Resolution of NADH:Ubiquinone Oxidoreductase from Bovine Heart Mitochondria into Two Subcomplexes, One of Which Contains the Redox Centers of the Enzyme†

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ABSTRACT: NADH:ubiquinone oxidoreductase (complex I) was purified from bovine heart mitochondria by solubilization with *n*-dodecyl β-D-maltoside (lauryl maltoside), ammonium sulfate fractionation, and chromatography on Mono Q in the presence of the detergent. Its subunit composition was very similar to complex I purified by conventional means. Complex I was dissociated in the presence of *N,N*-dimethyldodecylamine *N*-oxide and β-mercaptoethanol, and two subcomplexes, Iα and Iβ, were isolated by chromatography. Subcomplex Iα catalyzes electron transfer from NADH to ubiquinone-1. It is composed of about 22 different and mostly hydrophilic subunits and contains 2.0 nmol of FMN/mg of protein. Among its subunits is the 51-kDa subunit, which binds FMN and NADH and probably contains a [4Fe-4S] cluster also. Three other potential Fe-S proteins, the 75- and 24-kDa subunits and a 23-kDa subunit (N-terminal sequence TYKY), are also present. All of the Fe-S clusters detectable by EPR in complex I, including cluster 2, are found in subcomplex Iα. The line shapes of the EPR spectra of the Fe-S clusters are slightly broadened relative to spectra measured on complex I purified by conventional means, and the quinone reductase activity is insensitive to rotenone. Similar changes were found in samples of the intact chromatographically purified complex I, or in complex I prepared by the conventional method and then subjected to chromatography in the presence of lauryl maltoside. Subcomplex Iβ contains about 15 different subunits. The sequences of many of them contain hydrophobic segments that could be membrane spanning, including at least two mitochondrial gene products, ND4 and ND5. The role of subcomplex Iβ in the intact complex remains to be elucidated.

NADH:ubiquinone oxidoreductase (complex I, EC 1.6.5.3), the first and largest enzyme of the mitochondrial respiratory chain, plays a central role in oxidative phosphorylation by catalyzing the transfer of two electrons from NADH to ubiquinone-10, with concomitant translocation of 3–5 protons from the matrix to the cytosolic side of the inner mitochondrial membrane (Rottenberg & Gutman, 1977; DeJonge & Westerhoff, 1982; Wikström, 1984; Scholes & Hinkle, 1984; Lemasters, 1984; Brown & Brand, 1988). It is the least studied respiratory enzyme, and its size, polypeptide composition, and number and types of iron–sulfur clusters are not known with certainty [for reviews see Beinert and Albracht (1982), Ohnishi and Salerno (1982), Ragan (1987), Weiss et al. (1991), and Walker (1992)]. Previously, bovine complex I was thought to be composed of about 25 different polypeptides, including 7 hydrophobic proteins coded in mitochondrial DNA, but 34 different nuclear coded subunits have been characterized recently by sequence analysis (Fearnley et al., 1989, 1991; Pilkington & Walker, 1989; Runswick et al., 1989, 1991; Pilkington et al., 1991a,b; Dupuis et al., 1991a,b; Skehel et al., 1991; Walker et al., 1992; Arizmendi et al., 1992a,b). Therefore, it has at least 41 different subunits.

Complex I contains one FMN and a binuclear Fe-S cluster which was first detected by EPR[1] spectroscopy by Beinert and Sands (1960). This cluster is usually referred to as cluster 1b or N-1b (Ohnishi, 1979; Hearshen et al., 1981). A second [2Fe-2S] cluster, named N-1a, was reported by Ohnishi and co-workers (Ohnishi, 1979; Ingledew & Ohnishi, 1980; Ohnishi et al., 1981). The signal of this cluster has not been reported or observed by other workers (Orme-Johnson et al., 1971, 1974; Albracht et al., 1977; Beinert & Albracht, 1982; Kowal et al., 1986). The presence of three tetranuclear clusters, called clusters 2–4 (or N-2, N-4, and N-3 in the nomenclature of Ohnishi), has been established by work from several laboratories (Ohnishi et al., 1970; Orme-Johnson et al., 1971, 1974; Albracht & Slater, 1971; Ohnishi, 1975, 1979; Albracht, et al., 1977; Kowal et al., 1986). Complex I also shows an additional EPR signal at 4.2 K (Albracht, 1974a; Ohnishi, 1975) with $g_{x,y,z}$ = 1.89, 1.92, 2.06, now known as cluster 5 or N-5 (Ingledew & Ohnishi, 1980). Due to the apparent low-spin intensity of this signal (Beinert & Ruzicka, 1975; Albracht et al., 1977; Ohnishi, 1979) and the absence of such a signal in submitochondrial particles of *Candida utilis* (Albracht & Subramanian, 1977) and plant mitochondria (Rich & Bonner, 1978), it remains uncertain whether this cluster is essential for function of complex I.

There is still a dispute about the precise stoichiometry of clusters 1b, 2, 3, and 4 [see Beinert and Albracht (1982) and references cited therein; Kowal et al., 1986; Wang et al., 1991; Van Belzen et al., 1992]. In addition, there is an increasing

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[1] Abbreviations: LDAO, *N,N*-dimethyldodecylamine *N*-oxide; EPR, electron paramagnetic resonance; ES-MS, electrospray mass spectrometry.

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amount of evidence that complex I might be a functional dimer (Van Belzen et al., 1990, 1992).

It is not clear yet with which subunits the various clusters are associated. One approach to study this has been the disruption of complex I, for example, with chaotropic agents (Ragan et al., 1982; Ohnishi et al., 1985; Ragan, 1987) or by treatment with phospholipase at high pH (Paech et al., 1982), and the characterization of smaller fragments. The disruption of the bovine enzyme with chaotropes has proved to be particularly valuable. Two water-soluble fractions have been characterized after treatment with perchlorate; the FP (flavoprotein) fraction is a subcomplex of the 51-, 24-, and 10-kDa subunits and transfers electrons from NADH to ferricyanide and several other electron acceptors (Galante & Hatefi, 1979); the IP (iron–sulfur protein) fraction contains at least the 75-, 49-, 30-, 18-, 15-, and 13-kDa subunits [see Ragan (1987)] and subunit B13 (Masui et al., 1991a) and appears to contain some of the Fe-S clusters. The remainder of the subunits after perchlorate treatment are present in an insoluble precipitate known as the HP (hydrophobic protein) fraction.

As described below, complex I has been purified from bovine heart mitochondria by a new procedure based on chromatography. The purified enzyme was disrupted by treatment with a nondenaturing detergent, and two subcomplexes, named Iα and Iβ, were isolated and characterized. Subcomplex Iα is an assembly of about 22 predominantly hydrophilic subunits and contains all of the redox centers detected by EPR in the intact complex I. It transfers electrons from NADH to ubiquinone-1. In some respects it resembles a small isoform of complex I isolated from *Neurospora crassa* mitochondria after inhibition of protein synthesis in the organelle (Friedrich et al., 1989), but the *N. crassa* small complex lacks Fe-S cluster 2, whereas it is present in bovine Iα. Subcomplex Iβ contains about 15 other subunits, and some of them appear to be hydrophobic and membrane spanning. No biochemical function has yet been attributed to subcomplex Iβ.

MATERIALS AND METHODS

Chemicals. Lauryl maltoside and sodium cholate were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). LDAO (MicroSelect grade) and ethylene glycol were obtained from Fluka (Buchs, Switzerland). Samples of ubiquinone-1 were donated by Hofmann-La Roche (Basel, Switzerland) and Dr. P. Rich (Glynn Research Institute, Bodmin, U.K.). Rotenone was obtained from Sigma Biochemicals, Poole, Dorset, U.K.

Analytical Methods. Protein concentrations were measured by the BCA method (Pierce and Warriner Ltd., Chester, U.K.), using bovine serum albumin as a standard. FMN was determined according to Ragan and Racker (1973). Polyacrylamide gels containing a 12–22% acrylamide gradient [acrylamide:bis(acrylamide) 50:1.6] were prepared and run in the buffer system of Laemmli (1970). The gel system described by Schägger and von Jagow (1987) was also employed. Both types of gel were cast in minigel (10-cm × 10-cm) and conventional (16-cm × 20-cm) formats.

Purification of Complex I from Bovine Heart Mitochondria. Mitochondria, prepared according to Smith (1967), were divided into portions corresponding to ca. 1.5 hearts and stored at −20 °C. Complex I was purified either by the conventional method (Hatefi & Rieske, 1967; Ragan et al., 1987) or by the new method as follows. All of the steps, including chromatography, were conducted at 0–4 °C. Mitochondria were disrupted by mixing them for 5 min in cold water (2 L/portion) in a Waring blender. Then solid KCl was added (final concentration 150 mM), and mixing was prolonged for 2 min. The pellet arising from centrifugation of the suspension (40 min, 8600*g*) was homogenized in a buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10% (v/v) glycerol, and the homogenate was diluted with the same buffer to a protein concentration of 12 mg/mL. This corresponds to a cytochrome oxidase concentration in these membranes of about 7 μM, as estimated from the absorbance difference between a dithionite reduced and oxidized sample at 604 nm minus 630 nm, using a millimolar extinction coefficient of 27 per 1-cm light path (Vanneste, 1966). The membrane suspension was divided into 50-mL portions; one was solubilized as described below, and the others were stored at −20 °C.

Lauryl maltoside was added to the membrane suspension (final concentration 1%, w/v). The suspension was stirred for 5 min and then centrifuged (30 min, 30000*g*). The resultant pellet was discarded, and to the supernatant were added sodium cholate from a 20% stock solution (final concentration 1.6%) and saturated neutralized ammonium sulfate (final concentration 40% saturation). The suspension was stirred for 10 min and then centrifuged (10 min, 30000*g*). The pellet was discarded and ammonium sulfate was added to the supernatant to 52% saturation. After 10 min of stirring, the precipitate was collected by centrifugation as before for 10 min. The pellet contained ATP synthase, complex I, and some other proteins, but most of the respiratory complexes II–IV remained in the supernatant. After removal of the supernatant, the precipitate was solubilized in 15 mL of a solution consisting of 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 1.5% cholate, 1% lauryl maltoside, and 10% (v/v) ethylene glycol. Ammonium sulfate was added to 36% saturation, and the suspension was stirred for 10 min and centrifuged (10 min, 30000*g*). A small pellet was discarded, ammonium sulfate was added to the supernatant to 48% saturation, and the suspension was stirred and centrifuged as above. The resulting pellet was solubilized in 10 mL of buffer A–lauryl maltoside (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.1% lauryl maltoside, and 10% ethylene glycol) to which lauryl maltoside had been added to a final concentration of 1%. The solution was diluted with an equal volume of buffer A–lauryl maltoside, filtered through a Sartorius Minisart NML filter (0.2-μm porosity), and loaded onto a Mono Q HR 10/10 column (Pharmacia, Milton Keynes, U.K.) equilibrated with the buffer A–lauryl maltoside (flow rate 1.5 mL/min). Bound proteins were eluted with a gradient of NaCl, as shown in Figure 1. Complex I eluted at 250–320 mM NaCl, and its purity in the fractions was examined by minigel electrophoresis under denaturing conditions. Appropriate fractions, which were pale yellow, were pooled, and complex I was precipitated by addition of lauryl maltoside to 1%, cholate to 1.8%, and ammonium sulfate to 45% saturation. The precipitated material was dissolved in a small volume of buffer A–LM, divided into portions, and stored at −20 °C.

Isolation of Subcomplexes Iα and Iβ. Complex I was diluted to a protein concentration of about 3 mg/mL with buffer A–LDAO (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.1% LDAO, and 10% ethylene glycol), and an equal volume of 0.1 M LDAO stock solution was added, followed by β-mercaptoethanol (final concentration 1% v/v). The solution was left on ice for 60 min and then loaded onto a Mono Q HR 10/10 column equilibrated in buffer A–LDAO (flow rate 1.5 mL/min). The protein complexes were eluted with a gradient of NaCl (see Figure 2) and concentrated by ammonium sulfate precipitation as described above for complex I.

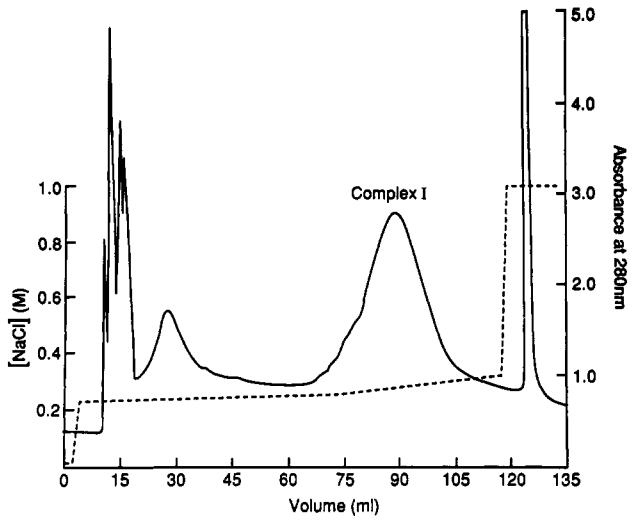

FIGURE 1: Purification of complex I from bovine heart mitochondria by chromatography on Mono Q in the presence of lauryl maltoside. About 100 mg of protein was loaded onto the column. (—) Absorbance at 280 nm; (- - -) NaCl gradient. For further details see Materials and Methods section.

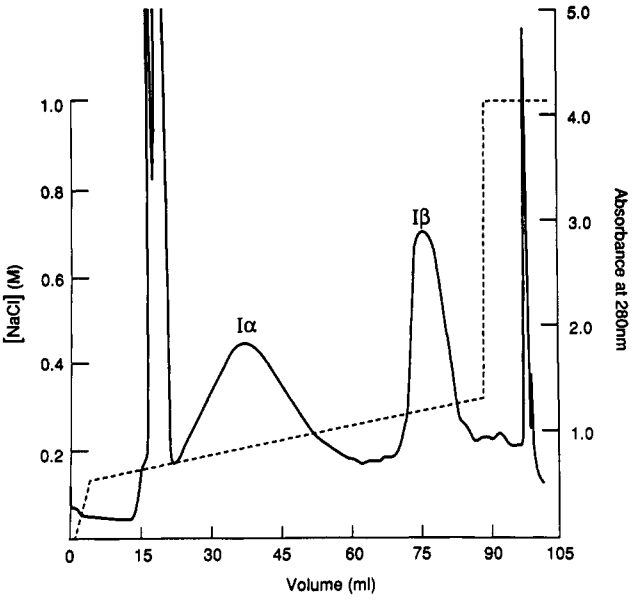

FIGURE 2: Isolation of subcomplexes Iα and Iβ by ion-exchange chromatography on Mono Q in the presence of LDAO. Bovine complex I (40 mg) was treated by LDAO as described in Materials and Methods and was then loaded onto the column. For meaning of symbols see legend to Figure 1.

Soluble NADH Dehydrogenase. This was prepared according to Cremona and Kearney (1964) and was a generous gift from Dr. T. P. Singer to S.P.J.A.

Enzyme Assays. Activities were measured at 35 °C in a buffer containing 50 mM potassium phosphate, pH 7.4, and soybean phospholipids (phosphatidylcholine type IV-S from Sigma), added to 1 mg/mL from a suspension (40 mg/mL) that had been sonicated to clarity in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 2% cholate. Reduction of ferricyanide was followed spectroscopically at 420 nm, and oxidation of NADH in the presence of ubiquinone-1, at 340 nm. All the reactions were started by addition of 200 μmol of NADH to solutions containing enzyme and either 1.3 mM ferricyanide or 65 μM ubiquinone-1. The enzyme concentration was adjusted to give a linear rate for 60 s since the spectrophotometer used (Philips PU 8740) was unsuitable for measurement of initial rates. Inhibition by rotenone (50 μM final concentration) was assayed after incubation at 35 °C for 10 min with or without NADH (1 μM).

Characterization of Subunits of Complex I and Subcomplexes Iα and Iβ. Proteins were separated by gel electrophoresis in the presence of SDS, transferred to poly(vinylidene difluoride) membranes, stained with Coomassie blue dye, and sequenced (Matsudaira, 1987; Fearnley et al., 1989). Mitochondrial gene products ND1, ND2, ND4, ND5, and ND4L were recovered by electroelution from gels (Runswick et al., 1989), treated with methanolic HCl to remove α-*N*-formyl groups, and sequenced (Fearnley & Walker, 1987). Subunits of subcomplexes Iα and Iβ were fractionated by HPLC in 0.1% trifluoroacetic acid on an Aquapore C_8 column (300-Å pore size, 7-μm particles, 100-mm × 2.1-mm i.d.; Applied Biosystems, Warrington, U.K.) with a linear gradient of acetonitrile. In the fractionation of Iα and Iβ subunits B14, B13, B9, and B8, and subunits B22, B18, B17, B15, and B12, eluted at acetonitrile concentrations of 44%, 48%, 56%, and 43% and 54%, 45%, 51%, 57%, and 50%, respectively. The purified subunits were subjected to electrospray mass spectrometry in a VG BIO-Q triple quadrupole instrument with electrospray ionization (VG Biotech, Altrincham, Cheshire, U.K.) as described before (Skehel et al., 1991).

Subunit Nomenclature. A number of subunits of complex I are named according to their apparent molecular weights. These are 3 proteins present in the FP fraction (the 51-, 24-, and 10-kDa subunits), 6 proteins in the IP fraction (the 75-, 49-, 30-, 18-, 15-, and 13-kDa subunits), and the 39- and 42-kDa subunits; 9 nuclear coded proteins with modified α-amino groups and estimated molecular masses of 8, 9, 12, 13, 14, 15, 17, 18, and 22 kDa are called B8, B9, B12, B13, B14, B15, B17, B18, and B22, respectively. Other nuclear coded subunits with unmodified α-NH_2 groups are named according to the one-letter code of the sequence of amino acids 1–4. Subunits encoded in mitochondrial DNA are known as ND1–ND6 and ND4L.

EPR Spectroscopy. Concentrated samples of protein in EPR tubes were kept in ice in the presence of 5 mM NADH for 30 s before freezing in liquid nitrogen. The samples were transported in liquid nitrogen to Amsterdam. EPR measurements were performed on a Varian E-9 EPR spectrometer equipped with a home-built He-flow cryostat (Lundin & Aasa, 1972; Albracht, 1974b). The magnetic field was calibrated with an AEG NMR field meter, and the microwave frequency was measured with an electronic counter (HP 5246L), supplemented with a frequency converter (HP 5255A). Spectra were stored on a personal computer using a 12-bit A/D converter. The Fe-S clusters of complex I are defined as follows: cluster 1b, $g_{x,y,z}$ = 1.92, 1.94, 2.02; cluster 2, $g_{x,y,z}$ = 1.92, 1.92, 2.05; cluster 3, $g_{x,y,z}$ = 1.88, 1.94, 2.10; cluster 4, $g_{x,y,z}$ = 1.86, 1.93, 2.04.

RESULTS

Purification of Complex I. Bovine complex I was purified by a new method using chromatographic steps. Its subunit composition is very similar to that of complex I purified by the conventional method (see Figure 3). One difference demonstrated by gel analyses is that transhydrogenase, which is usually present in conventional preparations and can be seen as a faint band near the top of the gel (slot a, Figure 3A), is absent from the sample made by the new procedure. It was also removed from a conventional preparation by chromatography on Mono Q, and this latter preparation is similar to the enzyme made by the new procedure in other respects also (see below). Another difference between the conventional

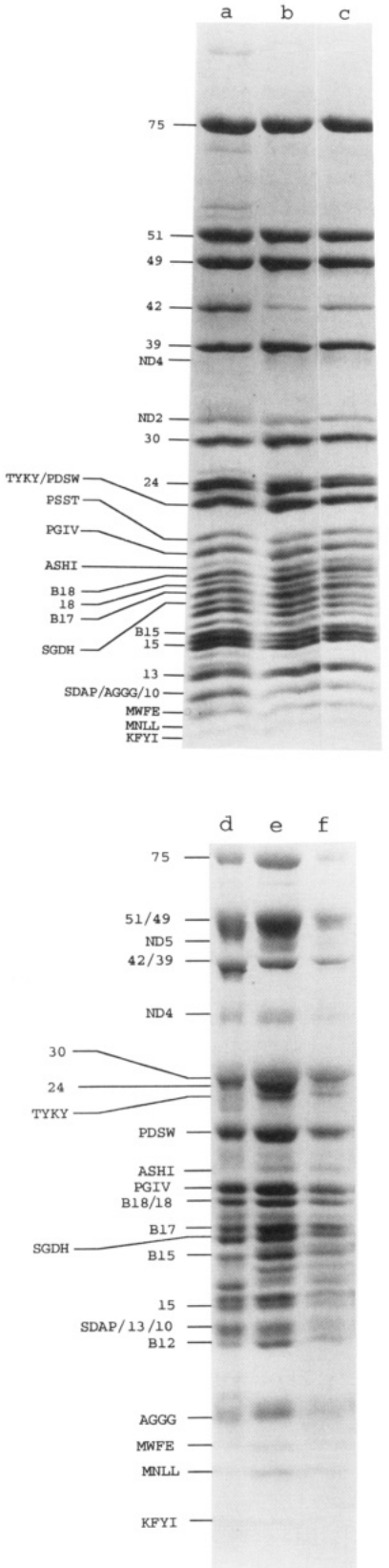

FIGURE 3: Comparison of the subunit compositions of samples of complex I purified from bovine heart mitochondria by different methods. In slots a–c and d–f the samples were analyzed by SDS/PAGE using the gel systems of Laemmli (1970) and Schägger and von Jagow (1987), respectively. Complex I purified by (a and d) the conventional method (Hatefi & Rieske, 1967; Ragan et al., 1987); (b and e) the conventional method followed by chromatography on Mono Q; and (c and f) a new method described here. The identities of many subunits in all three samples have been determined by sequence analysis, and their positions are indicated on the left-hand side of the photograph.

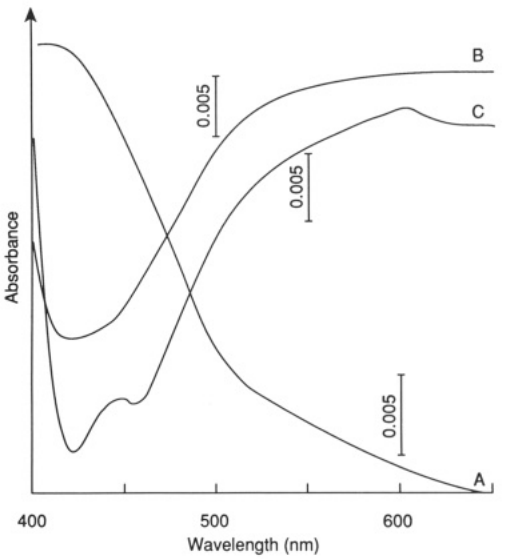

FIGURE 4: Optical spectra of complex I. The enzyme was purified by the new chromatographic method. The spectra were measured on a sample with a protein concentration of 0.73 mg/mL. (A) Oxidized enzyme minus buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, and 0.1% lauryl maltoside); (B) complex I reduced with NADH minus oxidized; (C) the sample in (B) further reduced with dithionite minus the oxidized spectrum.

and new preparations is that the 42-kDa subunit is relatively diminished in the sample prepared by the new procedure; it appears to be rather loosely attached to complex I and can be completely removed by chromatographing the enzyme at room temperature on Mono Q in the presence of lauryl maltoside, as discussed below. A third difference between the two preparations is that traces of other respiratory complexes are almost entirely absent from the material made by the new method. The visible spectrum of the purified enzyme (Figure 4) shows no cytochromes either in the oxidized sample or in the NADH-reduced minus oxidized difference spectra. Even upon reduction by dithionite, no *b*- or *c*-type cytochromes were detected at all, but a small amount of cytochrome oxidase was visible as a shoulder around 445 nm (Figure 4, curve C). Chromatography on Mono Q also removes contaminants from the conventionally purified enzyme (see Figure 3).

The similarity in subunit composition of the two preparations of enzyme was confirmed by N-terminal sequence analysis. Of the 32 nuclear coded subunits of the enzyme of known sequence (Fearnley et al., 1989, 1991; Pilkington & Walker, 1989; Runswick et al., 1989, 1991; Pilkington et al., 1991a,b; Dupuis et al., 1991a,b; Skehel et al., 1991; Walker et al., 1992; Arizmendi et al., 1992a,b), 23 have free α-NH_2 groups, and their presence in both preparations of the enzyme was readily demonstrated by N-terminal sequencing. The nine other nuclear coded subunits of known sequence (subunits B22, B18, B17, B15, B14, B13, B12, B9, and B8) have modified N-terminals, which makes their identification in samples of complex I more problematical. Some of them have been isolated from the conventionally prepared enzyme (B13, B14, B8, and B9); others (B22, B18, B17, B15, and B12) have been obtained from samples of subcomplex Iβ (see below) derived from enzyme prepared by the new method. Some of them can be recognized with reasonable certainty from their migration positions on gels, but others comigrate, making their identification difficult, even in 2D gels.

The identification in complex I of the seven hydrophobic subunits that are encoded in mitochondrial DNA is also difficult and is incomplete. All of them stain weakly with

Table I: FMN Contents and Activities of Different Complex I Preparations and of Subcomplex Iα

preparation	FMN	NADH to ferricyanide	NADH to Q-1	% inhibition of Q-1 reduction by rotenone
complex I (H)[b]	1.2–1.3	200	2.0	75
complex I (HMQ)	1.6	110	1.4	0
complex I (MQ)	1.5	110	1.5	0
subcomplex Iα	2.0–2.1	220	3.0	0

[a] FMN content is in nmol/mg of protein. Activities are in μmol of NADH oxidized per minute per milligram of protein. [b] The abbreviations denote different preparations of complex I: (H) made according to Ragan et al. (1987); (HMQ) by chromatography of preparation H on Mono Q; and (MQ) by chromatography on Mono Q according to the new method.

Coomassie blue dye and give rise to diffuse bands on gels, and in addition, their N-terminals are formylated and the formyl group must be removed with acid before N-terminal analysis. Six of them (ND1, ND2, ND3, ND4, ND5, and ND4L) have been identified in conventionally purified complex I by sequence analysis (J. M. Skehel and J. E. Walker, unpublished work), but a band on a gel of complex I containing ND6 has not yet been discovered. ND2 and ND4 have also been identified in complex I prepared by the new procedure, and ND2, ND4, and ND5 have been found in subcomplexes prepared from it (see below).

The activities of the enzyme prepared by the new procedure were somewhat lower than those measured on enzyme prepared by the conventional method, but were within the range of values reported previously (Table I). The major difference was that the new preparation was found not to be inhibited by rotenone, a classical inhibitor of complex I. The reason for this is unknown at present. The conventionally made enzyme also became insensitive to the inhibitor after chromatography in the presence of lauryl maltoside on Mono Q. The FMN content of the chromatographically purified enzyme was slightly higher than the conventionally purified one, possibly because impurities have been removed during purification.

Resolution of Complex I into Subcomplexes Iα and Iβ. After treatment of purified complex I with LDAO, two subcomplexes were separated by chromatography on Mono Q in the presence of LDAO. The first to elute, subcomplex Iα, was yellow; the second, subcomplex Iβ, was colorless and was very slightly contaminated with traces of subcomplex Iα (Figure 5). Subcomplex Iα contains FMN and, inter alia (see below), the 51-kDa subunit, the site of binding of both NADH and FMN.

The EPR spectra of chromatographically purified complex I and subcomplex Iα are rather similar (Figure 6) and demonstrate that this subcomplex contains all of the Fe-S clusters detected in complex I, including cluster 2, which is thought to transfer electrons to ubiquinone. However, there are certain significant differences in the signals of the clusters 2 and 3. When complex I prepared according to Ragan et al. (1987) was chromatographed on Mono Q [complex (HMQ)], the g_z line of cluster 2 at $g = 2.05$ shifted upfield by 0.3 mT, whereas its line width increased by 0.35 mT. In subcomplex Iα this g_z line was shifted 0.5 mT and broadened by 0.7 mT. In addition, the g_z line became asymmetric and tailed to the high-field side in subcomplex Iα. These values were obtained from detailed recordings of the individual lines. Also the width of the $g_{x,y}$ line of cluster 2 at $g = 1.92$ increased by 0.3 mT (measured at half-depth of the trough). This strongly influenced the overall line shape of the spectrum in the $g = 1.90$–1.95 region, since the line shapes of the clusters 1b and 4 did not change. A downfield shift of the g_z line from 0.4 mT in complex I (HMQ) to 0.6 mT in subcomplex Iα was observed in cluster 3. Similar changes were also noticed in the line shapes of the soluble high-molecular-weight NADH dehydrogenase (Figure 6, trace F). No other signals could be detected in the field span of 0–1 T at any temperature, except for the appearance in all preparations at 4.2 K and high microwave power of a signal of low intensity corresponding

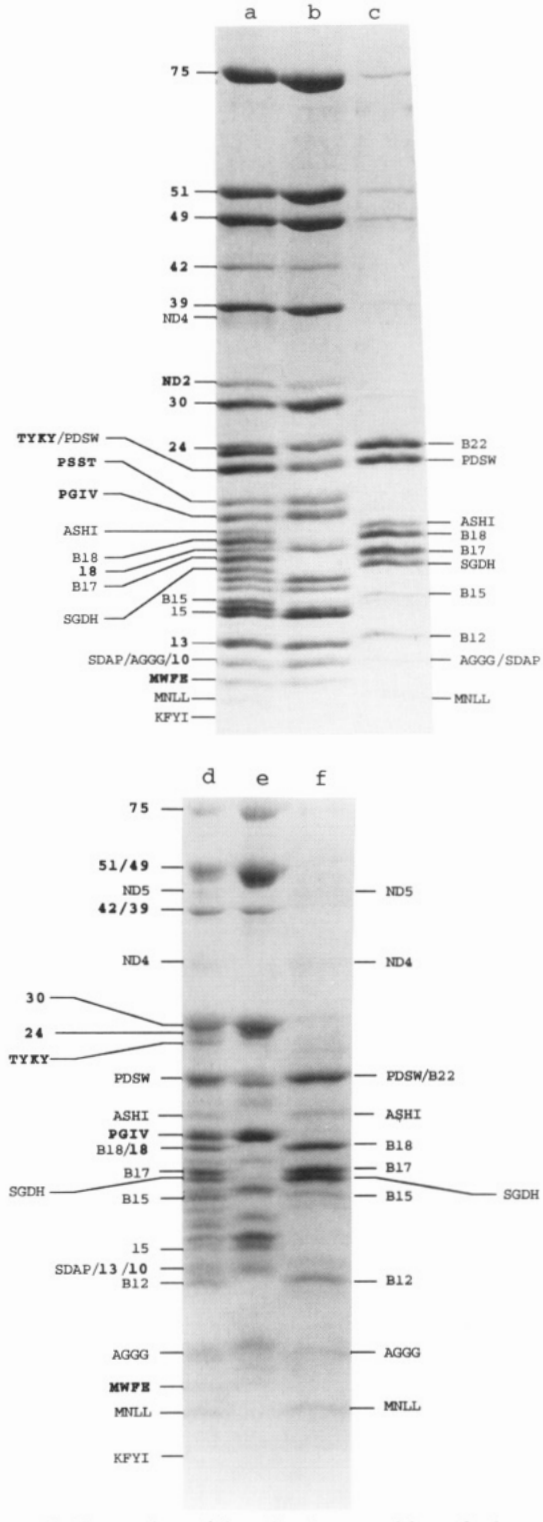

FIGURE 5: Comparison of the subunit compositions of subcomplexes Iα and Iβ and complex I prepared by the new method. As in Figure 3, the SDS/PAGE gel systems of Laemmli (1970) (slots a–c) and of Schägger and von Jagow (1987) (slots d–f) were employed. (a and d) Complex I; (b and e) subcomplex Iα; (c and f) subcomplex Iβ.

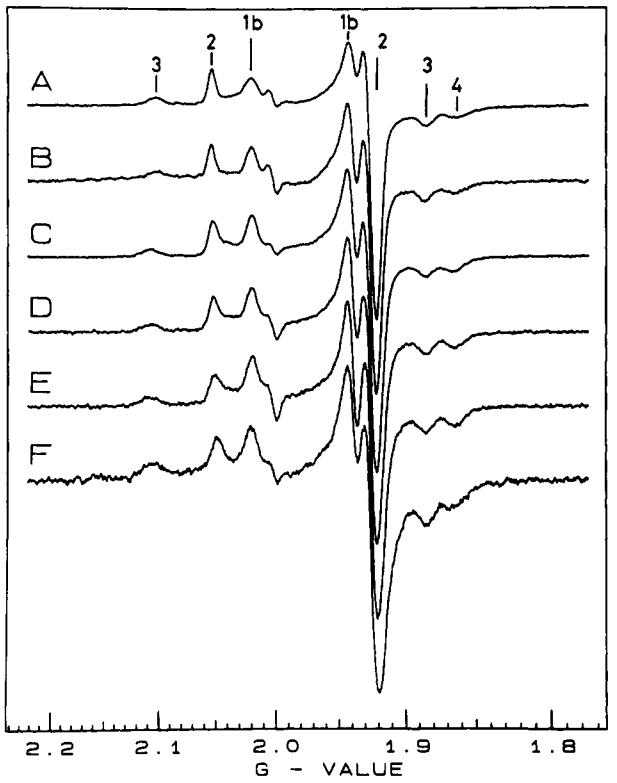

FIGURE 6: EPR spectra of different preparations of complex I and subcomplex Iα. Lines belonging to clusters 1–4 are indicated. Samples were reduced with 5 mM NADH in EPR tubes for 30 s at 0 °C and frozen and stored in liquid nitrogen. (A) Complex I prepared in Amsterdam (Hatefi & Rieske, 1967) dissolved in 0.66 M sucrose, 50 mM Tris-HCl, pH 8.0 (73 mg/mL, 0.96 nmol of FMN/mg); (B) complex I prepared in Cambridge according the same procedure as in (A); (C) complex I prepared according to Hatefi and Rieske (1967), and then chromatographed on Mono Q; (D) complex I prepared according to the new chromatographic method; (E) subcomplex Iα; (F) soluble high-molecular-weight NADH dehydrogenase dissolved in 30 mM potassium phosphate, pH 7.8 (10 mg/mL, 0.82 nmol FMN/mg). Spectra were plotted such that the amplitude of the trough of the $g_{x,y}$ line of cluster 2 was the same in all cases. Spectra were corrected to the same microwave frequency (Albracht, 1984). EPR conditions: microwave frequency, 9263 MHz; temperature, 10 K; microwave power incident to the cavity, 0.2 mW; modulation amplitude, 0.63 mT; modulation frequency, 100 kHz.

to cluster 5 (Albracht, 1974a; Ohnishi, 1975; Ingledew & Ohnishi, 1980; Beinert & Albracht, 1982).

Subcomplex Iα transfers electrons from NADH to both ubiquinone-1 and ferricyanide, with rates that are similar to those observed with complex I purified by the new method (Table I). The ubiquinone-1 reductase activity of subcomplex Iα is insensitive to rotenone, as is that of the chromatographically purified complex I. Subcomplex Iβ has no known enzymic activities and contains no detectable Fe-S clusters, other than traces of activity and EPR signals attributable to low amounts of contaminating subcomplex Iα (not shown).

Subunit Compositions of Subcomplexes Iα and Iβ. Seventeen subunits have been identified in subcomplex Iα by N-terminal sequencing (see Table II) and six more in subcomplex Iβ (see Table III). In subcomplex Iα, they include the three proteins that constitute the water-soluble FP fraction (the 51-, 24-, and 10-kDa subunits) and seven of the proteins (75-, 49-, 30-, 18-, 15-, and 13-kDa subunits and subunit B13) that are found in the water-soluble IP fraction. Subunits PSST (20 kDa) and TYKY (23 kDa), which have been detected in some preparations of the IP fraction (Masui et al., 1991a,b) but not in others, are also in subcomplex Iα. With the exceptions of the 42-kDa subunit and subunits SDAP (the acyl carrier protein), the yields of amino acids observed in sequencing experiments suggest that these proteins are present in approximately equimolar quantities. The 42-kDa subunit is clearly present in substoichiometric quantities. The amount of it found differs from one preparation to another, and it is completely absent from some preparations. Subunit SDAP is the only subunit that identified in both subcomplexes Iα and Iβ; the relative amounts are 40:60, respectively. The stoichiometries of subunits in intact complex I are not known. It is assumed that there is one copy of each polypeptide per complex, but other stoichiometries are possible and could not be distinguished with certainty in the experiments that are described here.

Among the subunits of bovine complex I are found at least nine nuclear coded subunits that have modified α-N-terminals (Walker et al., 1992). The modifying groups appear to be mostly acetyl groups except for subunit B18, which is α-N-myristylated. These subunits are more difficult to identify than those with free N-terminals, and the task is made harder by the large number of such subunits that have similar apparent molecular masses. One approach that is being used to overcome these problems is to separate the subunits in the subcomplex by HPLC and to then measure their molecular masses by electrospray mass spectrometry. The estimates of molecular mass by this technique are sufficiently accurate to allow proteins to be identified unambiguously. In this way subunits B14, B13, B8, and B9 have been detected in subcomplex Iα, and the presence of B22, B18, B17, B15, and

Table II: Subunits of Bovine Complex I Detected in Subcomplex Iα by N-Terminal Sequencing or Electrospray Mass Spectrometry

subunit	molecular weight: gel (K)	molecular weight: sequence[a]	molecular weight: ES-MS	comments	ref
75 kDa (IP)	75	76 960.2		[4Fe-4S] and possibly [2Fe-2S] cluster	*f*
51 kDa (FP)	51	48 416.1		NADH, FMN sites; [4Fe-4S] cluster	*g*
49 kDa (IP)	49	49 174.4			*h*
42 kDa	42	36 692.7			*i*
39 kDa	39	39 115.1			*i*
30 kDa (IP)	30	26 431.8			*j*
24 kDa (FP)	24	23 814.4		probably [2Fe-2S] cluster	*k*
TYKY	23	20 195.9		2 × [4Fe-4S] clusters	*l*
PSST	20	20 077.5		Fe-S protein?	*m*
PGIV	19	19 959.9		Fe-S protein?	*n*
18 kDa (IP)	18	15 337.2			*o*
B14[b]	14	14 964.2	14 963.7		*o*
15 kDa (IP)	15	12 536.4			*o*
B13[b]	13	13 226.4	13 229.5		*o*
13 kDa (IP)	13	10 535.7			*o*
B8[b]	8	10 990.6[a]	10 991.7		*o*
SDAP[c]	8	10 751.6[a]		acyl carrier protein	*p*
MLRQ	9	9 324.7		1 hydrophobic segment	*o*
B9[b,d]	9[f]	9 217.7[a]	9 295	1 hydrophobic segment	*o*
10 kDa (FP)	10	8 438.3			*q*
MWFE	7.5	8 135.4		1 hydrophobic segment	*o*
ND2[e]	30	39 282.1		9–10 hydrophobic segments	*r*

[a] The values include posttranslational modifications, with the exception of Fe-S clusters. SDAP includes both phosphopantetheine and the acyl group. The value for ND2 includes an N-formyl group. [b] The standard devations of the mass spectrometry measurements for B14, B13, B8, and B9, respectively, were 1.1, 4.4, 1.6, and 0.8. [c] This subunit is found in subcomplexes Iα and Iβ in the proportions 40:60, respectively. [d] Subunit B9 is modified in an unknown way, but the mass determined on other authentic samples of this protein was 9298. [e] Present at a level of about 10% relative to complex I. [f] Runswick et al., 1989. [g] Pilkington et al., 1991a. [h] Fearnley et al., 1989. [i] Fearnley et al., 1991. [j] Pilkington et al., 1991b. [k] Pilkington et al., 1989. [l] Dupuis et al., 1991a. [m] Arizmendi et al., 1992a. [n] Dupuis et al., 1991b. [o] Walker et al., 1992. [p] Runswick et al., 1991. [q] Skehel et al., 1991. [r] Anderson et al., 1982; Fearnley & Walker, 1992.

Table III: Subunits of Bovine Complex I Detected in Subcomplex Iβ

subunit	molecular weight gel[a]	molecular weight sequence	molecular weight ES-MS (SD)	hydrophobic segments
B22	22	21 700.6[b]	21 701.5 (5.2)	0
PDSW	22	20 833.6		1
ASHI	19	18 737.0		1
SGDH	16	16 726.3		1
B18	18	16 476.7[b]	16 477.9 (2.1)	0
B17	16.5	15 434.9[b]	15 438.5 (0.6)	1
B15	15	15 095.1[b]	15 095.2 (0.7)	1
B12	12	11 009.5[b]	11 039.0 (1.0)[c]	0
SDAP[d]	8	10 751.6[b]		0
AGGG	7.9	8 493.3		1
MNLL	7	6 966.1		1
ND4	39	52 127.1[b]		12
ND5	50	68 341.5[b]		14–16

[a] Subunits B22, B18, B17, B15, and B12 were isolated from subcomplex Iβ by HPLC (see Materials and Methods) and characterized by sequencing peptides (Walker et al., 1992). ND4 and ND5 were recovered from Iβ after fraction of the subunits in polyacrylamide gels, deformylated, and subjected to N-terminal sequencing. Other subunits were detected by N-terminal sequencing after transfer of proteins to a PVDF membrane. [b] The values include posttranslational modifications (except for B12, where the nature of the modification is not known). Subunit SDAP includes both phosphopantetheine and an acyl group (Runswick et al., 1991). The values for ND4 and ND5 include an *N*-formyl group. B18 has an *N*-α-myristyl group, and B22, B17, and B15 are N-α-acetylated (Walker et al., 1992). [c] The value measured on an authentic sample of B12 was 11 041.3 (standard deviation 0.8). [d] Subunit SDAP is the only subunit found in both subcomplexes Iα and Iβ in the approximate relative amounts of 40:60, respectively.

B12 in Iβ has been demonstrated in a similar way (see Tables II and III). However, it is not possible by this technique to obtain quantitative estimates of the amounts of each protein in the subcomplex. During sequence analysis of complex I, subunits B22, B18, B17, B15, and B12 were also purified from subcomplex Iβ by chromatography and extensively characterized by protein chemical analysis (Walker et al., 1992).

The seven subunits of complex I that are encoded in mitochondrial DNA (ND1–ND6 and ND4L) also have modified N-terminals. Their hydrophobicity poses different problems of detection that have not been overcome so far by mass spectrometry. In addition, they usually give rise to diffuse and weakly staining bands in polyacrylamide gels. A number of these subunits can be extracted selectively with organic solvents from intact complex I made by the classical method. When detergents have been introduced, the selectivity is lost, as it is, for example, in samples of either complex I made by the new chromatographic procedure or the subcomplexes. Therefore, these subunits have been recovered from samples fractionated in polyacrylamide gels, and after removal of the N-formyl group with mild acid, N-terminal sequences have been determined. In this way the presence of subunit ND2 has been demonstrated in subcomplex Iα. However, it is considerably diminished in some preparations of subcomplex Iα in comparison with complex I. This matter requires further investigation. Subunits ND4 and ND5, the two largest hydrophobic subunits of complex I encoded in mitochondrial DNA, are in subcomplex Iβ. The three other hydrophobic mitochondrial gene products in complex I have not been detected in the subcomplexes, although their presence is not excluded.

The sequences of the identified subunits of subcomplexes Iα and Iβ differ in their contents of hydrophobic segments with the potential to be folded into membrane-spanning α-helices. In subcomplex Iα, subunit ND2 contains about 9–10 such hydrophobic regions (Fearnley & Walker, 1992), and subunits MWFE, MLRQ, and B9 have 1 each (see Table II). In contrast, subcomplex Iβ contains not only ND4 and ND5, with possibly 12 and 14–16 potential transmembrane α-helices, respectively (Fearnley & Walker, 1992), but also 7 nuclear subunits which appear to be likely also to have 1 span each (see Table III). Therefore, subcomplexes Iα and Iβ represent predominantly hydrophilic (globular) and hydrophobic (membrane) domains of intact complex I.

Table IV: Subunits of Bovine Complex I Not (Yet) Detected in Subcomplexes Iα and Iβ

subunit	molecular weight by gel[a] (K)	molecular weight from sequence	hydrophobic segments
ND1	30	35 698.0[b]	8
ND3	15	13 082.6[b]	3
ND4L	10	10 825.2[b]	3
ND6	nd	19 105.6[b]	4–5
KFYI	6	5 828.7	1

[a] Determined on gels made according to Laemmli (1970). [b] Calculated with an α-N-formyl group.

Assuming that the subunits are present in unit stoichiometries, the molecular masses of subcomplexes Iα and Iβ are about 523 000 and 282 000 Da, respectively, representing approximately at least 50% and 33% of the entire complex. (This calculation assumes that the 42-kDa and ND2 subunits are part of subcomplex Iα, although, as mentioned above, they appear to be present in substoichiometric amounts.) Subcomplex Iα was estimated from the FMN content per milligram of protein of complex I and of subcomplex Iα (Table II) to contain about 70% of the protein mass of complex I.

Several subunits of intact complex I are not present or have not been detected in either subcomplex (see Table IV). At least subunit KFYI, the smallest protein detected in complex I, seems to be absent from both subcomplexes. Subunit ND6, which is encoded in mitochondrial DNA and is thought to be part of complex I, has not been detected so far by sequencing in either complex I or the subcomplexes.

DISCUSSION

Purification of Complex I. The main advantages of the new method of purifying mitochondrial complex I over earlier methods are its rapidity and reproducibility. Also, the enzyme is devoid of traces of other mitochondrial membrane enzymes (mainly transhydrogenase, ATP synthase, cytochrome bc_1) that are usually found in enzyme made by other means. Since the new method is based on simple precipitations and chromatographic steps, it potentially lends itself to small-scale purification, such as is required in study of defective forms of the enzyme associated with some human diseases (Morgan-Hughes et al., 1988; Schapira et al., 1989; Walker, 1992).

The subunit compositions of the preparations of enzyme made by the conventional and new procedures are very similar. Some minor differences in subunit composition are evident, but whether these are significant is not known at present. For example, the 42-kDa subunit appears to be depleted in the new preparation.

Another difference between the standard and new preparations of complex I is that the activity of enzyme made by the new route is not sensitive to the inhibitor rotenone (Table I). Similar changes were induced in complex I prepared by the conventional method by chromatography on Mono Q (Figure 3, Table I). The lack of sensitivity of the activity to inhibitors of both complex I prepared by the new method and

of subcomplex Iα requires further investigation. It is possible, for example, that some critical subunit is absent from the preparations or that the sensitivity depends upon a particular phospholipid composition or the presence of Q_{10} (van Belzen et al., 1990). Another possible explanation is that, after chromatography, ubiquinone-1 accepts electrons at a different site than in the native enzyme, thereby bypassing the rotenone inhibited site. It has been noted previously that the sensitivity to rotenone of complex I prepared by the conventional route is rather unstable in the purified enzyme (Yagi, 1986), and we have observed in similar preparations that a progressive loss of sensitivity accompanies repetitive freezing and thawing cycles. In addition, it should be noted that there was no cytochrome *c* reductase activity in either complex I or subcomplex Iα unless ubiquinone-1 was also added to the reaction mixture. In soluble NADH dehydrogenase prepared by perchlorate treatment, the cytochrome *c* reductase activity almost equals that of ubiquinone-1 reduction and is about half of the ferricyanide reduction rate (Galante & Hatefi, 1978). This indicates that the site accessible to cytochrome *c* in the flavoprotein (FP) fraction is not exposed in subcomplex Iα.

The line shapes of the EPR spectra of clusters 2 and 3 in the new preparation are slightly broadened relative to either native complex I in mitochondria or complex I isolated by the conventional route (Figure 6). The line widths of the spectra at 9 GHz of the individual clusters in complex I prepared according to Hatefi and Rieske (1967) receive contributions from both *g* strain and unresolved superhyperfine interactions (Albracht, 1984). In the case of cluster 2 the contribution from unresolved superhyperfine interaction to the line width at 9 GHz of its g_z line at 2.05 is still comparable to the contribution from the *g* strain (Albracht, 1984). At 35 GHz the width of this line (in units of *g*) is smaller than at 9 GHz and is also considerably smaller than the width of the g_z line of the EPR spectrum at 35 GHz of the binuclear cluster in spinach ferredoxin preparations (Hagen & Albracht, 1982; Albracht, 1984). This means that there is very little *g* strain (Fritz et al., 1971) in cluster 2. It is less likely that the increase in line width of the signal of cluster 2 is caused by an increase of superhyperfine interaction. Therefore, it is assumed that the increased width and the asymmetry of the line shape of cluster 2 in the new preparation is due to a spread in *g* values caused by structural changes in the vicinity of the individual clusters, resulting from the removal of components (subunits and/or phospholipids) by chromatography. The line shapes of the clusters 1b and 4 were not noticeably affected. Cluster 3 was slightly affected in that its g_z line shifted downfield.

Subcomplexes Iα and Iβ. The dissociation of bovine complex I into subcomplexes Iα and Iβ provides important information about both the structure of the enzyme and the electron pathway from NADH to ubiquinone. The sequences of the subunits of subcomplex Iα indicate that it is predominantly, although not exclusively, a hydrophilic globular domain. It contains among its 22 or so subunits the 3 subunits of the water-soluble FP fraction and 7 subunits of the water-soluble IP fraction. In addition, subunits TYKY and PSST have been detected in some preparations of the IP fraction (Masui et al., 1991a,b) and so can also be considered to be water-soluble proteins. Likewise, subunits B13 and B14 have been isolated from a water-soluble fraction prepared from complex I (Walker et al., 1992). Of its 22 or so subunits only 4 of them appear from their sequences to be likely to contain membrane-spanning segments, and one of those, ND2, appears to be present at a diminished level relative to complex I.

As summarized in Table II, subcomplex Iα contains all of the subunits with which prosthetic groups are thought to be associated in the intact complex I, and only one of them, subunit SDAP (the acyl carrier protein), is present in both subcomplexes. In addition to being the site of FMN and NADH binding, the 51-kDa subunit also appears to contain a [4Fe-4S] cluster, possibly cluster 4 (Ragan et al., 1982; Ohnishi et al., 1985; Ragan, 1987), and it has been proposed that the 24-kDa subunit contains the [2Fe-2S] cluster 1b (Ragan et al., 1982; Ohnishi et al., 1985; Ragan, 1987). A [4Fe-4S] cluster is likely to be in the 75-kDa subunit and possibly a binuclear cluster also (Runswick et al., 1989; Walker, 1992), and the sequence of subunit TYKY, also present in subcomplex Iα, suggests that it contains two further [4Fe-4S] clusters (Dupuis et al., 1991a). Another protein detected in subcomplex Iα, subunit PGIV, contains eight conserved cysteines regularly spaced through its sequence and could be another Fe-S protein (Dupuis et al., 1991b), although there is no direct evidence to support this suggestion at present. Lastly, subunit PSST contains three cysteines that are conserved across a wide range of species and could possibly be an iron–sulfur protein also (Arizmendi et al., 1992). In contrast, and in accord with the EPR spectra, the sequences of the subunits of subcomplex Iβ contain no sequence motifs that suggest the presence of Fe-S clusters.

The most likely interpretation of these observations is that the bulk of subcomplexes Iα lies mostly in the aqueous phase, presumably on the matrix side. In this respect at least, it appears to have much in common with the small NADH dehydrogenase produced in *N. crassa* mitochondria by inhibition of the organellar protein synthesis (Friedrich et al., 1989). The extent of overlap between the subunit compositions of bovine subcomplex Iα and the fungal small complex cannot be assessed precisely at present as the *N. crassa* complex has not been so extensively characterized in this respect, although it is known that homologues of the bovine 75-, 51-, 49-, 39-, 30-, 24-, and 19-kDa and SDAP (acyl carrier protein) subunits are present in the fungal complex I (Preis et al., 1991, 1990; Röhlen et al., 1991; Videira et al., 1990a,b; Weidner et al., 1991; van der Pas, 1991; Sackmann et al., 1991).

An important difference between bovine subcomplex Iα and the *N. crassa* small complex is that the latter lacks cluster 2. It has been suggested that, in the *N. crassa* enzyme, cluster 2 is associated with the hydrophobic subunit ND5 (Weiss et al., 1991), whereas sequence studies presented here show that the bovine ND5 subunit forms part of subcomplex Iβ and is absent from subcomplex Iα. Since subcomplex Iα contains cluster 2 (Figure 6), it cannot be bound by ND5, at least in the bovine enzyme. It is also worth noting that no cysteines are conserved in ND5 subunits characterized from a range of species (Fearnley & Walker, 1992).

The soluble high-molecular-weight NADH dehydrogenase prepared from bovine mitochondria by treatment with phospholipase and incubation at elevated pH (Cremona & Kearney, 1964) also appears to contain all of the Fe-S clusters detected in complex I by EPR measurements, and the line shapes in its spectra are similar to those of both chromatographically purified complex I and subcomplex Iα (Figure 6). Like subcomplex Iα this preparation can also reduce ferricyanide, but in contrast to subcomplex Iα it cannot reduce quinone analogs such as ubiquinone-1 (Kowal et al., 1986). The reason for this difference is not known, but the subunit composition of the soluble high-molecular-weight NADH dehydrogenase has not been studied in detail, and it is possible that it lacks

an essential subunit for transfer of electrons to quinone which is present in subcomplex Iα.

Subcomplex Iβ appears to represent a predominantly hydrophobic intrinsic membrane domain of complex I. With the exception of the phosphopantetheine moiety attached to the acyl carrier protein subunit, SDAP (Runswick et al., 1991), it has no known prosthetic groups, and the role of this domain in the functioning of complex I and its interactions with complex Iα remain to be elucidated.

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REFERENCES

Albracht, S. P. J. (1974a) *Biochim. Biophys. Acta 347*, 183–192.
Albracht, S. P. J. (1974b) *J. Magn. Reson. 13*, 299–303.
Albracht, S. P. J. (1984) *Curr. Top. Bioenerg. 13*, 79–106.
Albracht, S. P. J., & Slater, E. C. (1971) *Biochim. Biophys. Acta 245*, 503–507.
Albracht, S. P. J., & Subramanian, J. (1977) *Biochim. Biophys. Acta 462*, 36–48.
Albracht, S. P. J., Dooijewaard, G., Leeuwerik, F. J., & van Swol, B. (1977) *Biochim. Biophys. Acta 459*, 300–317.
Anderson, S., de Bruijn, M. H. L., Coulson, A. R., Eperon, I. C., Sanger, F., & Young, I. G. (1982) *J. Mol. Biol. 156*, 683–717.
Arizmendi, J. M., Runswick, M. J., Skehel, J. M., & Walker, J. E. (1992a) *FEBS Lett. 301*, 237–242.
Arizmendi, J. M., Skehel, J. M., Runswick, M. J., Fearnley, I. M., & Walker, J. E. (1992b) *FEBS Lett.* (in press).
Beinert, H., & Sands, R. H. (1960) *Biochem. Biophys. Res. Commun. 3*, 41–46.
Beinert, H., & Ruzicka, F. J. (1975) in *Electron transfer chains and oxidative phosphorylation* (Papa, S., Palmieri, F., Slater, E. C., & Siliprandi, N., Eds.) pp 37–42, North-Holland, Amsterdam.
Beinert, H., & Albracht, S. P. J. (1982) *Biochim. Biophys. Acta 683*, 245–277.
Brown, G. C., & Brand, M. D. (1988) *Biochem. J. 252*, 473–479.
Cremona, T., & Kearney, E. B. (1964) *J. Biol. Chem. 239*, 2328–2334.
De Jonge, P. C., & Westerhoff, H. V. (1982) *Biochem. J. 204*, 515–523.
Dupuis, A., Skehel, J. M., & Walker, J. E. (1991a) *Biochemistry 30*, 2954–2960.
Dupuis, A., Skehel, J. M., & Walker, J. E. (1991b) *Biochem. J. 277*, 11–15.
Fearnley, I. M., & Walker, J. E. (1987) *Biochemistry 26*, 8247–8251.
Fearnley, I. M., & Walker, J. E. (1992) *Biochim. Biophys. Acta Bioenerg. Rev.* (in press).
Fearnley, I. M., Runswick, M. J., & Walker, J. E. (1989) *EMBO J. 8*, 665–672.
Fearnley, I. M., Finel, M., Skehel, J. M., & Walker, J. E. (1991) *Biochem. J. 278*, 821–829.
Friedrich, T., Hofhaus, G., Ise, W., Nehls, U., Schmitz, B., & Weiss, H. (1989) *Eur. J. Biochem. 180*, 173–180.
Fritz, J., Anderson, R., Fee, J., Palmer, G., Sands, R. H., Tsibris, J. C. M., Gunsalus, I. C., Orme-Johnson, W. H., & Beinert, H. (1971) *Biochim. Biophys. Acta 253*, 110–133.
Galante, Y. M., & Hatefi, Y. (1978) *Methods Enzymol. 53*, 15–21.
Galante, Y. M., & Hatefi, Y. (1979) *Arch. Biochem. Biophys. 192*, 559–568.
Hagen, W. R., & Albracht, S. P. J. (1982) *Biochim. Biophys. Acta 702*, 61–71.
Hatefi, Y., & Rieske, J. S. (1967) *Methods Enzymol. 10*, 235–239.
Hearshen, D. O., Dunham, W. R., Albracht, S. P. J., Ohnishi, T., & Beinert, H. (1981) *FEBS Lett. 133*, 287–290.
Ingledew, W. J., & Ohnishi, T. (1980) *Biochem. J. 186*, 111–117.
Kowal, A. T., Morningstar, J. E., Johnson, M. K., Ramsey, R. R., & Singer, T. P. (1986) *J. Biol. Chem. 261*, 9239–9245.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lemasters, J. J. (1984) *J. Biol. Chem. 259*, 13123–13130.
Lundin, A., & Aasa, R. (1972) *J. Magn. Reson. 8*, 70–73.
Masui, R., Wakabayashi, S., Matsubara, H., & Hatefi, Y. (1991a) *J. Biochem. 109*, 534–543.
Masui, R., Wakabayashi, S., Matsubara, H., & Hatefi, Y. (1991b) *J. Biochem. 110*, 575–582.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.
Morgan-Hughes, J. A., Schapira, A. H. V., Cooper, J. M., & Clark, J. B. (1988) *J. Bioenerg. Biomembr. 20*, 365–382.
Ohnishi, T. (1975) *Biochim. Biophys. Acta 387*, 475–490.
Ohnishi, T. (1979) in *Membrane proteins in energy transduction* (Capaldi, R. A., Ed.) pp 1–87, Dekker, New York.
Ohnishi, T., & Salerno, J. C. (1982) in *Iron–Sulfur Proteins* (Spiro, T. G., Ed.) Vol. 4, pp 285–327, John Wiley & Sons, New York.
Ohnishi, T., Asakura, T., Wohlrab, H., Yonetani, T., & Chance, B. (1970) *J. Biol. Chem. 245*, 901–902.
Ohnishi, T., Blum, H., Galante, Y. M., & Hatefi, Y. (1981) *J. Biol. Chem. 256*, 9216–9220.
Ohnishi, T., Ragan, C. I., & Hatefi, Y. (1985) *J. Biol. Chem. 260*, 2782–2788.
Orme-Johnson, N. R., Orme-Johnson, W. H., Hansen, R. E., Beinert, H., & Hatefi, Y. (1971) *Biochem. Biophys. Res. Commun. 44*, 446–452.
Orme-Johnson, N. R., Hansen, R., & Beinert, H. (1974) *J. Biol. Chem. 249*, 1922–1927.
Paech, C., Friend, A., & Singer, T. P. (1982) *Biochem. J. 203*, 477–481.
Pilkington, S. J., & Walker, J. E. (1989) *Biochemistry 28*, 3257–3264.
Pilkington, S. J., Skehel, J. M., Gennis, R. B., & Walker, J. E. (1991a) *Biochemistry 30*, 2166–2175.
Pilkington, S. J., Skehel, J. M., & Walker, J. E. (1991b) *Biochemistry 30*, 1901–1908.
Preis, D., van der Pas, J. C., Nehls, U., Röhlen, D., Sackmann, U., Jahnke, U., & Weiss, H. (1990) *Curr. Genet. 18*, 59–64.
Preis, D., Weidner, U., Conzen, C., Azevedo, J. E., Nehls, U., Röhlen, D. A., van der Pas, J., Sackmann, U., Schneider, R., Werner, S., & Weiss, H. (1991) *Biochim. Biophys. Acta 1090*, 133–138.
Ragan, C. I. (1987) *Curr. Top. Bioenerg. 15*, 1–36.
Ragan, C. I., & Racker, E. (1973) *J. Biol. Chem. 248*, 6876–6884.
Ragan, C. I., Galante, Y. M., Hatefi, Y., & Ohnishi, T. (1982) *Biochemistry 21*, 590–594.
Ragan, C. I., Wilson, M. T., Darley-Usmar, V. M., & Lower, P. N. (1987) in *Mitochondria: A Practical Approach* (Darley-Usmar, V. M., Rickwood, D., & Wilson, M. T., Eds.) pp 79–112, IRL Press, Oxford and Washington.
Rich, P., & Bonner, W. D. (1978) in *Functions of alternative respiratory oxidases* (Lloyd, D., Degn, H., & Hill, G. C., Eds.) pp 61–68, Pergamon Press, New York.
Röhlen, D. A., Hoffmann, J., van der Pas, J. C., Nehls, U., Preis, D., Sackman, U., & Weiss, H. (1991) *FEBS Lett. 278*, 75–78.
Rottenberg, H., & Gutman, M. (1977) *Biochemistry 16*, 3220–3227.
Runswick, M. J., Gennis, R. B., Fearnley, I. M., & Walker, J. E. (1989) *Biochemistry 28*, 9452–9459.
Runswick, M. J., Fearnley, I. M., Skehel, J. M., & Walker, J. E. (1991) *FEBS Lett. 286*, 121–124.
Sackmann, U., Zensen, R., Röhlen, D., Jahnke, U., & Weiss, H. (1991) *Eur. J. Biochem. 200*, 463–469.
Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.
Schapira, A. H. V., Cooper, J. M., Dexter, D., Jenner, P., Clark, J. B., & Marsden, C. D. (1989) *Lancet*, 1269.

Scholes, T. A., & Hinkle, P. C. (1984) *Biochemistry 23*, 3341–3345.
Skehel, J. M., Pilkington, S. J., Runswick, M. J., Fearnley, I. M., & Walker, J. E. (1991) *FEBS Lett. 282*, 135–138.
Smith, A. L. (1967) *Methods Enzymol. 10*, 81–86.
Van Belzen, R., van Gaalen, M. C. M., Cuypers, P. A., & Albracht, S. P. J. (1990) *Biochim. Biophys. Acta 1017*, 152–159.
Van Belzen, R., de Jong, A. M. Ph., & Albracht, S. J. P. (1992) *Eur. J. Biochem.* (in press).
van der Pas, J. C., Röhlen, D. A., Weidner, U., & Weiss, H. (1991) *Biochim. Biophys. Acta 1089*, 389–390.
Vanneste, W. H. (1966) *Biochemistry 5*, 838–848.
Videira, A., Tropschüg, M., & Werner, S. (1990a) *Biochem. Biophys. Res. Commun. 171*, 1168–1174.
Videira, A., Tropschüg, M., Wachter, E., Schneider, H., & Werner, S. (1990b) *J. Biol. Chem. 265*, 13060–13065.
Walker, J. E. (1992) *Q. Rev. Biophys.* (in press).
Walker, J. E., Arizmendi, J. M., Dupuis, A., Fearnley, I. M., Finel, M., Medd, S. M., Pilkington, S. J., Runswick, M. J., & Skehel, J. M. (1992) *J. Mol. Biol. 226*, 1051–1072.
Wang, D.-C., Meinhardt, S. W., Sackmann, U., Weiss, H., & Ohnishi, T. (1991) *Eur. J. Biochem. 197*, 257–264.
Weidner, U., Sackmann, U., Nehls, U., & Weiss, H. (1991) *Biochim. Biophys. Acta 1089*, 391–392.
Weiss, H., Friedrich, T., Hofhaus, G., & Preis, D. (1991) *Eur. J. Biochem. 197*, 563–576.
Wikström, M. (1984) *FEBS Lett. 169*, 300–304.
Yagi, T. (1986) *Arch. Biochem. Biophys. 250*, 302–311.