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Purification and Preliminary Characterization of Mitochondrial Complex I (NADH:Ubiquinone Reductase) from Broad Bean (*Vicia faba* L.)[1]

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NADH:ubiquinone reductase (EC 1.6.19.3), or complex I, was isolated from broad bean (*Vicia faba* L.) mitochondria. Osmotic shock and sequential treatment with 0.2% (v/v) Triton X-100 and 0.5% (w/v) [3-cholamidopropyl)dimethylammonio]-1-propanesulfate (CHAPS) removed all other NADH dehydrogenase activities. Complex I was solubilized in the presence of 4% Triton X-100 and then purified by sucrose-gradient centrifugation in the presence of the same detergent. The second purification step was hydroxylapatite chromatography. Substitution of CHAPS for Triton X-100 helped remove contaminants such as ATPase. The high molecular mass complex is composed of at least 26 subunits with molecular masses ranging from 6000 to 75,000 kD. The purified complex I reduced ferricyanide and ubiquinone analogs but not cytochrome c. NADPH could not substitute for NADH as an electron donor. The K_M for NADH was 20 μM at the optimum pH of 8.0. The NH_2-terminal sequence of several subunits was determined, revealing the ambiguous nature of the 42-kD subunit.

Plant mitochondria have complex metabolic pathways for oxidizing both exogenous and endogenous NADH. Whereas mammalian mitochondria contain only two oxidation systems—one in the outer membrane and complex I in the inner membrane—plant mitochondria possess at least two and maybe three additional NAD(P)H dehydrogenases (for reviews, see Douce and Neuburger, 1989; Weiss et al., 1991). These multiple enzyme activities have been widely studied in purified mitochondria and their isolated membranes, but their location, subunit composition, and role in mitochondrial respiration remain unclear. Their purification to homogeneity should bring progress in this direction and make it possible to develop molecular tools such as antibody production and gene cloning. Recent investigations have been carried out on various plant tissues, but the essential focus of these studies was the external NADH dehydrogenases and not the matrix-facing enzymes of the inner membrane (Cook and Cammack, 1984; Chauveau and Lance, 1991).

Complex I, or mitochondrial NADH-ubiquinone reductase (EC 1.6.99.3), is the first of the three proton-translocating complexes of the mitochondrial respiratory chain. It was initially purified from mammalian mitochondria and more recently from *Neurospora crassa* (Hatefi et al., 1962). This complex, composed of 35 different subunits ranging from 5 to 75 kD, catalyzes electron transfer from endogenous NADH to ubiquinone by a large number of redox groups (one flavin mononucleotide and six iron-sulfur clusters) whose operational sequence is still unknown (Walker et al., 1992). Complex I can be fractionated with chaotropic agents into a flavoprotein, an iron-sulfur protein, and a hydrophobic protein moiety (for a review, see Møller and Palmer, 1982).

Very little has been reported to date about the isolation and polypeptide composition of plant mitochondrial complex I. Cottingham and Moore (1988) purified a complex I from mung bean mitochondria by band excision of a nondenaturing polyacrylamide gel, but only five major polypeptides were recovered. Because these were comparable in size to several subunits of beef heart complex I (75, 46, 39, 33, and 27 kD), this suggests that part of the complex was split off during electrophoresis (Cottingham and Moore, 1988). In immunological analyses, on the other hand, antisera raised against beef heart complex I subunits cross-reacted with four polypeptides of solubilized submitochondrial particles from mung bean (Cottingham et al., 1986). Recently, a complex I form was partly purified from beetroot mitochondria (Soole et al., 1992). However, the isolated complex contained only 14 major polypeptides and lacked the approximately 75- kD polypeptide typically found in complex I from other organisms.

We report here the purification and primary characterization of complex I of the respiratory chain of broad bean mitochondria. The solubilized high molecular mass complex contains 26 subunits including the expected approximately 75-kD polypeptide and shows remarkable similarity to the mammalian enzyme.

MATERIALS AND METHODS

Materials

Broad beans (*Vicia faba* L.) were obtained from the Station d'Amélioration des Plantes, Gembloux. Detergents were purchased from Boehringer Mannheim Biochemicals. Coenzyme

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Abbreviation: CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfate.

Q analogs and rotenone were obtained from Sigma. Hydroxylapatite Bio-Gel HT was from Bio-Rad, and bicinchoninic acid was from Pierce.

Preparation of Mitochondria

Mitochondria were prepared from 10-d-old etiolated seedlings of broad bean and purified by a procedure described previously (Boutry and Briquet, 1982). The yield was around 35 mg of mitochondrial protein per kg of fresh material. Unless used for oxygen uptake measurements, the mitochondria were frozen in liquid nitrogen and kept at −70°C until use.

Purification of Complex I

All of the purification steps were carried out at 4°C. Mitochondrial membranes were prepared by diluting thawed mitochondria (typically 200 mg) with TE buffer (20 mM Tris-HCl, 5 mM EDTA [pH 7.5]) to a final protein concentration of 1 mg mL^{-1}. The suspension was homogenized with a Piston homogenizer and centrifuged for 20 min at 35,000 rpm in a Sorvall A-641 rotor. The pellet was washed in the same buffer and centrifuged under the same conditions. Supernatants were discarded, and the membrane pellet was stripped with Triton X-100 in TE buffer. In this mixture, the detergent's final concentration was 0.2% (v/v) and the detergent:protein ratio was 0.2. The mixture was stirred for 15 min and then centrifuged for 20 min at 35,000 rpm in the Beckman Ti70 rotor. The supernatant was discarded and the membranes were stripped again, this time with CHAPS (final concentration, 0.5% [w/v] in TE; detergent:protein ratio, 1).

Complex I was solubilized from the stripped membranes by stirring for 30 min on ice in buffer (20 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl [pH 7.5]) containing 5% (w/v) Suc and 4% (v/v) Triton X-100 at a detergent:protein ratio of 5. The yellow supernatant obtained after a 30-min centrifugation at 35,000 rpm in a Beckman Ti70 was layered onto a 70-mL 10 to 35% (w/v) Suc gradient in buffer (20 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl [pH 7.5]) supplemented with 0.2% (v/v) Triton X-100. Approximately 20 mg of protein (3.5 mg mL^{-1}) was loaded per gradient. The samples were then centrifuged for 13 h at 35,000 rpm in a Sorvall AH-641 angular rotor at 2°C. Fractions containing the highest NADH dehydrogenase activity were pooled, concentrated 5- to 10-fold in an Amicon Diaflo apparatus equipped with a YM-10 filter, and finally diluted twice with the hydroxylapatite chromatography washing buffer (20 mM Mops, 5% [w/v] Suc, 0.5% [w/v] CHAPS [pH 7.2]).

The sample was loaded onto a hydroxylapatite column (1.6 × 7 cm) at a flow rate of 18 mL h^{-1}. The column was washed with 5 volumes of washing buffer, after which a 10-volume linear gradient (0–400 mM sodium phosphate) in the same buffer was run. NADH dehydrogenase activity was eluted between 150 and 300 mM phosphate. Fractions containing at least half the activity of the peak fraction were concentrated and dialyzed against the washing buffer, using the same filter as above. Thereafter, complex I was frozen in liquid nitrogen and stored at −70°C until use.

NADH:Acceptor Oxidoreductase Activity Assay

The standard assay medium for NAD(P)H:acceptor oxidoreductase activity contained 20 mM Tris-HCl (pH 8.0), 100 μM NADH or NADPH or deamino-NADH, 0.05% (v/v) Triton X-100, and, when mitochondria or membranes were used in the assay, 1 mM KCN and 1 mM salicylhydroxamic acid. Reduction of quinones (ubiquinone-0, duroquinone, menadione, ubiquinone-10, and ubiquinone-30; 100 μM) was monitored by recording the measured NADH oxidation at 340 nm. Reduction of ferricyanide (1 mM) and of Cyt *c* (50 μM) was monitored at 420 and 550 nm, respectively. The following extinction coefficients were used: $\epsilon_{340} = 6.22$ mM^{-1} cm^{-1}; $\epsilon_{420} = 1.03$ mM^{-1} cm^{-1}; $\epsilon_{550} = 21.0$ mM^{-1} cm^{-1}. In all experiments, measurement began with the addition of protein (1–10 μg), and results were corrected for the chemical reduction of acceptors.

Gel Electrophoresis and Electroblotting

Proteins were precipitated by the chloroform-methanol method (Wessel and Flügge, 1984) or, for large volumes, by the TCA method (Bensadoun and Weinstein, 1976). They were then separated by SDS-PAGE according to the procedure of Laemmli (1970) on a 13 to 17% polyacrylamide gradient gel and stained with Coomassie blue. Proteins to be sequenced were electroblotted as described by Matsudeira (1987) on a polyvinylidene difluoride-type membrane (Problot, Applied Biosystems).

NH_2-Terminal Microsequencing of Proteins

Amino acid sequencing of electroblotted proteins was carried out in a pulsed-liquid phase sequencer (model 477A, Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid derivative analyzer (model 120A).

Determination of Proteins

Proteins were determined by the bicinchoninic acid method (Smith et al., 1985) using BSA as a standard. Membrane proteins were first solubilized in 100 μL of 0.1% (w/v) SDS.

RESULTS

Preparation of Stripped Mitochondrial Membranes

As a starting material, we chose etiolated shoots of broad bean (*V. faba* L.). We have previously shown that mitochondria centrifuged on a Suc gradient yield a highly purified fraction characterized by good respiratory control and phosphate:oxygen ratio and proficiency in in vitro translation (Boutry and Briquet, 1982).

Preparation of mitochondrial membranes before solubilization of complex I involved three sequential steps. First, the thawed mitochondria were subjected to osmotic shock. One-third of the protein and 16% of the original NADH:ferricyanide oxidoreductase activity were discarded with the soluble fraction. The discarded fraction probably contained soluble NADH dehydrogenases similar to the enzymes purified from *Arum maculatum* and *Beta vulgaris* mitochondria (Chauveau and Lance, 1991; Luethy et al., 1991).

Complex I from other organisms is very hydrophobic, and its solubilization requires high concentrations of detergent. Therefore, we introduced a stripping step to remove dehydrogenases and other proteins loosely bound to the membranes. The membrane pellet was washed with 0.1% Triton X-100 at a low detergent:protein ratio (0.2), followed by 0.5% (w/v) CHAPS at a detergent:protein ratio of 1. This double stripping removed half of the protein and 40% of the NADH:ferricyanide reductase activity present in the membrane fraction. CHAPS stripping was introduced to minimize the frequently encountered contamination of complex I preparations by the α, β, and probably other subunits of the mitochondrial ATP synthase complex (Heron et al., 1979). McEnery et al. (1984) used CHAPS under these same conditions to solubilize ATP synthase from rat liver mitochondria, and preliminary experiments in this laboratory showed this detergent to extract at least 70% of the ATP synthase complex from broad bean mitochondria. The NADH dehydrogenase activity released by the double stripping was mainly due to CHAPS solubilization (Table I). This activity was analyzed together with that of the purified complex I (see below).

Solubilization of Complex I

The next step was solubilization of complex I. At this stage of purification, the number of remaining dehydrogenases was unknown. Therefore, the detergent was chosen to favor the complete extraction of activity rather than the highest specific activity (Fig. 1). Solubilization was highest with Triton X-100, Genapol, and Thesit (the latter is not shown in Fig. 1). These all belong to the same PEG ether family of detergents. With deoxycholate, used to solubilize the mammalian complex I, extraction of the plant enzyme was poor. As shown in Figure 1, the best choice appeared to be Triton X-100 at a detergent:protein ratio of 5. The working concentration was set at 4% (w/v), and the protein concentration was kept at 7 to 9 mg mL^{-1} to minimize the loading volume on the Suc gradient in the subsequent step. Under these conditions, more than 75% of the activity of the stripped membranes was solubilized in a typical experiment.

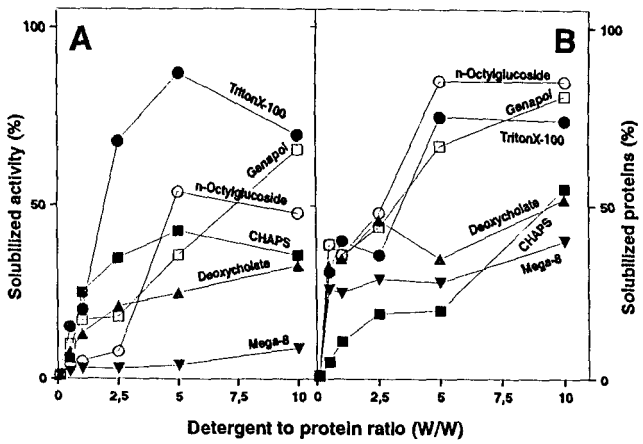

Figure 1. Solubilization of complex I by different detergents. Aliquots (1 mg) of stripped membranes obtained as described in "Materials and Methods" were suspended in TE buffer (100 μL) containing 5% (w/v) Suc and detergents at indicated concentrations. After 15 min on ice, samples were centrifuged in an Airfuge (Beckman) centrifuge at maximum speed for 30 min. In the supernatant and pellets, ferricyanide reductase activity (A) and proteins (B) were assayed as described in "Materials and Methods." ●, Triton X-100; □, Genapol; ○, *n*-octylglucoside; ■, CHAPS; ▲, sodium deoxycholate; ▼, Mega-8 (octanoyl-*N*-methylglucamide). Values were normalized to the total activity (supernatant + pellet).

Table I. *Recovery of protein and activity during purification of complex I from broad bean mitochondria*

Purification Step	Protein	NADH:Ferricyanide Reductase Activity			Purification Factor
		Total[a]	Yield	Specific[b]	
	mg		%		-fold
Mitochondria	168	1710	100	10.2	1.0
Matrix	50	280			
Membranes	110	1400	82	12.7	1.2
Triton X-100 (0.2%) extract	22	105			
CHAPS extract	38	520			
Stripped membranes	50	660	38	13.2	1.3
Triton X-100 (4%) extract	41	672	39	16.4	1.5
Suc gradient	6.5	311	18	47.8	4.5
Hydroxylapatite[c]	1.2	125	7	104	10.2

[a] Total activity is expressed as μmol of ferricyanide reduced min^{-1}. [b] Specific activity is expressed as μmol of ferricyanide reduced min^{-1} mg^{-1}. [c] Average values for pool 1 and pool 2 (see Fig. 3) are presented.

Suc-Gradient Centrifugation

After solubilization, complex I was further purified by Suc-gradient centrifugation in the presence of 0.1% Triton X-100. The high partial specific volume of the protein-Triton X-100 complexes implies a low sedimentation coefficient and a high Stoke's radius (Helenius and Simons, 1975). Therefore, an angular rotor and a high speed were required to isolate this complex, whose expected molecular mass is greater than 600,000 kD, whereas many Suc-gradient separations are typically carried out in a swinging-bucket rotor at a lower speed.

A typical profile is shown in Figure 2A. Ninety percent of the NADH dehydrogenase activity was concentrated in a relatively sharp peak in the lower third of the gradient. Very little activity was detected in the upper part of the gradient, which accounted for more than 80% of the loaded protein. NaCl (150 mM) was added to the Suc gradient, preliminary experiments having shown that the activity peak was more symmetrical in the presence of salt. Even so, a slight shoulder was often observed. Polypeptide compositions (Fig. 2B) were similar on the right and left sides of the peak. The dissymmetry could thus appear to reflect a variable lipid composition, because the detergent concentration was not sufficient to ensure complete removal of lipids from the complex.

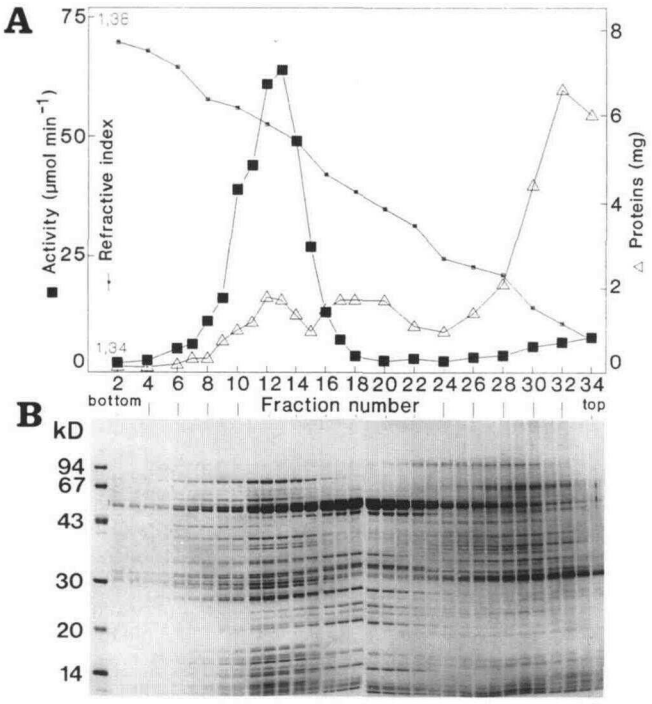

Figure 2. Suc-gradient analysis of solubilized complex I. A, Thirty milligrams of solubilized proteins in 4% Triton X-100 were layered onto a 70-mL Suc gradient (10–35%, w/v), run, and analyzed as described in "Materials and Methods." ■, NADH:ferricyanide reductase activity (µmol min^{-1}); △, protein (mg); ⊡, refractive index. B, SDS-polyacrylamide gel analysis of proteins separated on a Suc gradient. Proteins (100 µg) from each fraction were precipitated by the chloroform-methanol method, electrophoresed on a 12 to 16% polyacrylamide gel, and stained with Coomassie blue as described in "Materials and Methods." Fractions 9 to 16 were pooled and concentrated for the next purification step.

Chromatography on Hydroxylapatite

Anion exchange chromatography was attempted (not shown) according to the procedure used to purify the NADH:ubiquinone reductase of *N. crassa* (Ise et al., 1985). However, hydroxylapatite was found to more effectively remove contaminants such as the ATP synthase complex (see below). Chromatography of hydrophobic proteins on hydroxylapatite requires a relatively high detergent concentration (>0.5%) to separate effectively the various proteins (Riccio, 1983). However, elution of complex I from hydroxylapatite in the presence of 0.5% Triton X-100 and phosphate led to the loss of most of the activity, probably caused by enzyme dissociation: indeed, the elution profile exhibits a separation of the 37- and 42-kD subunits typically found in complex I (not shown). This problem was not encountered when chromatography was carried out in the presence of 0.5% CHAPS (Fig. 3A). When this was done, the peak eluted at low salt concentration was found to contain four major polypeptides (75, 65, 52, and 51 kD) exhibiting electrophoretic mobilities comparable to those of known complex I subunits (results not shown). Their NH_2-terminal amino acid sequence was determined (Fig. 4A). The 75-kD polypeptide did not share any homology with the bovine 75-kD subunit (Runswick et al., 1989) but did with a carboxylase enzyme (Browner et al., 1989). The 65-kD polypeptide was similar to a plant mitochondrial HSP60 protein (Prasad and Stewart, 1992). The 52- and 51-kD polypeptides probably correspond to the α and β subunits of the mitochondrial ATPase (Boutry and Chua, 1985; Chaumont et al., 1988). The NADH:ferricyanide activity was eluted by higher phosphate concentrations. The corresponding peak was asymmetric and overlapped with two protein peaks (Fig. 3A). The fractions of both peaks were separately pooled and electrophoretically

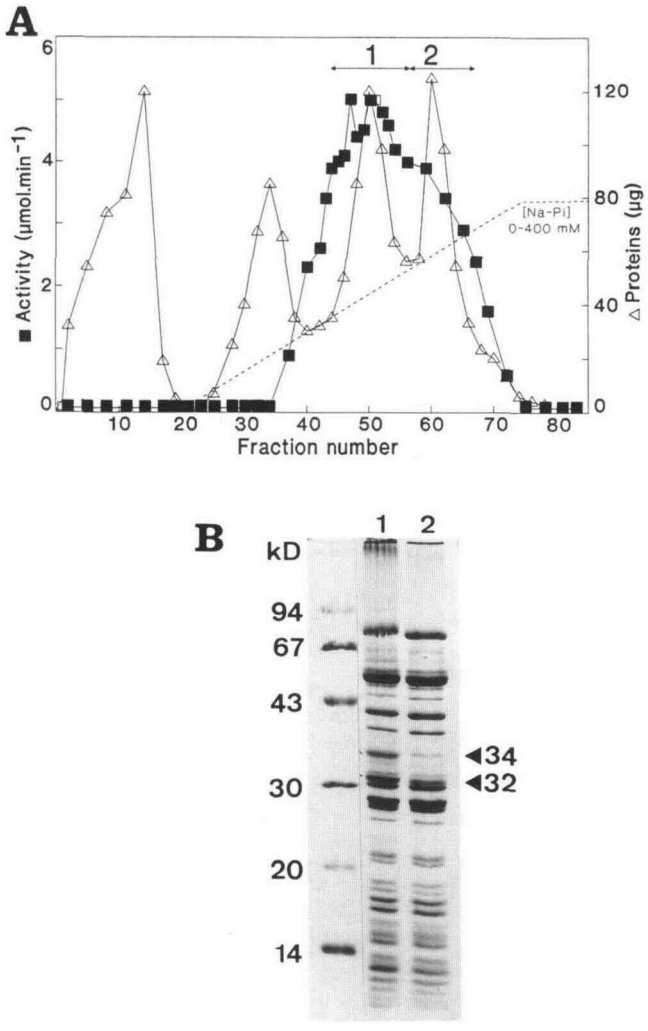

Figure 3. Chromatography of complex I on hydroxylapatite. A, The pooled fractions from the Suc gradient were loaded onto a 1.6- × 7-cm hydroxylapatite column and eluted in the presence of CHAPS as described in "Materials and Methods." ■, NADH:ferricyanide reductase activity; △, protein. Arrows indicate the pooled fractions. B, SDS-polyacrylamide gel analysis of proteins from pool 1 (fractions 42–53) and pool 2 (fractions 54–66). Proteins (60 µg) from both fractions were precipitated by TCA, electrophoresed on a 13 to 17% polyacrylamide gel, and stained with Coomassie blue as described in "Materials and Methods." Arrows indicate the 34- and 32-kD subunits, the intensity of which differs between both pools.

analyzed (Fig. 3B). The two preparations were found to differ only by the intensity of two polypeptide bands (34 and 32 kD).

The results from each purification step are summarized in Table I. The final yield from 160 mg of mitochondrial protein was 1.2 mg of NADH dehydrogenase. The enrichment factor (10.2) has little significance, because several enzymes other than complex I contribute to the total NADH:ferricyanide reductase activity in the whole mitochondrial fraction. Furthermore, complex I may lose some activity during purification, a phenomenon observed by Ise et al. (1985) for the *Neurospora* enzyme and probably due to slow and progressive depletion of lipids from the enzyme complex.

Subunit Composition of Complex I

Electrophoretic analysis of complex I (Fig. 3B) revealed 26 distinct subunits. This accords with observations of the mammalian (Heron et al., 1979) and *Neurospora* (Ise et al., 1985) enzymes. The preparation is totally devoid of polypeptides larger than 75 kD and thus of transhydrogenase polypeptide (90 kD) (Carlenor et al., 1988), a frequent contaminant of complex I preparations (Chen and Guillory, 1981). The staining ratio between subunits is not constant: it has been shown for other organisms that subunit stoichiometry departs from 1.0 (Ragan, 1987). Furthermore, some subunits are expected to be hydrophobic and thus possibly weakly stained. Nevertheless, minor bands may be interpreted as contaminants or proteolytic by-products of complex I subunits. By analogy with the *N. crassa* enzyme, for instance, the 46-kD polypeptide could be a proteolytic fragment of the 49-kD subunit. During purification, Preis et al. (1990) observed a similar 46-kD band reacting with an anti-49-kD-subunit antibody and disappearing when Lubrol PX was used instead of Triton X-100.

We have undertaken to sequence systematically the NH_2-terminal regions of each polypeptide isolated from purified complex I. Many of them were blocked and thus gave no information. The partial sequences obtained for the others (Fig. 4B) should enable us to design polynucleotide probes for gene cloning. Because the NH_2-terminal sequence is seldom conserved between proteins of various organisms, the sequences obtained so far are not of much help in subunit identification. However, the sequence obtained for the 42-kD subunit was found to be an exception (Fig. 4C): a stretch of 16 amino acid residues is identical with a sequence of the 49-kD subunit of the bovine mitochondrial complex I. Moreover, the 42-kD subunit is quite homologous (17 identical residues of 25) to the putative product of the mitochondrial NAD 7 pseudogene from *Marchantia polymorpha* (Oda et al., 1992) and also to open reading frames 392 and 393 of the *M. polymorpha* and *Nicotiana tabacum* chloroplast genomes, respectively (Fearnley et al., 1989).

Enzymic Activities of the Purified Complex I

The reduction of ferricyanide by NADH is the most widely used assay to follow NADH dehydrogenase purification because it is barely affected by aging or by slight damage to the enzyme.

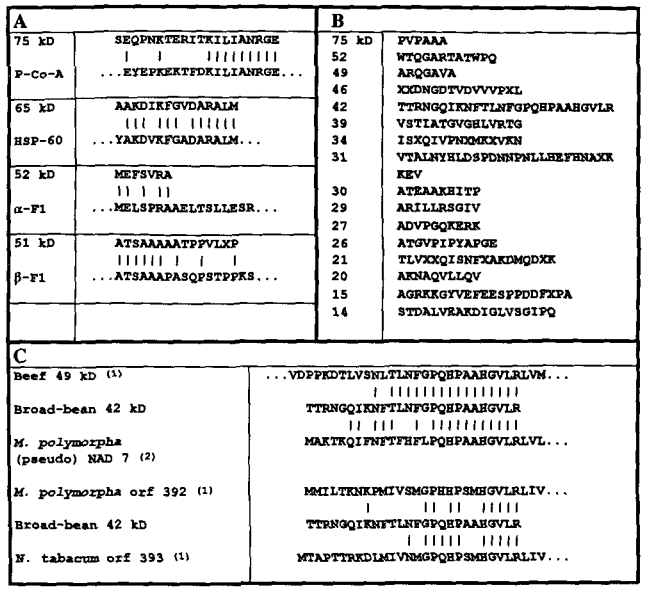

Figure 4. Amino-terminal sequences of complex I subunits and some contaminants. A, Identification of four contaminants eluted at low salt concentrations from the hydroxylapatite chromatography (fractions 32–36 of Fig. 3A). B, Amino acid sequences of broad bean complex I subunits. C, Sequence homology between the broad bean complex I 42-kD subunit and other proteins. P-Co-A, Rat liver propionyl-CoA carboxylase (Browner et al., 1989). HSP-60, *Zea mays* mitochondrial chaperonin HSP60 (Prasad and Stewart, 1992). α-F1 amd β-F1, α and β subunits of *Nicotiana plumbaginifolia* mitochondrial F1-ATPase (Boutry and Chua, 1985; Chaumont et al., 1988). 1, Fearnley et al., 1989. 2, Oda et al., 1992. orf, Open reading frame.

The purified complex exhibited K_M values of 20 μM for NADH (Fig. 5) and 2.5 mM for ferricyanide (not shown). These values are in accord with those found for beef complex I: 17 μM (NADH) and 2.2 mM (ferricyanide) (Paech, 1982). High substrate and acceptor concentrations (>100 μM NADH or >1 mM ferricyanide) inhibited the plant enzyme, as with the mammalian enzyme. The Suc-gradient step appears to reduce the affinity of the broad bean complex for NADH, because the K_m value obtained for the preparation before the hydroxylapatite chromatography is about 11 μM. This lower K_m is closer to the apparent K_M of 7 μM measured by Møller and Palmer (1982) on submitochondrial particles of Jerusalem artichoke for a rotenone-sensitive NADH dehydrogenase.

Other electron acceptors were more slowly or not at all reduced by the enzyme (Table II). The reduction rates for ubiquinone-0 and ubiquinone-10 and for analogs such as duroquinone were 40 to 50 times lower than the rate for ferricyanide. A ratio of 150 was obtained for a preparation enriched in human mitochondrial complex I (Chomyn et al., 1985). In the present work, and up to loading onto the Suc gradient, about 90% of the initial ubiquinone-0 and ubiquinone-10 activities was recovered, as estimated by summing the activities of the retained and discarded fractions. Recovery decreased in the last two steps, but there was no drastic change in the ferricyanide:ubiquinone-0 reductase ratio (not shown).

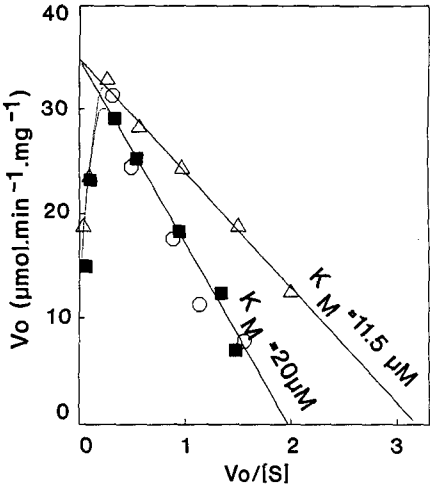

Figure 5. Eadie-Hofstie plot showing K_M values of complex I at different steps of the purification. Values were normalized to the same V_{max} value. NADH:ferricyanide reductase activity was measured as described in "Materials and Methods," using 1 to 5 µg of protein per assay. Δ, Sample after solubilization; O, sample from Suc gradient; ■, complex I from hydroxylapatite. Vo, Initial velocity of NADH oxidation; S, substrate (NADH) concentration.

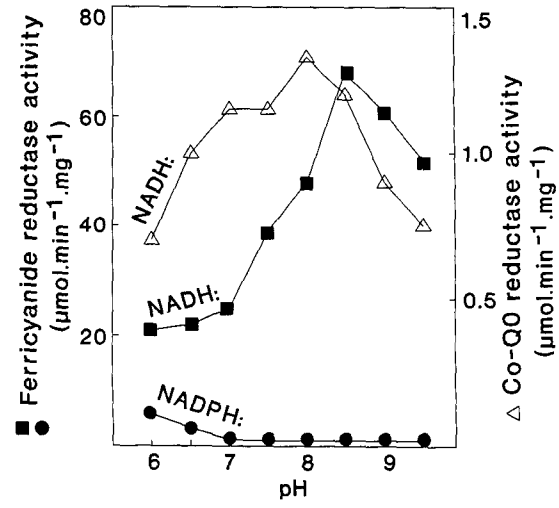

Figure 6. Effect of pH on NADH and NADPH oxidase activities. Potassium phosphate buffer (20 mM) adjusted to the indicated pH values replaced Tris in the standard assay medium, and 1 to 5 µg of protein per assay were used. Activities are expressed in µmol min^{-1} mg^{-1}. ■, NADH-ferricyanide reductase; Δ, NADH-ubiquinone-0 reductase; ●, NADPH-ferricyanide reductase.

For NADH oxidation, the optimal pH values for reduction of ubiquinone-0 and ferricyanide were 8.0 and 8.5, respectively (Fig. 6). At pH 8.0, no activity was detected with Cyt *c* or ubiquinone-30 as acceptors or with NADPH as the electron donor. However, NADPH oxidase activity was observed when the pH of the assay was lowered below 6.0 (Fig. 6). The latter activity could be attributed, as with the mammalian complex I, to the fact that, at acidic pH values, NADPH becomes a closer electronic analog of NADH (Galante and Hatefi, 1979). Another preparation of plant complex I showed an NADPH:ubiquinone-1 reductase activity equal to 11% of that observed with NADH as the electron donor and ubiquinone-5 as the acceptor (Soole et al., 1992). Using inside-out submitochondrial particles from potato, Rasmusson and Møller (1991) measured a deamino-NADPH:oxygen reductase activity of only 5% compared to the activity observed when deamino-NADH was used as the donor.

Finally, high ferricyanide and ubiquinone reductase activities were measured with deamino-NADH as the electron donor, arguing that the purified enzyme is indeed complex I.

Table II. *Activity of the purified complex I with various acceptors and donors*

Acceptor or Donor	Activity[a]
NADH:ferricyanide	52.10
NADH:menadione	2.60
NADH:duroquinone	2.20
NADH:ubiquinone-0	1.54
NADH:ubiquinone-0 + rotenone (40 µM)	1.54
NADH:ubiquinone-10	1.58
NADH:ubiquinone-30	<0.01
NADH:Cyt c	<0.01
deamino-NADH:ferricyanide	31.26
deamino-NADH:ubiquinone-0	1.06
NADPH:ferricyanide	<0.01

[a] All of the activities, expressed as µmol of (deamino-)NAD(P)H oxidized min^{-1} mg^{-1} of proteins, were measured at pH 8.0 as described in "Materials and Methods."

Comparison between Complex I and the CHAPS Extract

CHAPS stripping of mitochondrial membranes released 37% of the NADH dehydrogenase activity. Although the latter was not further purified, it is interesting to compare its properties with those of complex I. Electrophoretic analysis (Fig. 7) shows the presence of many polypeptides, some of them corresponding to complex I subunits. However, the CHAPS extract does not contain a bona fide complex I, because typical subunits of the latter, such as the 75-kD polypeptide, were not found. Enzymic parameters also distinguished the CHAPS extract from complex I (Table III). For instance, the ferricyanide:ubiquinone-0 reductase ratios were 6.5 and 33.7, respectively. In addition, no inhibition by high concentrations of NADH was observed with the CHAPS extract. However, both complex I and the CHAPS-released enzyme exhibited activity with deamino-NADH as an electron donor.

DISCUSSION

No complete complex I from a plant source had ever been isolated. A major problem was the presence of several other NADH dehydrogenases in plant mitochondria (Møller and Lin, 1986). Thus, our first task was to separate complex I from these various other enzymes.

NADH dehydrogenase activities were found in the soluble fraction after osmotic shock as well as in the fraction solubi-

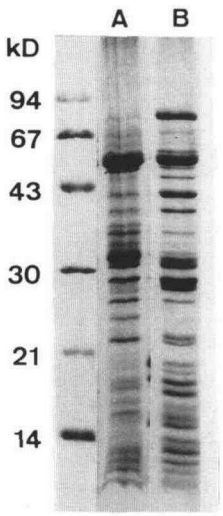

Figure 7. SDS-polyacrylamide gel analysis of the CHAPS extract. A, Seventy micrograms of proteins were precipitated by the chloroform-methanol method from the supernatant obtained after CHAPS stripping. B, Fifty micrograms of purified complex I (see Fig. 3A). Samples were electrophoresed on a 12 to 16% polyacrylamide gel and stained with Coomassie blue.

lized by low detergent concentrations. This stripping was particularly effective in removing most of the ATPase activity and a third of the starting NADH dehydrogenase activity. The fraction released by the CHAPS stripping contained 37% of the initial membrane-bound activity. Electrophoretic analysis indicated the presence of polypeptides whose size was similar to that of complex I subunits. Further purification of this fraction is required to determine which polypeptides are bound to the activity.

We cannot rule out the possibility that this NADH dehydrogenase represents the flavoprotein moiety solubilized from the complex, a moiety capable of catalyzing NADH oxidation by a wide variety of acceptors (Galante and Hatefi, 1979). However, this hypothesis is unlikely for two reasons. First, solubilization of the flavoprotein moiety of animal complex I requires strong treatments with urea and chaotropic agents. It would be surprising that the same results would be achieved with the mild CHAPS treatment of plant mitochondrial membranes. Second, an additional CHAPS stripping did not release any more NADH activity, indicating that the CHAPS-released enzyme and complex I are distinct enzymes. The CHAPS-solubilized activity is reminiscent of that recently characterized by Soole et al. (1992). The latter, solubilized by deoxycholate (detergent:protein ratio of 0.6 compared to 1.0 in our case), contains 14 major polypeptides, some of which cross-reacted with antibodies raised against beef heart complex I. The authors suggested that complex I from plants is simpler than that of other organisms and lacks, for instance, the 75-kD subunit. Acceptor specificity (Table III; Soole et al., 1992) also pointed to a similarity between our CHAPS-released enzyme and the complex obtained from beetroot. However, because, in addition, we found a complex I released by higher detergent concentrations and similar to complex I from other organisms, we suggest that plant organisms contain two complexes capable of oxidizing NADH and sharing identical subunits. Purification of the CHAPS-released enzyme will allow us to perform a more accurate characterization of this dehydrogenase and to evaluate our hypothesis.

Triton X-100 was chosen as one of the more suitable detergents for solubilizing complex I, despite the fact that its use strongly affected the NADH:ubiquinone-10 and NADH:duroquinone reductase activities but not the NADH:ferricyanide reductase activity in beef heart mitochondria (Ruzicka and Crane, 1971). In broad bean mitochondria, the NADH:quinone reductase activity was not affected, the ratio of the quinone and ferricyanide reductase activities remaining fairly constant throughout the purification. The low activities measured for ubiquinone-10, -30, and -50 may be due in part to the enzyme specificity for the unknown natural acceptor in plant mitochondria and partly to the extremely low solubility of ubiquinone-*n* in the assay medium when *n* is greater than 2. This phenomenon was studied in mammalian mitochondria by Ragan (1978), who showed the importance of phospholipids in solubilizing the ubiquinone in the environment of its reduction site. In our case, preliminary experiments with phosphatidylcholine did not significantly increase the activity with ubiquinone-30, but more extensive investigations are needed, notably to determine which phospholipids should be used.

We presume that phospholipids are responsible for the doublets or shoulders observed after the Suc-gradient centrifugation and the hydroxylapatite chromatography. It could be that lipids are progressively removed or replaced by the detergent, leading to heterogeneity of charge, and, to a lesser extent, size and causing a loss of activity. The similar polypeptide composition in different pools derived from these asymmetric peaks argues in favor of this hypothesis.

An accurate determination of the molecular mass of the whole complex was not possible, because there are, to date, few chromatographic means of obtaining a good molecular mass scale for large protein complexes solubilized in the

Table III. *Comparison between complex I and CHAPS extract*

	Complex I	CHAPS Extract
Molecular mass (kD)	>670,000	>232,000 <670,000
Electron donors	NADH deamino-NADH	NADH deamino-NADH
Electron acceptors (%)		
Ferricyanide	100	100
Menadione	5	3
Duroquinone	4	6
Ubiquinone-0	3	15
Ubiquinone-10	3	8
Ubiquinone-30	<0.1	1
Cyt *c*	<0.1	13
K_M (NADH) (μM)	20	19
Inhibition by		
NADH	>100 μM	None
Ferricyanide	>1 mM	>1 mM
Optimal pH (Co-Q 0)	8.5 Shoulder at 7.5	8.5 Shoulder at 7.0

presence of a nondenaturing detergent. A more important question is whether the complex is pure and whether subunits were lost during purification. The exact identity of integral membrane protein complexes cannot easily be approached, and solubilization is liable to cause structural modifications. That we have indeed purified complex I from the respiratory chain of broad bean mitochondria is indicated by the similarities between our complex and the enzymes derived from *N. crassa* and beef heart mitochondria and by the deamino-NADH:ubiquinone-0 reductase activity. This electron donor has been reported to be specific for complex I (Rasmusson and Møller, 1991). Nevertheless, because the sensitivity to rotenone is lost during centrifugation into the Suc gradient (not shown), the identification of the purified enzyme as the rotenone-sensitive NADH dehydrogenase requires reconstitution of the complex in phospholipid vesicles, as reported for the beef heart and *N. crassa* purified complex I (Ragan, 1978; Ise et al., 1985).

It would probably be pointless to discuss the exact number of subunits in the complex and the likelihood that some of the polypeptides shown in Figure 3 are contaminants. The only argument in favor of their belonging to complex I is that the intensity of polypeptide staining after electrophoretic separation paralleled the activity curve throughout the various purification steps. To identify some of the complex I subunits, we attempted to obtain their NH_2-terminal sequences. Although the NH_2-terminal residue was blocked in many cases, thus requiring proteolytic fragmentation, our findings concerning the 42-kD subunit were interesting. This subunit's NH_2-terminal amino acid sequence exhibits strong homology with that of the 49-kD subunit of bovine complex I (from residues 38–62) and that of the putative translation products of two open reading frames derived from the *M. polymorpha* (open reading frame 392) and *N. tabacum* (open reading frame 393) chloroplast genomes. Thus, we presume that the 42-kD subunit of broad bean complex I corresponds to the mammalian 49-kD subunit, although this result should be interpreted cautiously when polypeptides of similar molecular mass are compared between organisms.

The failure to find any significant homology for the other subunits is probably related to the poor conservation of amino-terminal sequences of complex I subunits between different organisms (Burger and Werner, 1985; Rasmussen and Hanson, 1989; Xue et al., 1990). On the other hand, several plant mitochondrial genes encoding putative complex I subunits have been sequenced and should represent a better source for comparison. However, we only obtained amino-terminal sequences for half of the complex I subunits, and we generally observed that hydrophobic polypeptides, which include the mitochondrially encoded subunits, are more frequently subject to amino-terminal blockage. For the beef heart complex I, Walker et al. (1992) found that 9 of 20 subunits were also blocked: 6 by acetylation, 1 by myristoylation, and 1 by an unknown modification. Proteolytic or chemical fragmentation of blocked subunits of plant complex I should be carried out to clarify this point.

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