The Structure and Subunit Composition of the Particulate NADH–Ubiquinone Reductase of Bovine Heart Mitochondria

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1. Preparations of NADH–ubiquinone reductase from bovine heart mitochondria (Complex I) were shown to contain at least 16 polypeptides by gel electrophoresis in the presence of sodium dodecyl sulphate. 2. High-molecular-weight soluble NADH dehydrogenase prepared from Triton X-100 extracts of submitochondrial particles [Baugh & King (1972) Biochem. Biophys. Res. Commun. 49, 1165–1173] was similar to Complex I in its polypeptide composition. 3. Solubilization of Complex I by phospholipase A treatment and subsequent sucrose-density-gradient centrifugation did not alter the polypeptide composition. 4. Lysophosphatidylcholine treatment of Complex I caused some selective solubilization of a polypeptide of mol.wt. 33000 previously postulated to be the transmembrane component of Complex I in the mitochondrial membrane [Ragan (1975) in Energy Transducing Membranes: Structure, Function and Reconstitution (Bennun, Bacila & Najjar, eds.), Junk, The Hague, in the press]. 5. Chaotropic resolution of Complex I caused solubilization of polypeptides of molecular weights 75000, 53000, 29000, 26000 and 15500 and traces of others in the 10000–20000-mol.wt. range. 6. The major components of the iron–protein fraction from chaotropic resolution had molecular weights of 75000, 53000 and 29000, whereas the flavoprotein contained polypeptides of molecular weights 53000 and 26000 in a 1:1 molar ratio. 7. Iodination of Complex I by lactoperoxidase indicated that the water-soluble polypeptides released by chaotropic resolution, in particular those of the flavoprotein fraction, were largely buried in the intact Complex. 8. The polypeptides of molecular weights 75000, 53000, 42000, 39000, 33000, 29000 and 26000 were present in 1:2:1:1:1:1:1 molar proportions. The two subunits of molecular weight 53000 are probably non-identical.

The organization of proteins in biological membranes has been the subject of intensive study over the past few years. The inner membrane of mitochondria is particularly diverse in its polypeptide content (Hare & Crane, 1974) and, because of the possible relevance to the mechanism of oxidative phosphorylation, there is much interest in the 'sidedness' of the membrane and the vectorial organization of the constituent proteins (Harmon et al., 1974). Because membrane proteins have been separated primarily by gel electrophoresis in the presence of sodium dodecyl sulphate, positive identification of individual polypeptides with enzyme function has proved difficult, and there is still considerable disagreement in the literature as to the polypeptide composition of relatively simple membrane complexes such as Complex III (ubiquinone–cytochrome c reductase) (Gellerfors & Nelson, 1975). As far as polypeptide composition is concerned, the NADH dehydrogenase region of the respiratory chain has received little attention, and two reports of the composition of Complex I (NADH–ubiquinone reductase; Hatefi et al., 1962a) are in almost total disagreement (Hare & Crane, 1974; Capaldi, 1974). In addition to Complex I, soluble NADH dehydrogenases have been described of both the high- (Ringler et al., 1963; Huang & Pharo, 1971; Baugh & King, 1972) and low- (e.g. King & Howard, 1967; Hatefi & Stempel, 1967) molecular-weight varieties. Chaotropic resolution of Complex I (Hatefi & Stempel, 1967) gives rise not only to a low-molecular-weight NADH dehydrogenase, but to a soluble iron–protein fraction as well. The enzymic properties of all these preparations have been described in detail but their polypeptide compositions have not. In the present report, the constituent polypeptides of Complex I and the high-molecular-weight NADH dehydrogenase are described, and a detailed study of the chaotropic resolution of Complex I is presented.

Materials and Methods

Complex I (EC 1.6.5.3) (Hatefi et al., 1962a) and soluble NADH dehydrogenase (EC 1.6.99.3) (Baugh & King, 1972) were prepared from bovine heart as
described in the references. Phospholipase A (EC 3.1.1.4) was purified from *Naja naja* venom (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) by the method of King & Howard (1967). Protein (Lowry et al., 1951), iron (Smith et al., 1952), acid-labile sulphide (King & Morris, 1967) and FMN (Ragan & Racker, 1973) were assayed as described in the references. Bovine serum albumin (fraction V; Sigma Chemical Co.) was used as a standard for protein determination.

Iodination of proteins

Iodination was carried out as described by Phillips & Morrison (1971). Experimental details are given in the Figure and Table legends. Iodination was terminated by the addition of EDTA (1 mM) and 2-mercaptoethanol (0.1%). Lactoperoxidase (EC 1.11.1.7) was obtained from Sigma Chemical Co. and Na^{125}I from The Radiochemical Centre, Amersham, Bucks., U.K.

Polyacrylamide-gel electrophoresis

Samples (0.5–1 mg of protein/ml) were denatured in 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol for 12–20 h at room temperature (21°C). Gels containing 10% (w/v) acrylamide and 0.27% bisacrylamide were polymerized in glass tubes (12 cm × 6 mm internal diam.) as described by Weber & Osborn (1969). Electrophoresis was performed at 5 mA/gel for 16 h. Gels were fixed, stained and destained by the method of Weber & Osborn (1969) and scanned in a Joyce–Loebl densitometer (Joyce–Loebl and Co., Gateshead, U.K.). Gels containing radioactive bands were sliced in the semi-frozen state into approx. 1.8 mm slices with a stack of razor blades and each slice was counted directly for radioactivity in a Beckman gamma counter for 5 min. Molecular weights were estimated by co-electrophoresis or parallel electrophoresis with standard proteins, namely lactoperoxidase (EC 1.11.1.7, mol.wt. 82000), bovine serum albumin (mol.wt. 68000), pyruvate kinase (EC 2.7.1.40, mol.wt. 57000) (a gift from Dr. P. Poat), ovalbumin (mol.wt. 43000), lactate dehydrogenase (EC 1.1.1.27, mol.wt. 36000), trypsin (EC 3.4.21.4, mol.wt. 23300), haemoglobin (mol.wt. 15500) and cytochrome c (mol.wt. 11700), which were otherwise from commercial sources. With the gel system described, the mobility was linearly related to the logarithms of the molecular weights for all the marker proteins. The highest-molecular-weight band and the two lowest-molecular-weight bands of Complex I were outside the range covered by the markers. Their molecular weights were determined by extrapolation and are therefore not necessarily accurate.

Enzyme assays

NADH–ubiquinone-1 reductase was measured by the decrease in extinction at 340 nm of a solution containing 20 μmol of potassium phosphate, pH 8.0, 0.1 μmol of NADH, 0.06 μmol of ubiquinone-1, 0.1 μmol of soya-bean phosphatidylcholine (Ragan & Racker, 1973) and enzyme in 1 ml. Where indicated, 0.5 μg of rotenone was included. NADH–menadione reductase was measured similarly except that 0.2 μmol of menadione replaced the ubiquinone-1 and phosphatidylcholine. NADH–ferricyanide reductase was measured by the decrease in extinction at 420 nm of a solution containing 20 μmol of potassium phosphate, pH 8.0, 0.1 μmol of NADH, 1 μmol of K₃Fe(CN)₆ and enzyme in 1 ml. Nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) was measured by the decrease in extinction at 340 nm of a solution containing 0.1 mmol of Tris/acetate, pH 7.4, 0.07 mol of NADH, 10 μg of L-lactate dehydrogenase (Sigma Chemical Co.), 2 μmol of NADPH and enzyme in 1 ml. All measurements were made at room temperature. Where specific comparison of activities with literature values has been made, assays were performed exactly as described in the literature.

Results

Polypeptide composition of Complex I

Complex I preparations contained as a routine (per mg of protein) 28–29 nmol of iron or acid-labile sulphide and 1.1–1.3 nmol of FMN. Activities at 38°C ranged from 280 to 340 μmol of NADH oxidized/min per mg of protein (Vₘₐₓ, with ferricyanide as acceptor) and 7–15 μmol of NADH oxidized/min per mg of protein (Vₘₐₓ, with ubiquinone-1 as acceptor). The polypeptide composition of a typical preparation is shown in Fig. 1. The same position and relative amount of each component was observed in a large number of preparations, except for the content of the slowest-moving component, which was variable. The molecular weights of the bands are also indicated in Fig. 1, and agree closely with those given by Hare & Crane (1974), although only the major bands were resolved on their gels. Contamination by Complex III (ubiquinone–cytochrome c reductase; Hatefi et al., 1962b) was not significant, since the major components of Complex III, with mol.wts. of 47000 and 50000 (Gellerfors & Nelson, 1975), were clearly absent from Complex I. Complex I did not exhibit cytochrome c oxidase activity, consistent with the absence of cytochrome a+a₅ absorption bands in spectra of Complex I. The possibility that cytochrome oxidase apoproteins could contribute to the electrophoretic profile of Complex I is unlikely in view of the conditions used in the isolation of Complex I, which are considerably milder than those sometimes
used for the isolation of cytochrome oxidase [for example, treatment of cytochrome oxidase with 1.5 mg of cholate per mg of protein and 44% saturation with (NH₄)₂SO₄ led to negligible losses of haem a (Yu et al., 1975)]. Complex I did not contain any F₁ ATPase* (Penefsky et al., 1960), assayed either by its activity or by the presence of the major F₁ subunits with apparent mol.wts. of 59000 and 54000 as determined by electrophoresis in the presence of sodium dodecyl sulphate (Knowles & Penefsky, 1972). The non-identity of the polypeptide of mol.wt. 53000 with a subunit of F₁ ATPase was clearly established by co-electrophoresis of Complex I and F₁ ATPase (results not shown). Absence of a polypeptide of mol.wt. 70000 (Davis & Hatefi, 1971) indicated that Complex I did not contain succinate dehydrogenase.

**Polypeptide composition of soluble high-molecular-weight NADH dehydrogenases**

In the absence of any criteria for the purity of a particular multienzyme complex, it was decided to compare the polypeptide composition of similar preparations purified by radically different procedures. For the NADH dehydrogenase region of the respiratory chain, Complex I is the preparation of choice, since it exhibits all the properties expected of this segment. The soluble high-molecular-weight dehydrogenases lack rotenone-sensitive ubiquinone reductase activity (Singer & Gutman, 1970) and cannot recombine with the cytochromes to reconstitute NADH oxidase. Nevertheless they are similar to Complex I in their iron and flavin contents and in their electron-paramagnetic-resonance spectra (Gutman et al., 1971). A comparison of the polypeptide composition of Complex I and NADH dehydrogenase purified as described by Baugh & King (1972) (specific activity at Vₘₐₓ. for ferricyanide, 942μmol of NADH oxidized/min per mg of protein at 30°C) is shown in Fig. 2. The soluble dehydrogenase did not contain the 87000-mol.wt. polypeptide, and, compared with Complex I, contained relatively higher concentrations of polypeptides in the 30000- and 15000-mol.wt. regions. Direct comparison of peak heights was complicated by the consistently poor resolution of the polypeptides of the soluble dehydrogenase, particularly in the low-molecular-weight region (Hare & Crane, 1974), and the decreased staining intensity of the dehydrogenase subunits compared with Complex I (in Fig. 2, twice as much soluble dehydrogenase protein as Complex I protein was electrophoresed). Apart from these differences the profiles were quite similar despite the totally different isolation procedures for the two preparations.

The Baugh & King (1972) preparation is solubilized with Triton X-100. Another similar high-molecular-weight NADH dehydrogenase is solubilized by the action of phospholipase A (Ringler et al., 1963). In the experiment of Fig. 3, Complex I was incubated with phospholipase A, which caused complete loss of rotenone-sensitivity, and subjected to density-gradient centrifugation at pH10, as described for the final stage of purification of the soluble NADH dehydrogenase (Lusty et al., 1965). NADH dehydrogenase activity was located in a broad band, the less-dense material having slightly higher specific activity. Electrophoresis of representative fractions from across the gradient showed that in all cases the polypeptide composition was identical with that of Complex I, except for a progressive decrease in the concentration of the

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Fig. 1. *Subunit composition of Complex I*

Electrophoresis of Complex I on polyacrylamide gels was as described in the Materials and Methods section. Molecular weights in thousands are indicated by the postscript K, i.e. 87K is 87000.

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*Abbreviation: ATPase, adenosine triphosphatase.*
87000-mol. wt. polypeptide from heavier to lighter fractions (results not shown).

**Nicotinamide nucleotide transhydrogenase of Complex I**

Unlike the other respiratory complexes, Complex I contains nicotinamide nucleotide transhydrogenase activity (Hatefi & Hanstein, 1973). This activity does not appear to be a function of NADH dehydrogenase, but is due to a separate enzyme system (Ragan et al., 1974). It has been reported that transhydrogenase activity, but not dehydrogenase activity, can be solubilized from ox heart submitochondrial particles by low concentrations of lysophosphatidylcholine (Rydstrom et al., 1974). A partially purified transhydrogenase contains two polypeptides of mol. wt. approx. 30000, which taken together account for 70% of the total protein (Rydstrom, 1975). The effect of lysophosphatidylcholine on Complex I is shown in Fig. 4. Nicotinamide nucleotide transhydrogenase activity was solubilized completely by 0.1% lysophosphatidylcholine. However, 50–60% of the NADH dehydrogenase activity was also solubilized, although lower concentrations of lysophosphatidylcholine were effective. Despite the lack of separation of the two activities, Fig. 4 indicates that fractions with different ratios of the two activities could be obtained, and electrophoretic profiles of some of these are shown in Fig. 5. The supernatant fractions from either 0.02 or 0.1% lysophosphatidylcholine treatment contained increased concentrations of the 87000- and 30000-mol. wt. polypeptides. Soluble Complex I was dialysed against 200 vol. of 0.67 M sucrose/50 mM-Tris/HCl, pH 8.0, for 16 h at 4°C to remove bile salts. A portion (0.4 ml, containing 6.2 mg of protein) was incubated with 0.1 mg of purified phospholipase A at 30°C for 1 h. A parallel incubation received no phospholipase A. Samples were cooled to 4°C, mixed with 0.055 ml of 0.5 M glycine/NaOH, pH 10.0, and layered on 10 ml linear gradients containing 50 mM-glycine/NaOH, pH 10.0, and 28–65% (w/v) sucrose as described by Lusty et al. (1965). Centrifugation at 2°C was for 20 h at 35000 rev./min in the SW39 rotor of the Beckman L5-65 centrifuge. Tubes were punctured and approx. 0.3 ml fractions collected. Undigested Complex I was located in a tight band near the bottom of the tube. Fractions were assayed for protein (○) and NADH–ferricyanide reductase activity (●) as described in the Materials and Methods section.

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**Fig. 2. Polypeptide composition of Complex I and soluble NADH dehydrogenase**

Soluble NADH dehydrogenase, purified as described by Baugh & King (1972) (40 μg of protein) (a), and Complex I (20 μg of protein) (b), were electrophoresed as described in the Materials and Methods section except that 7 cm × 0.5 cm internal diam. gels were used.
SUBUNITS OF NADH-UBIQUINONE REDUCTASE

Complex I (0.5 mg) was incubated at 4°C in a final volume of 1 ml containing 0.1 M-Tris/acetate, pH 7.4, 3 mM-EDTA, 1 mM-dithiothreitol and the indicated concentrations of lysophosphatidylcholine. Total activity was unaffected by lysophosphatidylcholine in this concentration range. After 60 min, samples were centrifuged at 10000g for 4 h at 4°C in the 10×10 ml rotor of the MSE 65 centrifuge. Nicotinamide nucleotide transhydrogenase (C) and NADH-ferricyanide reductase (●) were assayed as described in the Materials and Methods section. Total (100%) activities were 0.68 μmol of NADP+ reduced/min per mg of original protein and 66 μmol of NADH oxidized/min per mg of original protein respectively.

Fig. 4. Effect of lysophosphatidylcholine on Complex I

33 000-mol.wt. polypeptides, whereas the pellet fraction from the 0.1% lysophosphatidylcholine treatment contained decreased concentrations of these bands. The 0.02% lysophosphatidylcholine supernatant and 0.1% lysophosphatidylcholine pellet contained a decreased transhydrogenase/dehydrogenase ratio, whereas the 0.1% lysophosphatidylcholine supernatant contained an increased ratio. Therefore presence of the 87 000- and 33 000-mol.wt. polypeptides did not correlate with the presence of transhydrogenase activity and it can therefore be concluded that transhydrogenase does not contribute significantly to the polypeptide profile of Complex I.

Chapotropic resolution of Complex I

Resolution of Complex I by NaClO₄ released relatively few polypeptides into solution, namely those of mol.wts. 75 000 (75 K), 53 K, 29 K, 26 K and 8 K, along with traces of others in the 10 K–20 K-molecular-weight range (Figs. 6a and 6b).

The iron–protein fraction (Fig. 6c) contained all of these components, but had a lesser content of the 53 K- and 26 K-mol.wt. bands, which were also found in the flavoprotein fraction (Fig. 6d). Chromatography of the iron–protein fraction on Sephadex G-200 in the presence of 1 M-urea caused removal of the residual 26 K-mol.wt. band, but not all of the 53 K-mol.wt. band (results not shown). This indicated that either the 53 K- and 26 K-mol.wt. components of the flavoprotein were relatively easily separated or that the 53 K-mol.wt. band was heterogeneous, a possibility that is discussed at greater length below.

The low-molecular-weight NADH dehydrogenase (flavoprotein) prepared by either urea or perchlorate resolution of Complex I contained components of molecular weights 53 K and 26 K and small amounts of a component of molecular weight about 8 K. Activities were as high as or higher than those claimed by Hatefi & Stempel (1969). The molar ratio of these two bands was estimated from the relative stain intensity and the molecular weights to be 1.0. The summed mol.wt. of 79 000 agreed well with the molecular weight calculated from the FMN content [13.5–14.5 nmol/mg of protein (Hatefi & Stempel, 1969)] or estimated by Sephadex G-200 chromatography, where a value of 76 000 was obtained (Fig. 7). It is proposed therefore that the low-molecular-weight NADH dehydrogenase contains two subunits in a 1:1 molar ratio. The 8 K-mol.wt. band is probably an impurity, since it is present in low and variable amounts.

The fraction precipitated from the perchlorate-soluble material between 27.5 and 36.4% saturation with (NH₄)₂SO₄ did not contain significant amounts of protein compared with the iron–protein fraction.

Fig. 5. Polypeptide composition of fractions from lysophosphatidylcholine treatment of Complex I

Portions (60 μl each) of fractions from the experiment of Fig. 4 were treated with 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol as described in the Materials and Methods section and electrophoresed. Fractions were as follows: (a) treated with 0.1% lysophosphatidylcholine, uncentrifuged; (b) treated with 0.02% lysophosphatidylcholine, supernatant fraction; (c) treated with 0.1% lysophosphatidylcholine, supernatant fraction; (d) treated with 0.1% lysophosphatidylcholine, pellet fraction.

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Complex I was iodinated as described in the legend to Fig. 8. Approx. 20mg of Complex I protein in a volume of 2ml containing 0.67M-sucrose and 50mM-Tris/HCl, pH8.0, was treated with 0.5M-NaClO4 at 35°C for 10min, cooled to 4°C and centrifuged at 100000g for 15min in the 10 x 10 ml rotor of the MSE 65 centrifuge. The supernatant was fractionated with (NH4)2SO4 as described by Hatefi & Stempel (1967) to give the iron-protein and flavoprotein fractions. The pellet from the NaClO4 treatment was re-extracted with NaClO4 under the same conditions to ensure complete removal of solubilized polypeptides. This second treatment only removes those polypeptides solubilized by the first NaClO4 extraction but trapped in the pellet volume (Table 1). Gel electrophoresis was performed on 100µg of unresolved Complex I (Fig. 1) and portions of the other fractions that would have been derived from 100µg of Complex I protein (Fig. 6). Apart from the handling losses, the profiles of Figs. 1 and 6 are directly comparable on a recovery basis. (a) NaClO4-insoluble material; (b) NaClO4-soluble material; (c) iron-protein fraction; (d) flavoprotein fraction.

(0-27.5% saturation) or the flavoprotein fraction (36.4-52.9% saturation). Almost no protein was left in the supernatant after the addition of (NH4)2SO4 to 52.9% saturation.

The distribution of NADH- and dithionite-reducible flavin and non-haem iron in the various fractions from perchlorate resolution was measured by the spectroscopic change at 450nm, as shown in Table 1. After perchlorate treatment, 98% of the NADH-reducible material was in the insoluble fraction, but only 74% of that reducible by dithionite after NADH. The remainder of the dithionite-reducible material remained in the insoluble residue, which contained 71% of the original protein, even after repeated perchlorate treatment. This clearly indicates two types of non-haem iron-protein in Complex I. Most (60%) of the NADH-reducible material was found in the flavoprotein fraction, which contained 5.7% of the original protein. The 10-11-fold purification of this material from

Complex I agrees exactly with the purification of the FMN (Hatefi & Stempel, 1969). Most of the soluble dithionite-reducible material was in the iron-protein fraction, which contained 22% of the original protein. Variable contamination of this fraction with NADH-reducible material was encountered, agreeing with the presence of some of the 26K-mol.wt. band in this fraction (Fig. 6).

Iodination of Complex I

Lactoperoxidase-catalysed iodination of Complex I caused incorporation of 125I into most of the polypeptides (Ragan, 1975). In Fig. 8, the distribution of radioactivity among the various polypeptides of Complex I and fractions from the perchlorate fractionation of Fig. 6 is shown. In Complex I (Fig. 8a), all polypeptides were apparently labelled, except for the 29K-, 26K- and possibly the 23.5K- and 15.5K-mol.wt. bands. The perchlorate-solubilized material (Fig. 8c), although containing 30% of the total protein, was labelled to only a limited extent, predominantly in the 75K- and 53K-mol.wt. bands. Despite almost complete solubilization of the polypeptide of molecular weight 53 K, most of the 125I at this position remained in the insoluble residue (Fig. 8b). Therefore there are at least two polypeptides of this molecular weight, a minor component that is heavily labelled, and a major component that is lightly labelled. The 29K-
Table 1. Chaotropic resolution of Complex I

<table>
<thead>
<tr>
<th>Recovery (%)</th>
<th>ΔA at 460–510 nm with indicated reductant</th>
<th>Fraction</th>
<th>NADH Dithionite Protein</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Complex I+ NaClO₄</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaClO₄ supernatant</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NaClO₄ pellet</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0–27.5%–satd. (NH₄)₂SO₄</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.5–36.4%–satd. (NH₄)₂SO₄</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.4–52.9%–satd. (NH₄)₂SO₄</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52.9%–satd.- (NH₄)₂SO₄</td>
<td>0</td>
</tr>
</tbody>
</table>

and 26K-mol.wt. polypeptides were not labelled to any significant extent. Fig. 8(d) shows that the radioactivity associated with the solubilized 75K- and 53K-mol.wt. bands was concentrated in the iron-protein fraction, whereas the 53K-mol.wt. band of the flavoprotein fraction was not labelled (Fig. 8e). Therefore the solubilized polypeptide of molecular weight 53K is also heterogeneous.

Although the 29K-mol.wt. band of the iron-protein fraction and both the 53K- and 26K-mol.wt. band of the flavoprotein fraction were unlabelled in this experiment, iodination after NaClO₄ treatment of Complex I caused incorporation of ¹²⁵I into all these polypeptides (Fig. 9). Because of different conditions of labelling in each case, the absolute values of incorporation in the two experiments of Fig. 9 and that of Fig. 8 are not strictly comparable. However, comparing the relative labelling of bands within one experiment, it is clear that the 29K-, 26K- and 53K-mol.wt. bands had been iodinated, as well as the minor components of the iron-protein and flavoprotein fractions. These experiments indicate that absence of labelling was
not due to absence of iodinatable residues, but to inaccessible
of these residues in the native multienzyme complex.

To substantiate further the finding that iodination of
Complex I did not cause incorporation of $^{125}$I
into the subunits of the flavoprotein fraction, the
experiment of Table 2 was undertaken. Complex I
was titrated with iodine in the presence of lacto-
peroxidase and excess of H$_2$O$_2$ until no further changes in enzyme activity were observed. Ragan (1975)
reported that rotenone-sensitive NADH–ubiquinone-
1 reductase activity declined, whereas NADH–mena-
dione reductase and rotenone-insensitive NADH–
ubiquinone-1 reductase activities increased. Flavo-
protein derived from urea treatment of the iodinated
Complex I exhibited activities not significantly different from flavoprotein isolated from non-
iodinated Complex I. In contrast, iodination of isolated flavoprotein resulted in parallel decreases in all activities. The spectra in Fig. 10 indicate that iodination of Complex I did not cause any alteration to the spectroscopic change caused by NADH reduction, except to decrease the extent of reduction of the cytochrome impurities. Iodination of the flavoprotein fraction (Fig. 11) completely prevented

\begin{table}
\centering
\caption{Effect of iodination on activity of Complex I and the low-molecular-weight flavoprotein}
\begin{tabular}{|c|c|c|c|}
\hline
Sample & Rotenone-Rotenone-sensitive & K$_3$Fe(CN)$_6$ & Mena-
dione \\
\hline
Complex I & 5.80 & 0.13 & 55 & 0.64 \\
Iodinated & 1.38 & 0.80 & 60 & 1.68 \\
Complex I & 0.0 & 52.1 & 122 & 77.6 \\
Flavoprotein & 0.0 & 48.9 & 110 & 70.6 \\
from iodinated & & & & \\
Complex I & 0.0 & 6.7 & 20 & 9.5 \\
Iodinated & & & & \\
flavoprotein & & & & \\
\hline
\end{tabular}
\end{table}

the NADH-induced spectroscopic change, but had less effect on the dithionite-induced change. Flavo-
protein isolated from iodinated Complex I exhibited unmodified spectroscopic properties.

One possible criticism of the experiment of Fig. 8 is that iodinated polypeptides may not behave in subsequent fractionations like uniodinated polypeptides. For example, it is possible that iodination caused the polypeptides to become mostly perchlorate-
soluble, thus explaining the low extent of labelling in the soluble fraction. Since carrier-free $^{125}$I had been used in this experiment, the proportion of these iodinated polypeptides would be very small, so that the amount of protein solubilized would be relatively unchanged.

The experiments of Table 2 and Figs. 10 and 11 show that this is not the case. First, the yield of
Fig. 10. Reduced-minus-oxidized difference spectra of Complex I and iodinated Complex I

Complex I (4 mg of protein) was suspended in 1.92 ml of buffer at room temperature containing 0.67 M sucrose/50 mM-Tris/HCl, pH 8.0, 5 µg of lactoperoxidase and 0.5 µmol of KI; 20 portions (4 µl each) of 10 mM-H$_2$O$_2$ were added at 30 s intervals. The final volume was therefore 2.0 ml. Untreated Complex I (2.0 mg of protein/ml) in both cuvettes, test side reduced with 2 µl of 20 mM-NADH. Iodinated Complex I (2.0 mg of protein/ml) in both cuvettes, test side reduced with 2 µl of 20 mM-NADH. Light-path was 1 cm.

Flavoprotein obtained in the experiment of Table 2 from extensively iodinated Complex I was not significantly different from that obtained from untreated Complex I. Secondly, the data of Table 2 and Fig. 10 show that extensive iodination of Complex I had little or no effect on the ability of NADH to reduce the chromophores of the enzyme. Since iodination of the isolated flavoprotein led to loss of reducibility by NADH, it is clear that the flavoprotein was not significantly iodinated when it was still part of Complex I. This latter conclusion does not depend on a subsequent fractionation of iodinated Complex I and is therefore not open to the above criticism.

Stoichiometry of the subunits of Complex I

On the basis of the FMN content of Complex I (1.2–1.3 nmol/mg of protein) or of the soluble high-molecular-weight NADH dehydrogenase (1.1 nmol/mg of protein; Baugh & King, 1972) the minimum protein molecular weight of the NADH dehydrogenase region, assuming 100% purity, is of the order of 850 000. The relative amounts of the constituent polypeptides of Complex I may be estimated from the intensity of the Coomassie Blue staining, and from the molecular weights of these polypeptides and total assumed molecular weight of Complex I, the molar proportions of some of the clearly resolved polypeptides have been calculated (Table 3). The presence of one molecule of the 26 K-mol.wt. band and one of the 53 K-mol.wt.

Fig. 11. Reduced-minus-oxidized difference spectra of the flavoprotein fraction

Flavoprotein was prepared from Complex I by the method of Hatefi & Stempel (1967). (a) Flavoprotein (0.47 mg of protein/ml in 50 mM-Tris/HCl, pH 8.0) in both cuvettes: 10 µl of 20 mM-NADH to test side; 10 µl of 20 mM-NADH to control side. (b) Flavoprotein (0.9 mg of protein) was incubated in a final volume of 1.94 ml at room temperature containing 50 mM-Tris/HCl, pH 8.0, 5 µg of lactoperoxidase and 0.4 µmol of KI, and 15 portions (4 µl each) of 10 mM-H$_2$O$_2$ were added at 30 s intervals. Final protein concentration was therefore 0.45 mg/ml. The sample was divided equally into two cuvettes: 10 µl of 20 mM-NADH to test side; 10 µl of 20 mM-NADH to control side. Light-path was 1 cm.

**Table 3. Molar proportions of Complex I subunits**

Complex I was electrophoresed as described in the Materials and Methods section except that a 6 h staining period was used to ensure complete and uniform binding of Coomassie Blue. Destained gels were scanned, and areas of the peaks were estimated by weighing the chart paper. Molar proportions were calculated on the basis of a protein mol.wt. of 850 000 for Complex I. The data are averages from three separate determinations.

<table>
<thead>
<tr>
<th>Mol.wt. of polypeptide</th>
<th>Concentration (% of total protein)</th>
<th>Molar proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 000</td>
<td>8.35 ± 0.3</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>53 000</td>
<td>14.0 ± 0.2</td>
<td>2.24 ± 0.04</td>
</tr>
<tr>
<td>42 000$^*$</td>
<td>10.1 ± 0.7</td>
<td>2.12 ± 0.15</td>
</tr>
<tr>
<td>39 000$^*$</td>
<td>3.45 ± 0.2</td>
<td>0.90 ± 0.05</td>
</tr>
<tr>
<td>29 000</td>
<td>3.10 ± 0.1</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>26 000$^+$</td>
<td>2.65</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* The total area of the 42000- and 39000-mol.wt. peaks was measured and the total molar proportion calculated on an average mol.wt. of 40500.
† The molar proportion was based on analysis of the flavoprotein fraction as described in the text.
band in the flavoprotein fraction was established above. Since the flavoprotein contains (per mg of protein) about 11-fold more FMN than Complex I and the molecular weight is about one-eleventh that of Complex I, it is clear that a molecule of Complex I contains only one flavoprotein molecule, i.e. one subunit of mol.wt. 26 K and one subunit of mol.wt. 53 K. However, Table 3 indicates that Complex I contained two molecules of the 53 K-mol.wt. polypeptide. Since it was shown above that the 53 K-mol.wt. band was heterogeneous, these results are compatible with one subunit of mol.wt. 53000 in the iron-protein fraction and one in the flavoprotein fraction. The molar content of the 75 K- and 29 K-mol.wt. bands strongly suggests that these (together with a 53 K-mol.wt. band) are integral components of the iron-protein. One molecule of the 33 K-mol.wt. component is present per molecule of Complex I, strengthening the conclusion that it is a true constituent. The incompletely resolved 39 K- and 42 K-mol.wt. bands are present in a total concentration of 2 mol/mol of Complex I. The 42 K-mol.wt. band appears to be present in lower concentrations than the 39 K-mol.wt. band (Fig. 1), but it is possible that spreading of the former band during electrophoresis is greater than that of the 39 K-mol.wt. band and that each is present at the same concentration, i.e. 1 mol/mol of Complex I.

The percentage of the protein of Complex I contained in those polypeptides of Table 3 that are solubilized by chaotropic agents is 28.1±1.5%, agreeing well with data of Table 1.

Discussion

The most serious problem in attempting to analyse the polypeptide composition of a multienzyme complex is the question of purity. It has been established in this report that Complex I is free from contamination by the major protein components of the respiratory complexes and from F1 ATPase. However, it cannot definitely be supposed that all the polypeptides found in Complex I preparations are necessary for the catalytic and membrane function of this enzyme. Since all chromophores are lost from the complex on treatment with sodium dodecyl sulphate, complete resolution of the polypeptides and functional reconstitution of the complex seems extremely unlikely. Non-destructive methods of resolution seem limited to the action of chaotropes which, even so, lead to an irreversible alteration in catalytic properties. Alternative methods to assign functions to specific polypeptides include covalent affinity labels and specific antibodies to individual polypeptides. Other evidence that a polypeptide is an integral component of a multi-enzyme complex may be provided by indirect means. Thus if a polypeptide is consistently associated with the complex in all preparations, if it remains associated with it after further attempts at resolution, if it is present in similar preparations made by different procedures, and if it is present in stoichiometric amounts it is unlikely that the polypeptide is an impurity. By these criteria, the polypeptides described in this report appear to be integral components of Complex I.

When Complex I is incorporated into phospholipid vesicles, oxidation of NADH by ubiquinone-I is linked to proton translocation across the membrane (Ragan & Racker, 1973; Ragan & Hinkle, 1975). Ragan (1975) proposed that the 33000-mol.wt. polypeptide of Complex I is the transmembrane component involved in proton translocation. With such a significant function assigned to this polypeptide, it is important to establish that it is not an impurity. First, this component is consistently observed in all Complex I preparations and is present in equimolar concentration with the major components of the iron-protein fraction and the subunits of the flavoprotein fraction. A component or components with similar molecular weight constitutes the major polypeptide of submitochondrial particles and is selectively solubilized by lysophosphatidylcholine (Capaldi et al., 1973). It is also a major component of the membrane sector of the proton-translocating ATPase (Kagawa & Racker, 1971), but is present in submitochondrial particles in a much higher mol concentration than the F1 ATPase (Capaldi et al., 1973). Oligomycin-sensitive ATPase exhibiting $^{32P}$F$_1$-ATP exchange activity has been isolated by Hafner et al. (1974). This preparation (Complex V) contains the 29000-mol.wt. polypeptide, and since the techniques of purification of Complex V were similar to those used for the other respiratory complexes, it would appear that these same techniques do not lead to resolution of the F1 ATPase from the membrane sector components. In view of this, and the absence of F1 ATPase components from Complex I, it is unlikely that the 33000-mol.wt. polypeptide of Complex I is a fortuitous impurity. It is proposed therefore that the polypeptide of this molecular weight is a component of the membrane sector of the proton-translocating NADH dehydrogenase and also possibly the proton-translocating nicotinamide nucleotide transhydrogenase (Rydstrom, 1975).

The results of iodination of Complex I indicated that the polypeptides solubilized by chaotropic agents were relatively inaccessible to labelling compared with the polypeptides in the insoluble (i.e. hydrophobic) fraction. Assuming that the solubility of these fractions reflects their hydrophilicity within the intact complex, it would appear that, as a generalization, the surface polypeptides of Complex I are hydrophobic, consistent with the insolubility of the intact complex except in the
presence of detergents. This unusual state of affairs would, however, be expected for a structure whose natural environment is a membrane. The inaccessibility of the hydrophilic polypeptides, one of which must be the site of action of NADH, to labelling suggests the possibility of a hydrophilic cleft allowing excess of the external aqueous phase to sites within the complex.

Some preliminary experiments on the subunit composition of Complex I were performed while I was at the Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, N.Y. 14850, U.S.A. Ubiquinone-I was kindly given by Professor K. Folker, Institute for Biomedical Research, The University of Texas at Austin, Austin, Tex. 78712, U.S.A.

References