Partial purification and properties of the external NADH dehydrogenase from cuckoo-pint (*Arum maculatum*) mitochondria

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The external NADH dehydrogenase has been purified from *Arum maculatum* (cuckoo-pint) mitochondria by phosphate washing, extraction with deoxycholate, ion-exchange and gel-filtration chromatography. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis shows, when the gel is silver-stained, that the purified enzyme contains two major bands of M_r 78 000 and 65 000 and a minor one of M_r about 76 000. It is not possible at present to determine which of these, or which combination, constitutes the dehydrogenase. The enzyme contains non-covalently bound FAD and a small amount of FMN. Since the conditions of purification lead to considerable loss of flavin and possibly iron-sulphur centres, it is not possible to decide with certainty whether the enzyme is a flavo- or ferroflavo-protein. The enzyme has been distinguished from the other NADH dehydrogenases on the basis of its substrate specificity, its capability of reducing electron acceptors such as ubiquinone-1 and 2,6-dichlorophenol-indophenol and its sensitivity towards Ca^{2+} , EGTA and dicoumarol.

Most plant mitochondria are capable of oxidizing exogenous NADH via an external dehydrogenase located on the outer surface of the mitochondrial inner membrane (Douce *et al.*, 1973). This membrane-bound dehydrogenase donates reducing equivalents to the respiratory chain at the level of the ubiquinone pool (Coleman & Palmer, 1971; Douce *et al.*, 1973; Cottingham & Moore, 1983). The physiological significance of this enzyme and the mechanism whereby it transfers reducing equivalents to ubiquinone remains to be elucidated (see Moore & Rich, 1984). There is no specific inhibitor of this dehydrogenase, but its activity is regulated by micromolar concentrations of Ca^{2+} , inhibited by calcium chelators such as EGTA (Coleman & Palmer, 1971; Møller *et al.*, 1981; Moore & Åkerman, 1982) and does not appear to involve calmodulin (Dunn *et al.*, 1984).

To date, relatively little information is available

on either the subunit or polypeptide composition of this dehydrogenase. There have been two recent abstracts, however, on its solubilization and partial purification from cauliflower (*Brassica oleracea*) (Klein *et al.*, 1983) and cuckoo-pint (*Arum maculatum*) (Cook & Cammack, 1983) mitochondria. Both groups obtained a solubilized preparation that was insensitive to rotenone and unaffected by EGTA or Ca^{2+} . Little evidence was presented on either the method of assay or the degree of contamination by the other NADH dehydrogenases (see Palmer & Møller, 1982). The present paper describes a partial purification, molecular characteristics and enzymic properties of the external NADH dehydrogenase from *Arum maculatum* mitochondria. It is distinguished from the other NADH dehydrogenases on the basis of its substrate specificity, its capability of reducing electron acceptors such as ubiquinone-1 and DCPIP and its sensitivity towards Ca^{2+} , EGTA and dicoumarol.

Materials and methods

Chemicals

Ubiquinone-1 was kindly given by both Hoffmann-La Roche, Basle, Switzerland, and Eisai Co., Koishikanafour, Tokyo, Japan. Trifluoperazine

Abbreviations used: ubiquinone-1, ubiquinone with a 5-carbon isoprenoid side chain; SHAM, salicylhydroxamic acid; Mops, 4-morpholinepropanesulphonic acid; DCPIP, 2,6-dichlorophenol-indophenol; SDS, sodium dodecyl sulphate; DCMU, dichloromethylurea; DBMIB, 2,5-dibromo-6-isopropyl-3-methyl-*p*-benzoquinone; QAE-, quaternary aminoethyl-

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was generously donated by Unilever Research, Sharnbrook, Bedford, U.K. Piericidin A and ox heart complex I were kindly given by Dr. C. I. Ragan, Department of Biochemistry, University of Southampton, Southampton, U.K. Tinopal was donated by Dr. D. B. Kell, University College of Wales, Aberystwyth, Wales, U.K. SHAM was obtained from Aldrich Chemical Co., and antimycin A from Boehringer-Mannheim. All other chemicals were obtained from Sigma.

Preparation of mitochondria

Mitochondria were prepared from *A. maculatum* spadices by the method of Moore & Proudlove (1983). If not used immediately, they were frozen in liquid N₂ and stored at -60°C until required. Mitochondria are referred to below as 'fresh' if used immediately and 'frozen and thawed' if used from -60°C storage. In the latter case, mitochondria were thawed slowly on ice before use.

Assays

NADH oxidase activity was measured polarographically with a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) in a closed 2 ml reaction vessel maintained at 22°C. The standard assay medium contained 2 mM-NADH, 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂ and 10 mM-Mops and was adjusted to pH 7.4 with KOH. The O₂ concentration of this medium, which was air-saturated, was assumed to be 240 μM. Spectrophotometric assays used the same medium for mitochondria, but, when assaying the solubilized enzyme, MgCl₂ and KCl were omitted. These assays were run at 22°C with 1 ml volume and a 1 cm light-path. NADH:ubiquinone-1 oxidoreductase activity, measured in the presence of 250 μM-NADH and 25 μM-ubiquinone-1, was monitored by the decrease in A₃₄₀ by using an absorption coefficient (ε) of 6300 litre·mol⁻¹·cm⁻¹. NADPH:ubiquinone-1 oxidoreductase activity was measured in the presence of 250 μM-NADPH instead of NADH. In the assay for NADH:DCPIP oxidoreductase activity, 40 μM-DCPIP was substituted for ubiquinone-1 and the reaction was monitored by the decrease in A₆₀₀, an absorption coefficient of 21 000 litre·mol⁻¹·cm⁻¹ being used.

NADH:cytochrome *c* oxidoreductase activity was measured in the presence of 50 μM-cytochrome *c*, 150 μM-NADH and 1 mM-SHAM. The antimycin-insensitive rate was obtained by comparing the rates before and after the addition of 2 μM-antimycin. Malate dehydrogenase activity was assayed as described by Ochoa (1955) in the presence of 250 μM-oxaloacetate, 150 μM-NADH, 1 mM-SHAM and 2 μM-antimycin. The flavin content of the purified enzyme was measured by the

method of Faeder & Siegel (1973), after acid extraction. FAD and FMN standards were prepared from commercial material by chromatography on Sephadex G-15. These were standardized by their A₄₅₀ by using absorption coefficients of 11 300 and 12 200 litre·mol⁻¹·cm⁻¹ for FAD and FMN respectively (Carletti & Siliprandi, 1953). The phospholipid content of fractions was measured as phosphate after HClO₄ digestion (Bartlett, 1969). For assays in which phospholipid was added, a purified soya-bean phospholipid fraction was used (Ragan & Racker, 1973). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, after trichloroacetic acid precipitation in the presence of deoxycholate. Desalting of samples before gel electrophoresis was carried out by centrifugation through a 0.5 ml-packed-volume Sephadex G-25 column.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis under dissociating conditions was carried out in the presence of SDS as described by Laemmli (1970). Gels were cast in 1 mm-thick slabs containing a 12.5–16% (w/v) polyacrylamide gradient. Proteins were detected by silver staining, the method of Eschenbruck & Burk (1982), involving a formaldehyde-fixative step, being used.

Results

Properties of external NADH dehydrogenase activity in fresh mitochondria

Arum maculatum mitochondria can support high rates of exogenous NADH oxidase activity (Table 1). The rate of NADH oxidation with the artificial electron acceptors ubiquinone-1 and DCPIP, in the presence of antimycin and SHAM to inhibit electron transport to oxygen, is even higher (Table 1). Ubiquinone-1 reductase activity was chosen to monitor the solubilization and subsequent purification of the dehydrogenase because it has a relatively low *K_m* (44 μM). DCPIP was not routinely used because, in addition to the high *K_m* (100 μM) for this acceptor, there is also a fast non-enzymic rate. However, the DCPIP assay was sometimes useful when using inhibitors with a large absorbance at shorter wavelengths. *Arum maculatum* mitochondria can also oxidize external NADPH at about 40% of the rate with NADH (Table 1). Interestingly, the NADPH:ubiquinone-1 oxidoreductase activity was only a tiny fraction (6%) of the equivalent activity towards NADH. This supports the notion, based on the differential effects of thiol-directed reagents, that NADH and NADPH are oxidized by different external dehydrogenases (Arron & Edwards, 1980).

Table 1. *Properties of external dehydrogenase activity in fresh mitochondria*

Assays were performed as described in the Materials and methods section. The concentration of NADH for the ubiquinone-1 and DCPIP assays was 250 μ M.

Substrate	Acceptor	Rate (μ mol/min per mg)	K_m (μ M)	$V_{max.}$ (μ mol/min per mg)
NADH	Oxygen	1.33	—	—
	Ubiquinone-1	10.9	44	11.1
	DCPIP	2.2	100	3.3
NADPH	Oxygen	0.47	—	—
	Ubiquinone-1	0.14	—	—

The effects of various inhibitors of NADH oxidase activity in plant mitochondria were examined in order to determine whether or not the ubiquinone-1 reductase activity corresponded to that of the external NADH dehydrogenase (Table 2). Piericidin A, which is an inhibitor of complex I, had no effect on either NADH oxidase activity or ubiquinone-1 reductase activity. The inhibition of exogenous NADH oxidase activity by Ca^{2+} chelators, such as EGTA and citrate, is well known (Palmer & Coleman, 1974). EGTA is equally efficacious in inhibiting the ubiquinone-1 reductase activity, suggesting that the same enzyme is responsible for both activities. This conclusion is supported by the observation that both activities have a comparable sensitivity to dicoumarol. A surprising difference between these activities is that EGTA inhibition of the ubiquinone-1 reductase activity is completely reversed by the re-addition of Ca^{2+} , whereas the oxidase activity is not. With other plant mitochondria, such as those of Jerusalem artichoke (*Helianthus tuberosus*) (Palmer & Coleman, 1974), mung bean (*Phaseolus aureus*) (Moore & Åkerman, 1982) and voodoo lily (*Saromatium guttatum*) (A. L. Moore & I. R. Cottingham, unpublished work), the inhibition of NADH oxidase activity by EGTA is completely reversed by the addition of excess Ca^{2+} .

Integrity of mitochondrial membranes and the effects of freezing and thawing

The proposal that NADH oxidase and NADH: ubiquinone-1 oxidoreductase activities reflect the operation of the external NADH dehydrogenase depends on the assumption that the mitochondrial inner membrane is intact and thus impermeable to NADH. In addition, it is important to establish that the outer membrane has been removed before solubilization of the dehydrogenase of interest, since this membrane possesses some NADH dehydrogenase activity of its own (Day & Wiskich, 1975). In view of the fact that freezing and thawing mitochondria is likely to disrupt both membranes, this treatment was selected as a convenient method to investigate the original membrane integrity, the contribution of

Table 2. *Modulators of external NADH dehydrogenase activity*

Assays were performed as described in Materials and methods section except that the effects of dicoumarol on the oxidase activity were measured at the lower-than-normal NADH concentration of 250 μ M. The ubiquinone-1 reductase assay mixtures contained about 1 μ g of mitochondrial protein, whereas 50 μ g was used for the oxidase assays.

Addition	Ubiquinone-1 relative rate	Oxidase relative rate
None	1.00	1.00
Piericidin A (50 nM)	0.98	1.01
EGTA (1 mM)	0.26	0.18
EGTA, then Ca^{2+} (3 mM)	0.97	0.25
Dicoumarol (10 μ M)	0.38	0.29

the internal NADH dehydrogenases to NADH: ubiquinone-1 oxidoreductase activity after membrane breakage and the removal of the outer membrane before attempting the purification of the external inner-membrane dehydrogenase.

The effect of freezing and thawing on both NADH oxidase activity and ubiquinone-1 reductase activity is shown in Table 3. The oxidase activity is decreased 80% by this treatment, whereas the ubiquinone-1 reductase activity is only 30% lower. However, both activities still have similar sensitivities to inhibition by EGTA and dicoumarol. This shows that the ubiquinone-1 reductase activity still measures the activity of the EGTA- and dicoumarol-sensitive external NADH dehydrogenase. It also suggests that the loss of oxidase activity reflects disruption of the membrane rather than damage to the dehydrogenase.

Antimycin-insensitive NADH:cytochrome *c* oxidoreductase activity was used as a marker for the mitochondrial outer membrane (Moreau & Lance, 1972; Douce *et al.*, 1973; Day & Wiskich, 1975). In fresh *Arum maculatum* mitochondria this activity (Table 3) was comparable with values reported in the literature for other species of plant mitochondria. Since this membrane is broken very easily, for instance by changes in osmolarity (Douce *et al.*, 1973), it is reasonable to predict

Table 3. *Comparison of the properties of fresh and frozen-thawed mitochondria*

Assays were performed as described in the Materials and methods section. The antimycin-insensitive NADH:cytochrome *c* oxidoreductase activity of frozen-thawed mitochondria was measured after phosphate washing as described under 'Purification of NADH dehydrogenase activity'. Mitochondria were pelleted for the malate dehydrogenase assays by centrifugation at 10000*g* for 10 min in a bench centrifuge.

Parameter	Fresh	Frozen-thawed
NADH oxidase activity ($\mu\text{mol}/\text{min}$ per mg)	1.33	0.31
NADH:ubiquinone-1 oxidoreductase activity ($\mu\text{mol}/\text{min}$ per mg)	10.93	7.5
Inhibition (%) of NADH:ubiquinone-1 oxidoreductase activity by 50 μM -dicoumarol at 250 μM -NADH	86	71
Inhibition (%) of NADH:ubiquinone-1 oxidoreductase activity by 1 mM-EGTA	82	81
Antimycin-insensitive NADH:cytochrome <i>c</i> oxidoreductase activity (nmol/min per mg)	130	<10
Malate dehydrogenase activity ($\mu\text{mol}/\text{min}$ per mg)		
Activity of mitochondrial suspension	2.5	27.6
Activity of supernatant after removal of mitochondrial by centrifugation	3.0	26.1
Activity of original mitochondrial suspension after addition of 0.1% (w/v) Triton X-100	28.0	26.1

membrane disruption on freezing and thawing. Thus when frozen-thawed mitochondria were phosphate-washed (as described under 'Purification of NADH dehydrogenase activity') the final pellet contained very low amounts of outer-membrane marker activity (Table 3). The most likely explanation for this, in view of its fragility, is that the outer membrane was broken by freezing and thawing and did not pellet with the mitochondrial fraction. However, we cannot eliminate the possibility that this treatment inactivates the enzymes that constitute this activity.

The integrity of the mitochondrial inner membrane can be assessed directly in terms of the latency of malate dehydrogenase activity (Douce *et al.*, 1973). Most of the malate dehydrogenase activity is located within the mitochondrial matrix and is thus only accessible to NADH, a substrate for malate dehydrogenase, after breakage of the inner membrane. The measurement of malate dehydrogenase activity in fresh mitochondria (Table 3) shows a relatively low activity that cannot be pelleted and is presumably due to broken mitochondria. However, the addition of detergent to a suspension of fresh mitochondria causes a 10-fold increase in activity due to the rupture of the inner membrane. This released activity was not pelleted with the mitochondrial fraction (results not shown). Freezing and thawing of mitochondria causes the complete loss of malate dehydrogenase latency, which indicates a large increase in inner-membrane permeability. The inner-membrane integrity of fresh mitochondria, assuming that all

malate dehydrogenase activity originates from the matrix, is about 90% intact. The total detergent-releasable malate dehydrogenase activity is comparable with values given in the literature for other plant mitochondria.

Purification of NADH dehydrogenase activity

Unless otherwise stated, all of the following operations were carried out at 4°C.

(a) *Phosphate-buffer wash.* The purpose of this stage is to remove soluble protein and outer-membrane fragments from the frozen-thawed mitochondrial fraction. Mitochondria were thawed rapidly at room temperature and then homogenized gently with 20 vol. of cold buffer, which contained 50 mM-potassium phosphate, pH 7.4. The washed mitochondrial fragments were pelleted by centrifugation at 10000*g* for 10 min. The supernatant was discarded and the pellet was resuspended in 20 ml of the same buffer and then re-pelleted. The final pellet was resuspended in 300 mM-mannitol/10 mM-Mops buffer, pH 7.4, to a protein concentration of 6 mg/ml. This treatment removed a considerable proportion (35%) of the total protein, with only a small loss of activity (13%) (Table 4).

(b) *Solubilization with deoxycholate.* NADH:ubiquinone-1 oxidoreductase activity was readily solubilized at quite a low detergent/protein ratio (Fig. 1). However, this ratio is critical, since activity is rapidly lost as it increases. Solubilization was routinely carried out at a detergent/protein ratio of 0.2 mg/mg. Deoxycholate, as a 10% (w/v)

Table 4. *Purification of NADH dehydrogenase*

The purification procedure is described in detail in the Results section. The rates measured were NADH:ubiquinone-1 oxidoreductase activities and 1 unit is a rate of 1 nmol/min. This Table shows values for a single purification with the range of specific activities from four different preparations shown in parentheses.

Fraction	Volume (ml)	Activity		[Protein]		Specific activity (units/mg of protein)	Purification (fold)
		(units/ml)	(% of initial)	(mg/ml)	(% of initial)		
Frozen-thawed mitochondria	1.25	272	100	30.9	100	8.8 (3.5–8.8)	1
Phosphate-buffer- washed mitochondria	3.21	179	87	7.8	65	11.8 (6.7–12.8)	1.3
Deoxycholate supernatant	2.8	61	50	1.3	9.3	46 (12–62)	5.4
Ion-exchange chromatography	2.5	30	22	0.15	1.0	200 (125–230)	22.0
Gel-filtration chromatography	2.5	1.8	1.3	0.04	0.2	45 (29–58)	5.1

Fig. 1. *Solubilization of NADH dehydrogenase activity with deoxycholate*

Solubilization of NADH dehydrogenase activity was carried out as described in the Results section. The supernatant fractions were assayed for ubiquinone-1 reductase activity (●) and protein (▲). The specific activity (■) was then calculated from these values. The original specific activity of the phosphate-washed frozen-thawed mitochondria was $7.5 \mu\text{mol/min per mg of protein}$.

Fig. 2. *Ion-exchange chromatography of the deoxycholate extract*

Chromatography was performed as follows. The deoxycholate fraction was applied directly to the column, which was then washed with 50 ml of buffer; the activity was eluted with a 200 ml linear KCl gradient from 0 to 150 mM, and 7.5 ml fractions were collected. These were assayed for ubiquinone-1 reductase activity (■) and protein (●). Fractions 24–29 were pooled and concentrated for the next purification step. The broken line shows the KCl gradient starting at zero on the left and rising to 150 mM on the right.

solution of the sodium salt, was added dropwise to a rapidly stirred suspension of phosphate-washed mitochondrial membranes. After 20 min the suspension was centrifuged at $100000g$ for 1 h to remove particulate material. The deoxycholate supernatant fraction was then carefully recovered from the loosely compacted pellet. Half of the total activity was solubilized by this stage with an approx. 5-fold purification.

(c) *Ion-exchange chromatography.* The deoxycholate supernatant fraction from the previous stage was adjusted to 0.1% (w/v) with respect to Triton X-100. It was then loaded on to a $10\text{ cm} \times 4\text{ cm}$ -diameter ion-exchange column (QAE-Sephadex, Pharmacia) that had been pre-equilibrated with 20 mM-Mops buffer, pH 7.4, containing 0.1% (w/v) Triton X-100. After loading of the sample was

completed, the column was washed with 50 ml of the same buffer. The dehydrogenase activity was eluted by using a 150 ml-total-volume linear KCl gradient from 0 to 150 mM. As Fig. 2 shows, a peak of activity was eluted at about 90 mM-KCl, and the fractions of high activity were pooled. This dilute solution was then concentrated by ultrafiltration over a $50000\text{-}M_r$ -cut-off cellulose filter (Amicon) under N_2 pressure. Although the specific activity and yield of activity were high at this stage, gel electrophoresis revealed that at least a dozen polypeptides were present (result not shown).

(d) *Gel-filtration chromatography.* In the final purification stage (Fig. 3) the concentrated frac-

Fig. 3. Gel-filtration chromatography of the ion-exchange purified fraction

Chromatography was performed as described in the Results section. Fractions (7.5 ml) were collected and assayed for ubiquinone-1 reductase activity (■) and protein (●). Fractions 34–37 were pooled, concentrated, and frozen for storage.

tion from the previous step was loaded on to a 100 cm × 1.5 cm-diameter gel-filtration column (Fractogel TSK HW-55, Merck) that had been pre-equilibrated with a buffer containing 20 mM-Mops, 50 mM-KCl and 0.1% (w/v) Triton X-100, pH 7.4. The fractions of high activity were pooled, concentrated by ultrafiltration, frozen in liquid N₂ and stored at -60°C until required. Under these conditions the enzyme activity was stable for at least 1 year. This final step in the procedure gave a 5-fold protein purification (Fig. 3), although the yield of activity is very low (1.3%) (Table 4).

Polypeptide composition of the purified dehydrogenase

The purified dehydrogenase was subjected to polyacrylamide-gel electrophoresis under dissociating conditions. When silver-stained for protein, three polypeptides were visible (Fig. 4). The two major bands have M_r values of approx. 78 000 and 65 000 as determined from a comparison of their R_F values with those of the protein standards, and the minor one of about 76 000. There is also a small amount of lower- M_r contamination. Without further studies it cannot be determined which of these, or which combination, constitute the dehydrogenase.

Catalytic properties of the purified dehydrogenase and the effects of inhibitors

Table 4 shows the activities and yields obtained at different stages of a single purification and the range of specific activities obtained with different purifications. The yield of activity at each stage is good until the last step, where 90% of the activity applied to the gel-filtration column is not recovered. However, as judged from a comparison of gel-electrophoresis profiles of the products from

Fig. 4. SDS/polyacrylamide-gel electrophoresis of the purified NADH dehydrogenase

This was performed as described in the Materials and methods section. The enzyme sample (1 μg of protein) was run adjacent to two M_r -marker tracks, one containing purified complex I from ox heart mitochondria and the other containing the commercial M_r markers [bovine albumin (66 000), egg albumin (45 000) and pepsin (37 700) obtained from Sigma]. The 75 000- M_r marker is the large iron-protein subunit from complex I (Ragan, 1980).

the last two stages (not shown), the protein purification was good.

The purified dehydrogenase has a K_m value for NADH, using ubiquinone-1 as the acceptor, of 28 μM. This is close to the value of 27 μM reported for a rotenone-insensitive NADH dehydrogenase that has been partially purified from *A. maculatum* mitochondria (Cook & Cammack, 1983). It is also within the range 19–31 μM reported for the external NADH dehydrogenase of intact mitochondria (Møller & Palmer, 1981). The reason for variation in the latter K_m value is because of changes in the ionic composition of the medium. The K_m value for ubiquinone-1 of the purified enzyme was measured as 18 μM, which is considerably less than the value of 44 μM found with intact mitochondria (Table 1). On the other hand, the K_m value for DCPIP of the purified enzyme was much higher (150 μM compared with 100 μM) than that for the membrane-bound one. The reasons for these variations are at present unclear. Ferricyanide did not act as an efficient acceptor for the purified enzyme.

In addition to the K_m value for NADH, there are two other properties of the dehydrogenase that remained unchanged after solubilization. The first of these was the optimal pH range, which for the purified enzyme was 7.0–7.5 (Fig. 5), and is very close to that of 7.1–7.2 reported for the membrane-bound enzyme (Møller & Palmer, 1981). The other unchanged property is the high sensitivity of the solubilized dehydrogenase to dicoumarol. The NADH:ubiquinone-1 oxidoreductase activity of the purified dehydrogenase was inhibited 50% by

Fig. 5. Activity of NADH dehydrogenase as a function of pH

The ubiquinone-1 reductase activity of the purified dehydrogenase (0.2 μg) was measured in the standard assay at various pH values.

9 μM-dicoumarol. This is slightly higher than for the membrane-bound enzyme, where an inhibitor concentration of 5 μM was required to give the same degree of inhibition. A Dixon plot of dicoumarol inhibition of the purified dehydrogenase at two different NADH concentrations (results not shown) showed that inhibition was competitive, with a K_i of 8 μM. The purified dehydrogenase was not capable of supporting NADPH:ubiquinone-1 oxidoreductase activity, which is not surprising in view of the very low rate measured in intact mitochondria (Table 1). One dramatic change brought about by solubilization is that ubiquinone-1 reductase is no longer inhibited by EGTA or stimulated by Ca^{2+} . This loss of Ca^{2+} -dependence occurs concomitantly with solubilization, since it could not even be detected with freshly solubilized dehydrogenase (A. L. Moore & I. R. Cottingham, unpublished work). This finding is in agreement with the two other reports of solubilized NADH dehydrogenase activity, both of which indicate insensitivity of the enzyme to EGTA inhibition (Klein *et al.*, 1983; Cook & Cammack, 1983).

Apart from dicoumarol, no other inhibitors of NADH:ubiquinone-1 oxidoreductase activity were found to have an effect on the purified dehydrogenase. Table 5 summarizes the effect of various inhibitors on purified dehydrogenase activity.

Flavin content of the purified dehydrogenase

Flavin analysis of the purified enzyme from two different preparations gave an FAD content of 2.24 nmol/mg of protein and an FMN content of 0.63 nmol/mg of protein. Flavin determinations were not performed on every batch of purified

Table 5. Effects of inhibitors on NADH:ubiquinone-1 oxidoreductase activity of the purified enzyme

Assays were performed as described in the Materials and methods section. Inhibitors were added in a 10 μl maximum volume of ethanol. The amount of purified dehydrogenase used in each assay was approx. 0.2 μg. The degree of inhibition is calculated as: 100 – (rate plus inhibitor/rate minus inhibitor) as a percentage.

Inhibitor	Concn. in assay	Inhibition (%)
Dicoumarol	50 μM	90
EGTA	1 mM	<5
Piericidin A	50 μM	<5
Trifluoperazine*	75 μM	<5
Tinopal*	125 μM	<5
SHAM*	1 mM	10
Atrazine	70 μM	<5
DCMU	70 μM	<5
DBMIB	250 μM	27

* Measured on NADH:DCPIP oxidoreductase activity. In all other assays ubiquinone-1 was used as electron acceptor.

enzyme because of the very low yields. It is for this reason also that no non-haem-iron assays were carried out. The presence of a flavin in the partially purified enzyme was confirmed from a visible-wavelength spectrum of the ion-exchange-chromatography fraction. Initially the effect of NADH on the absolute spectrum was examined and no change was observed (I. R. Cottingham, unpublished work). This was thought to be caused by the flavin already being reduced in the isolated enzyme. This idea was confirmed by running a NADH-reduced-minus-ferricyanide-oxidized difference spectrum (Fig. 6). The single, fairly regularly shaped, absorbance peak at 420 nm is quite probably due to the presence of a protein-bound flavin. There was no cytochrome contamination at this stage because the spectrum was unchanged by the addition of dithionite to the NADH-containing cuvette (results not shown).

Possible role of phospholipids and Ca^{2+}

The phospholipid content of the ion-exchange-chromatography fraction, as determined by phosphate analysis, was less than 1 μmol of protein. This low value might reasonably be expected to decrease during the subsequent gel-filtration chromatography, especially in the presence of Triton X-100. The possibility that the purified dehydrogenase was phospholipid-deficient was confirmed by experiments in which mixed soya-bean phospholipids were added (Table 6). The direct addition of phospholipids to the assay had no effect. However, when the enzyme was preincu-

Fig. 6. Visible absorption difference spectra of NADH dehydrogenase

The visible spectrum of the concentrated ion-exchange fraction, at a protein concentration of 1.5 mg/ml, was measured on a Cary 114 spectrophotometer. NADH and ferricyanide were added to final concentrations of 10 μ /ml where appropriate. The two traces, which have been corrected for baseline drift are: (a) no addition to either cuvette (this is actually reduced-minus-reduced); and (b) NADH-reduced-minus-ferricyanide-oxidized.

Table 6. Effect of phospholipid and Ca^{2+} on NADH: ubiquinone-1 oxidoreductase activity of the purified dehydrogenase

The amount of purified dehydrogenase protein in each assay was 0.2 μ g. Phospholipid (7.5 nmol) was added directly to the assay as an aqueous dispersion obtained by sonication. The concentration of Ca^{2+} in the assay was 1 mM. Preincubations were performed on ice as follows. Dehydrogenase protein (2 μ g), phospholipid (10.5 nmol) and cholate (1 mg as a solution neutralized with KOH) were mixed in a final volume of 100 μ l, left on ice for 20 min, then a sample (containing 0.2 μ g of protein) was added to the assay. The preincubation with Ca^{2+} was performed in an identical manner, except that 0.1 mM- Ca^{2+} was present. The addition of 1 mM- Ca^{2+} to this assay mixture did not further stimulate the rate.

Addition	Relative rate
None	1
Phospholipid	1.12
Phospholipid and cholate with 20 min preincubation before dilution into the assay mixture	1.60
Ca^{2+} alone	0.80
Ca^{2+} plus phospholipid	1.50
Ca^{2+} , phospholipid and cholate with preincubation	<0.1

bated with a concentrated cholate dispersion of phospholipids for 20 min on ice, the rate was stimulated. There is no evidence from these studies that Ca^{2+} stimulation is phospholipid-dependent

since, whenever calcium was added, it was found to be inhibitory (Table 6).

Discussion

This is the first report of the partial purification and characterization of the external NADH dehydrogenase from plant mitochondria. The identification of this enzyme is complicated by the fact that the exact number of membrane-bound NADH dehydrogenases has yet to be established. In addition to an outer-membrane dehydrogenase and the external NADH dehydrogenase on the inner membrane, plant mitochondria also possess at least one NADH dehydrogenase on the inner face of the inner membrane. The oxidation of internally generated NADH is insensitive to rotenone and piericidin A, which are classical inhibitors of the mammalian mitochondrial NADH dehydrogenase, complex I. However, in the presence of either inhibitor, the P/O ratio decreases from 3 to 2, which shows either a bypass of the inhibitor block or the presence of another dehydrogenase, presumably with a higher K_m for NADH. Despite this uncertainty, there is considerable evidence that our purified enzyme is the external NADH dehydrogenase. This is summarized as follows.

(1) In the tissue selected, the activity of the external NADH dehydrogenase is at least an order of magnitude greater than that of any other NADH dehydrogenase.

(2) The freezing-thawing and phosphate-buffer washing of mitochondria removes the outer membrane, as shown by the loss of the outer-membrane marker and the release of the matrix contents before solubilization of activity.

(3) The NADH:ubiquinone-1 oxidoreductase activity of intact mitochondria does not increase when the inner membrane is ruptured, showing that the contribution of the internal dehydrogenases to this activity is negligible. This is confirmed by the EGTA-sensitivity, which is high in both intact and broken mitochondria.

(4) The K_m of the purified dehydrogenase for NADH is the same as that reported for the external NADH dehydrogenase in intact mitochondria.

(5) The pH optimum is similar to that reported for the external dehydrogenase.

(6) The dicoumarol-sensitivity of NADH: ubiquinone-1 oxidoreductase activity is comparable for intact or broken mitochondria and the purified enzyme.

The design of a successful protocol for the purification of this enzyme has not been easy because, after a certain degree of purity has been obtained, i.e. after the ion-exchange-chromatography step, the enzyme quickly disintegrates.

This could be due to many factors, but some possibilities are: loss of tightly bound phospholipids with a concomitant unfavourable structural change; loss of a subunit; or loss of flavin. The stimulatory effect of phospholipid supports the first suggestion, although this may not be the whole story, since the minimum polypeptide composition of the enzyme has yet to be established. The possibility of flavin loss was checked by incubating the purified dehydrogenase with excess FAD. No stimulation of activity was detected (I. R. Cottingham, unpublished work), but this does not discount the possibility that flavin loss during purification was associated with an irreversible conformational change.

The purified enzyme contains only three polypeptides, as shown by the highly sensitive silver staining of polyacrylamide gels, and non-covalently bound flavin, a high proportion of which is FAD. The difference spectrum is typical of a flavoprotein rather than that of a ferroflavoprotein, but sufficient quantities of purified enzyme for conventional non-haem-iron analysis were not available. Although it is quite possible that an iron-sulphur centre has been lost during the purification, there is no reason why the dehydrogenase could not simply comprise a single polypeptide flavoprotein. This is not a novel suggestion, because glycerol 3-phosphate dehydrogenase from pig brain mitochondria has an M_r of 75000, contains non-covalently bound FAD and very low amounts of non-haem iron (Cottingham & Ragan, 1980). However, even here it was not possible to rule out the involvement of an iron-sulphur centre that was lost during the purification. If the external NADH dehydrogenase is lacking an iron-sulphur centre, its mechanism would be of considerable interest.

The mechanism of Ca^{2+} stimulation cannot be studied with the purified enzyme because it is lost on solubilization. This could be due to the loss of a subunit, a change in the protein's phospholipid environment, or a conformational change. Although phospholipid stimulation of the enzyme was shown, this did not involve Ca^{2+} . The possible participation of calmodulin is unlikely, since Ca^{2+} -calmodulin antagonists do not inhibit NADH oxidase activity in mitochondria (Dunn *et al.*, 1984). In addition, ox brain calmodulin failed to cause any stimulation when added with phospholipids and Ca^{2+} (I. R. Cottingham & A. L. Moore, unpublished work).

The determination of the minimum polypeptide composition of this enzyme and the elucidation of

the mechanism of calcium activation will only be possible if the final purification step can be considerably improved. This possibility requires further investigation.

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