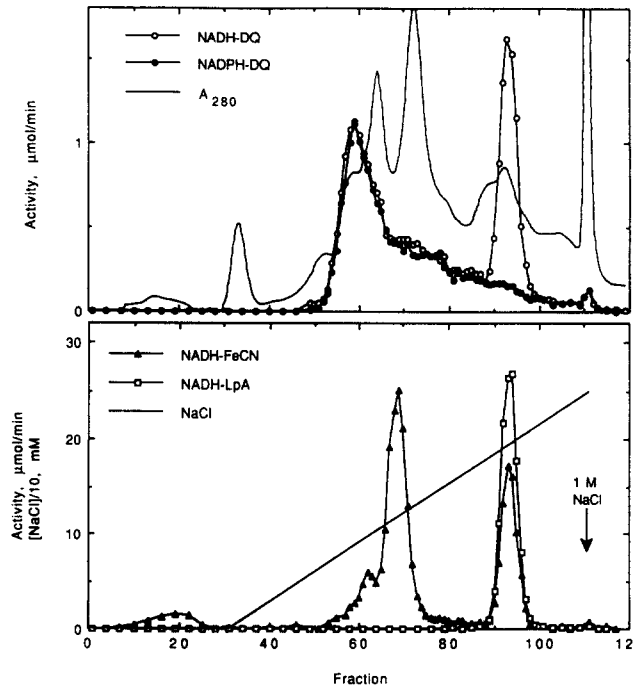

Fig. 1. Anion-exchange chromatography of soluble protein fraction from red beetroot mitochondria. The sample had not been precipitated with $(NH_4)_2SO_4$ before chromatography. A_{280} is given in arbitrary units.

For the purification of the Peak I dehydrogenase, the soluble fraction was precipitated between 35 and 60% ammonium sulphate prior to anion-exchange chromatography. The dehydrogenase was then purified by affinity chromatography (Fig. 2). The slow increase in A_{280} during the elution is due mainly to the absorbance of NADPH.

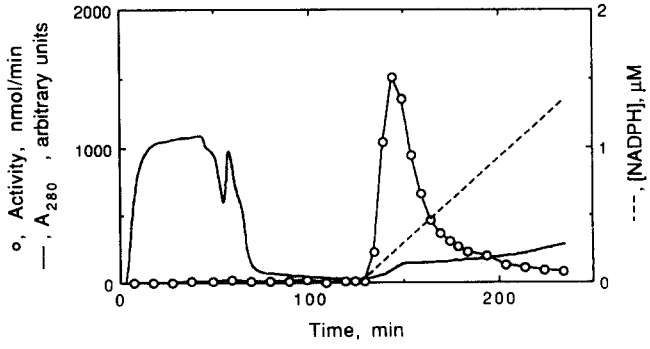

Fig. 2. Affinity chromatography of Peak I from anion-exchange-purified NAD(P)H-DQ reductase. The A_{280} peak at 60 min is due to washing with 100 mM KCl.

TABLE I

Purification of NADH-duroquinone reductase

Fraction	Protein (mg)	NADPH-DQ reductase activity			
		total (μmol min^{-1})	specific (μmol min^{-1} mg^{-1})	purification (fold)	yield (%)
Soluble proteins	83.5	22.3	0.267	1	100
$(NH_4)_2SO_4$ pellet	34.0	13.0	0.381	1.4	58
DEAE peak	2.38	5.2	2.2	8.2	23
Affinity peak	– [a]	3.1	–	–	14
SDS-PAGE [b]	0.0001	1.6	≈ 10000	≈ 40000	–

[a] Too little protein to measure by conventional means.

[b] Estimated from a band on a Coomassie-stained gel assuming that the detection limit is 0.1 μg.

SDS-PAGE of the peak fraction from the affinity column showed the presence of one main band at 26 ± 0.3 kDa ($n = 3$) (Fig. 3c) which correlated with activity whereas the weak band at 67 kDa did not correlate with activity (results not shown).

The NADPH-DQ reductase activity in Peak I after anion-exchange chromatography was stable for weeks at −80°C, whereas the affinity-purified enzyme lost about 60% of its activity after storage at −80°C for a couple of days.

A summary of the purification is shown in Table I. The enzyme has a turnover number of more than 4000 s^{-1} and is present at less than 0.1 μg per mg mitochondrial protein. It was necessary to load half a preparation into one lane on a minigel to be able to detect it by Coomassie staining (not shown).

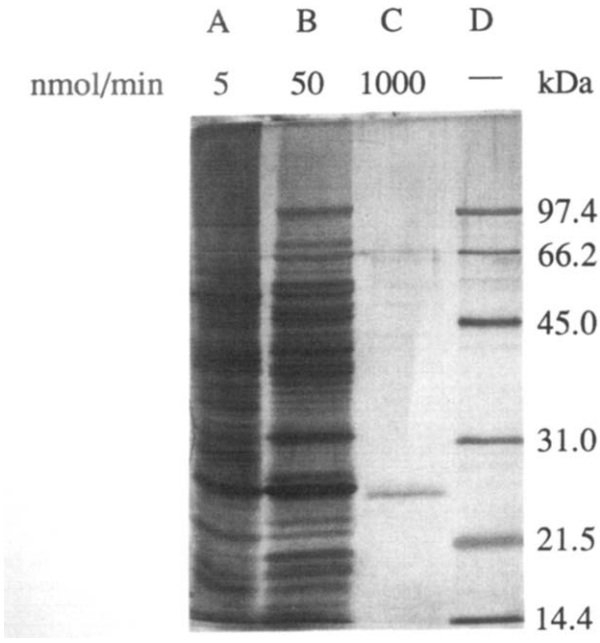

Fig. 3. SDS-PAGE illustrating the purification of the NAD(P)H dehydrogenase. The lanes contain ammonium sulphate-precipitated soluble fraction (A), Peak I from anion-exchange chromatography (B), peak from affinity chromatography (C) and molecular weight markers (D). Figures on top denote the amount of NADPH-DQ activity used for the lane. Figures on the right denote molecular masses of the standard proteins.

The affinity-purified enzyme had about the same activity with NADH and NADPH. As acceptor it preferred UQ_0 to DQ and UQ_1. It showed relatively high activity with ferricyanide and none with decylubiquinone and cytochrome *c* (Table II).

When the affinity-purified enzyme was run through a gel-filtration column it came out just before albumin (67 kDa), indicating that the native molecular mass was 70–80 kDa (results not shown). The average for three determinations was 76 ± 6 kDa. Thus, the internal NAD(P)H dehydrogenase of plant mitochondria appears to be a trimer or a tetramer in the native form.

A rotenone-insensitive NADH dehydrogenase of 53 kDa, or twice the size of our NAD(P)H dehydrogenase, has been isolated from the inner surface of the inner membrane of yeast mitochondria. It does not oxidize NADPH [19,20]. The enzyme is similar to an NADH dehydrogenase from *E. coli* and this enzyme has, in turn, been shown to contain two similar halves each with an NADH binding domains [21,22]. Although most rotenone-insensitive NAD(P)H dehydrogenases purified to date fall within the range 40–60 kDa [22], a low-molecular-mass NADH dehydrogenase (16 kDa) has been purified from the bacterium

TABLE II

Specificity of affinity-purified NADH-duroquinone reductase with NADPH, NADH or deamino-NADH as electron donor and various acceptors

Electron acceptor	Relative activity (%)		
	NADPH	NADH	deamino-NADH
DQ (200 μM)	100	103	78
UQ_0 (100 μM)	220	–	–
UQ_1 (80 μM)	32	–	–
Decyl-ubiquinone (50 μM)	0.5	–	–
Ferricyanide (1 mM)	68 [a]	–	–
Cytochrome *c* (50 μM)	0	–	–

[a] Measured as ferricyanide reduction and recalculated as NADPH oxidation.

Rhodopseudomonas capsulata [23]. Like our enzyme, this enzyme appears to be an homo-oligomer in its native form [23]. It is therefore possible that the low-molecular-mass dehydrogenases are products of a 'basic' dehydrogenase gene.

On the basis of our results we conclude the following. We have purified the NAD(P)H dehydrogenase from red beetroot mitochondria responsible for rotenone-insensitive oxidation of matrix NADH and NADPH by the respiratory chain [3,4]. It has a subunit size of 26 kDa and shows no preference for NADH or NADPH. The reasons previous attempts to identify and purify this enzyme and the external NAD(P)H dehydrogenase have failed are the following:

(a) Use of too little starting material. The enzyme has a high turnover number and is found at less than 0.1 μg/mg mitochondrial protein. For instance, had it been possible for Luethy et al. [8] to apply all their starting material on one SDS-PAGE lane, the internal NAD(P)H dehydrogenase would still have been only faintly detectable by Coomassie staining.

(b) The presence of an external and an internal rotenone-insensitive NAD(P)H dehydrogenase with similar properties in most plant mitochondria.

(c) The fact that the soluble dihydrolipoamide dehydrogenase present in all mitochondria has both NADH-DQ and -ferricyanide reductase activities.

(d) Use of NADH-ferricyanide reductase activity to detect the enzyme. The internal rotenone-insensitive dehydrogenase catalyzes only a minute proportion of the total NADH-ferricyanide reductase activity in plant mitochondria.

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