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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.*, 1962*a*) 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.*, 1962*a*) 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 (1mM) 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 (0.5–1mg of protein/ml) were denatured in 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol for 12–20h at room temperature (21°C). Gels containing 10% (w/v) acrylamide and 0.27% bisacrylamide were polymerized in glass tubes (12cm × 6mm internal diam.) as described by Weber & Osborn (1969). Electrophoresis was performed at 5mA/gel for 16h. 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. 1.8mm slices with a stack of razor blades and each slice was counted directly for radioactivity in a Beckman gamma counter for 5min. 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 340nm of a solution containing 20μmol of potassium phosphate, pH8.0, 0.1μmol of NADH, 0.06μmol of ubiquinone-1, 0.1μmol of soya-bean phosphatidylcholine (Ragan & Racker, 1973) and enzyme in 1ml. Where indicated, 0.5μg of rotenone was included. NADH–menadione reductase was measured similarly except that 0.2μmol of menadione replaced the ubiquinone-1 and phosphatidylcholine. NADH–ferricyanide reductase was measured by the decrease in extinction at 420nm of a solution containing 20μmol of potassium phosphate, pH8.0, 0.1μmol of NADH, 1μmol of $K_3Fe(CN)_6$ and enzyme in 1ml. Nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) was measured by the decrease in extinction at 340nm of a solution containing 0.1mmol of Tris/acetate, pH7.4, 0.07μmol of NADH, 10μg of L-lactate dehydrogenase (Sigma Chemical Co.), 2μmol of NADPH and enzyme in 1ml. 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) 28–29nmol of iron or acid-labile sulphide and 1.1–1.3nmol of FMN. Activities at 38°C ranged from 280 to 340μmol of NADH oxidized/min per mg of protein ($V_{max.}$ with ferricyanide as acceptor) and 7–15μmol of NADH oxidized/min per mg of protein ($V_{max.}$ 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.*, 1962*b*) 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 $a+a_3$ 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

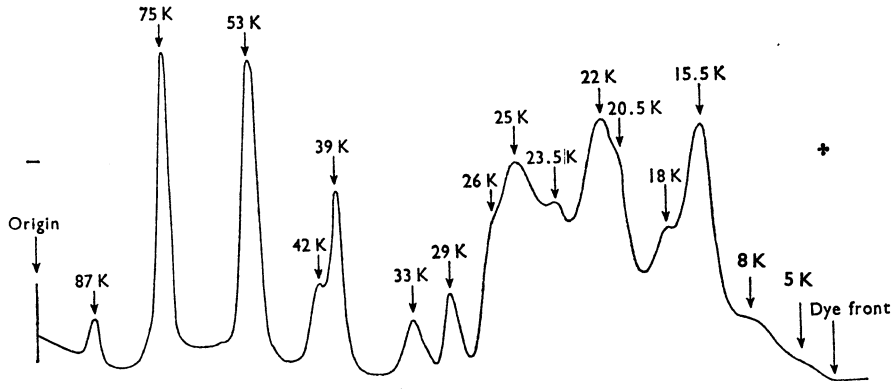

Fig. 1. *Subunit composition of Complex I*

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.

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 $(NH_4)_2SO_4$ led to negligible losses of haem a (Yu *et al.*, 1975)]. Complex I did not contain any F_1 ATPase* (Penefsky *et al.*, 1960), assayed either by its activity or by the presence of the major F_1 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 F_1 ATPase was clearly established by co-electrophoresis of Complex I and F_1 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 $V_{max.}$ for ferricyanide, 942 μmol 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 pH 10, 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

* Abbreviation: ATPase, adenosine triphosphatase.

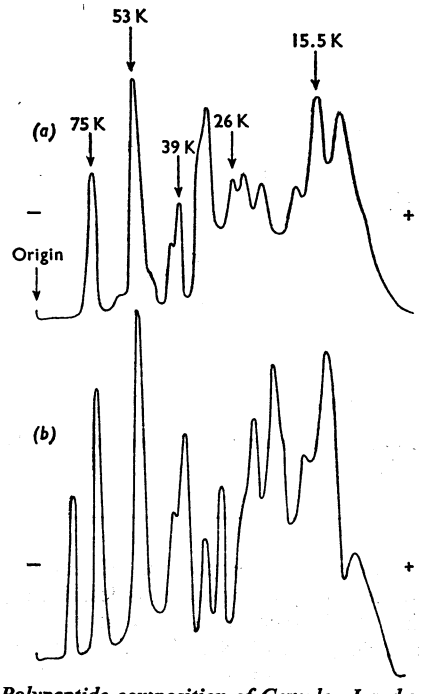

Fig. 2. *Polypeptide composition of Complex I and soluble NADH dehydrogenase*

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 7cm×0.5cm internal diam. gels were used.

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 (Rydström *et al.*, 1974). A partially purified transhydrogenase contains two polypeptides of mol.wt. approx. 30000, which together account for 70% of the total protein (Rydström, 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

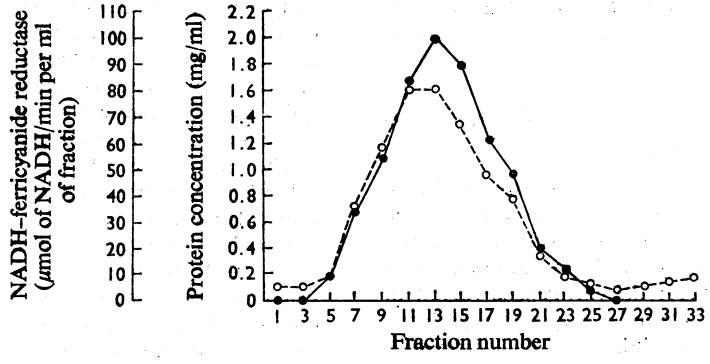

Fig. 3. *Effect of phospholipase A on Complex I*

Complex I was dialysed against 200vol. of 0.67M-sucrose/50mM-Tris/HCl, pH8.0, for 16h at 4°C to remove bile salts. A portion (0.4ml, containing 6.2mg of protein) was incubated with 0.1mg of purified phospholipase A at 30°C for 1h. A parallel incubation received no phospholipase A. Samples were cooled to 4°C, mixed with 0.055ml of 0.5M-glycine/NaOH, pH10.0, and layered on 10ml linear gradients containing 50mM-glycine/NaOH, pH10.0, and 28–65% (w/v) sucrose as described by Lusty *et al.* (1965). Centrifugation at 2°C was for 20h at 35000rev./min in the SW39 rotor of the Beckman L5-65 centrifuge. Tubes were punctured and approx. 0.3ml 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.

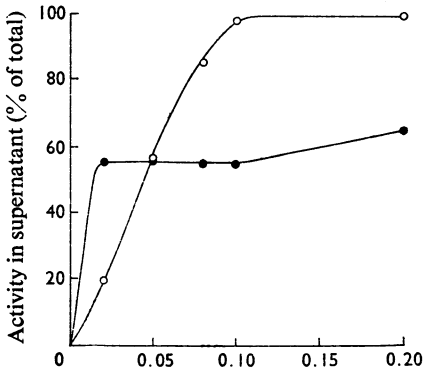

Fig. 4. *Effect of lysophosphatidylcholine on Complex I*

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 100000*g* for 4 h at 4°C in the 10 × 10 ml rotor of the MSE 65 centrifuge. Nicotinamide nucleotide transhydrogenase (○) 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.

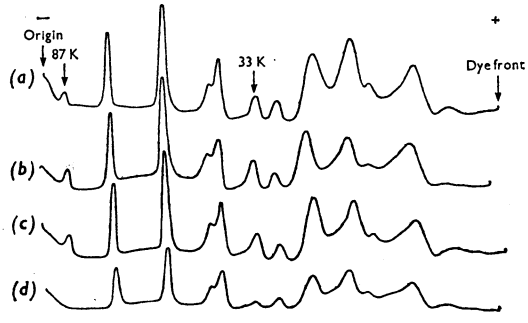

Fig. 5. *Polypeptide composition of fractions from lysophosphatidylcholine treatment of Complex I*

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.

33000-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 87000- and 33000-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.

Chaotropic resolution of Complex I

Resolution of Complex I by $NaClO_4$ released relatively few polypeptides into solution, namely those of mol.wts. 75000 (75K), 53K, 29K, 26K and 8K, along with traces of others in the 10K–20K-molecular-weight range (Figs. 6*a* and 6*b*).

The iron–protein fraction (Fig. 6*c*) contained all of these components, but had a lesser content of the 53K- and 26K-mol.wt. bands, which were also found in the flavoprotein fraction (Fig. 6*d*). Chromatography of the iron–protein fraction on Sephadex G-200 in the presence of 1 M-urea caused removal of the residual 26K-mol.wt. band, but not all of the 53K-mol.wt. band (results not shown). This indicated that either the 53K- and 26K-mol.wt. components of the flavoprotein were relatively easily separated or that the 53K-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 53K and 26K and small amounts of a component of molecular weight about 8K. 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 79000 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 76000 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 8K-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 $(NH_4)_2SO_4$ did not contain significant amounts of protein compared with the iron–protein fraction

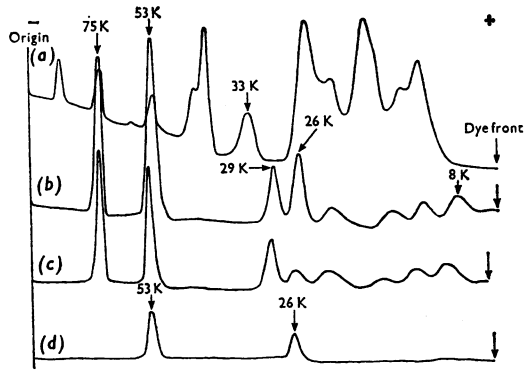

Fig. 6. *Chaotropic resolution of Complex I*

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-$NaClO_4$ at 35°C for 10min, cooled to 4°C and centrifuged at 100000*g* for 15min in the 10×10ml rotor of the MSE 65 centrifuge. The supernatant was fractionated with $(NH_4)_2SO_4$ as described by Hatefi & Stempel (1967) to give the iron–protein and flavoprotein fractions. The pellet from the $NaClO_4$ treatment was re-extracted with $NaClO_4$ under the same conditions to ensure complete removal of solubilized polypeptides. This second treatment only removes those polypeptides solubilized by the first $NaClO_4$ 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*) $NaClO_4$-insoluble material; (*b*) $NaClO_4$-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 $(NH_4)_2SO_4$ 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 soluble 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

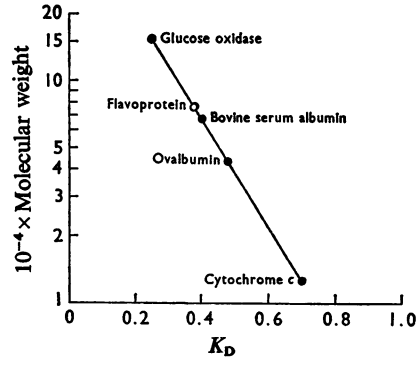

Fig. 7. *Molecular weight of the low-molecular-weight flavoprotein from Complex I*

Flavoprotein was isolated as described by Hatefi & Stempel (1967). Chromatography was performed on a column (27cm×1.7cm internal diam.) of Sephadex G-200 equilibrated with 50mM-Tris/HCl, pH9.0 at 4°C. The column was calibrated with Blue Dextran, glucose oxidase (EC 1.1.3.4, mol.wt. 154000), bovine serum albumin, ovalbumin, cytochrome *c* and Bromophenol Blue and the elution of these marker proteins and flavoprotein was monitored at 280nm, Dextran Blue at 600nm and Bromophenol Blue at 592nm.

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 ^{125}I 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. 8*a*), 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. 8*c*), 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 53K, most of the ^{125}I at this position remained in the insoluble residue (Fig. 8*b*). 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*

Complex I (20mg of protein) was diluted to 2.0ml with medium containing 0.67M-sucrose, 50mM-Tris/HCl, pH8.0, 1mM-histidine and 0.5M-$NaClO_4$, and incubated at 35°C for 10min. After centrifugation at 100000*g* for 15min in the 10×10ml rotor of the MSE 65, the supernatant was fractionated with $(NH_4)_2SO_4$ at 27.5, 36.4 and 52.9% saturation (Hatefi & Stempel, 1967), and the pellet fraction was resuspended in $NaClO_4$ containing buffer, incubated at 35°C for 10min and re-centrifuged. The material in the supernatant from the second $NaClO_4$ treatment completely accounted for that proportion of the first supernatant calculated to be trapped in the pellet, i.e. the second $NaClO_4$ treatment caused no further solubilization of protein. All precipitates were resuspended to the original volume and samples removed for assay of protein concentration and bleaching by NADH and dithionite, which were measured as follows. Portions (0.1 or 0.2ml) of samples were diluted to 1.1ml with 0.67M-sucrose/50mM-Tris/HCl (pH8.0)/1mM-histidine. Extinction changes at 460–510nm were measured at room temperature after sequential addition of 2μl of 25mM-NADH and solid dithionite. Measurements are presented as the percentage of the total measured in Complex I after $NaClO_4$ treatment. Appropriate corrections were made for losses incurred by sampling. The protein concentration of the 52.9%-satd.-$(NH_4)_2SO_4$ supernatant was too low for measurement. Unless otherwise stated, all operations were performed at 4°C.

	Recovery (%)		
	ΔE at 460–510nm with indicated reductant		
Fraction	NADH	Dithionite	Protein
Complex I+$NaClO_4$	100	100	100
$NaClO_4$ supernatant	98	74	30
$NaClO_4$ pellet	0	22	71
0–27.5%-satd. $(NH_4)_2SO_4$	24	52	22
27.5–36.4%-satd. $(NH_4)_2SO_4$	1.8	1.6	2.0
36.4–52.9%-satd. $(NH_4)_2SO_4$	60	3.6	5.7
52.9%-satd.-$(NH_4)_2SO_4$ supernatant	0	4	—

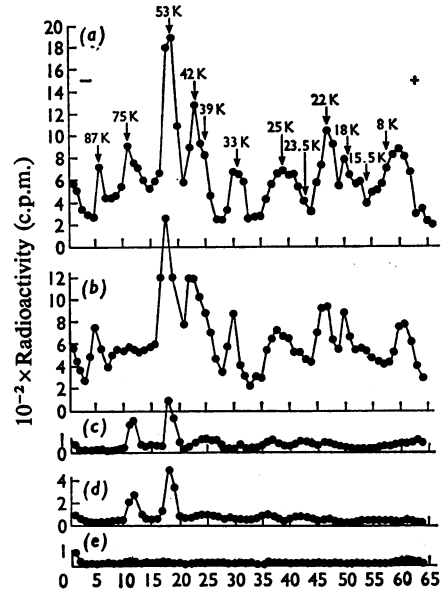

Fig. 8. *Iodination of Complex I subunits*

Complex I (19.6mg of protein) was incubated at room temperature in a final volume of 20ml containing 0.67M-sucrose/50mM-Tris/HCl, pH8.0, 20μg of lactoperoxidase and 160μCi of $Na^{125}I$ (carrier-free). Iodination was performed by the addition of 10μl portions of 20mM-H_2O_2 at 30s intervals (total of ten additions). The sample was diluted to 40ml with the sucrose/Tris medium, EDTA (1mM) and 2-mercaptoethanol (0.1%) were added and the mixture was centrifuged at 100000*g* for 1h at 4°C in the 8×50ml rotor of the MSE 65 centrifuge. The pellet was homogenized in sucrose/Tris medium at 4°C with a motor-driven Potter–Elvehjem homogenizer, washed again by centrifugation as before and finally homogenized in sucrose/Tris medium to a final volume of 2ml. Chaotropic resolution, isolation of fractions and gel electrophoresis were performed as described in the legend to Fig. 6. The gels that gave rise to the densitometer profiles of Figs. 1 and 6 were sliced and counted for radioactivity as described in the Materials and Methods section (*a*) Complex I; (*b*) $NaClO_4$-insoluble material; (*c*) $NaClO_4$-soluble material; (*d*) iron–protein fraction; (*e*) flavoprotein fraction.

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. 8*e*). 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 $NaClO_4$ treatment of Complex I caused incorporation of ^{125}I 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

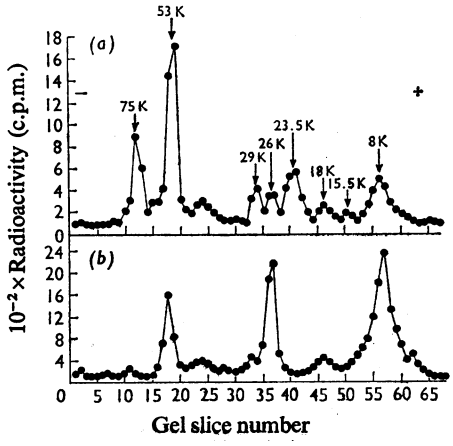

Fig. 9. *Iodination of the iron–protein and flavoprotein fractions of Complex I*

Iron–protein (3.9mg of protein) or flavoprotein (0.95mg of protein) was incubated at room temperature in a final volume of 2ml containing 5μg of lactoperoxidase and 40μCi of carrier-free $Na^{125}I$. Ten portions (10μl each) of 20mM-H_2O_2 were added at 30s intervals. These amounts of iron–protein and flavoprotein were derived from the same amount of Complex I that was iodinated in the experiment of Fig. 8 by 160μCi of $Na^{125}I$. Therefore, for comparison, four times as much protein was electrophoresed in the experiments of Fig. 9 as in the respective experiments of Fig. 8.

not due to absence of iodinatable residues, but to inaccessibility 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 lactoperoxidase and excess of H_2O_2 until no further changes in enzyme activity were observed. Ragan (1975) reported that rotenone-sensitive NADH–ubiquinone-1 reductase activity declined, whereas NADH–menadione reductase and rotenone-insensitive NADH–ubiquinone-1 reductase activities increased. Flavoprotein 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

Table 2. *Effect of iodination on activity of Complex I and the low-molecular-weight flavoprotein*

Complex I (30mg of protein) was incubated at room temperature in a final volume of 30ml containing 0.67M-sucrose/50mM-Tris/HCl, pH8.0, 30μg of lactoperoxidase and 3μmol of KI, and 15 portions (10μl each) of 60mM-H_2O_2 were added at 30s intervals. These concentrations of KI and H_2O_2 produced maximum changes in activities. At the end of the incubation, Complex I was collected by centrifugation at 100000*g* for 60min at 4°C and resuspended to a final volume of 3ml in the sucrose/Tris medium. Chaotropic resolution was performed by the addition of $NaClO_4$ to a final concentration of 0.5M and incubation at 35°C for 10min. The flavoprotein fraction was isolated as described by Hatefi & Stempel (1967). Flavoprotein from untreated Complex I was isolated in parallel. Iodination of flavoprotein was performed by incubation of 0.1mg of protein at room temperature in a final volume of 1ml containing 0.67M-sucrose/50mM-Tris/HCl, pH8.0, 5μg of lactoperoxidase and 30nmol of KI, and eight portions (2μl each) of 5mM-H_2O_2 were added at 30s intervals. The KI and H_2O_2 concentrations were sufficient for maximum changes in activities.

	Rate of NADH oxidation with indicated acceptor (μmol/min per mg of protein)			
	Ubiquinone-1			
Sample	Rotenone-sensitive	Rotenone-insensitive	$K_3Fe(CN)_6$	Menadione
Complex I	5.80	0.13	55	0.64
Iodinated Complex I	1.38	0.80	60	1.68
Flavoprotein	0.0	52.1	122	77.6
Flavoprotein from iodinated Complex I	0.0	48.9	110	70.6
Iodinated flavoprotein	0.0	6.7	20	9.5

the NADH-induced spectroscopic change, but had less effect on the dithionite-induced change. Flavoprotein 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-insoluble, 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

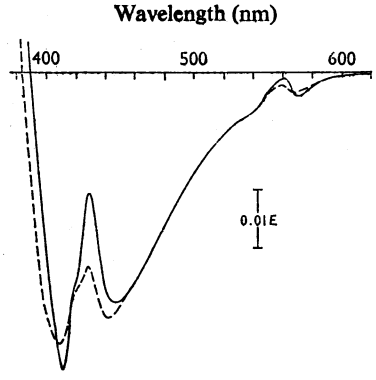

Fig. 10. *Reduced-minus-oxidized difference spectra of Complex I and iodinated Complex I*

Complex I (4mg of protein) was suspended in 1.92ml of buffer at room temperature containing 0.67M-sucrose/50mM-Tris/HCl, pH8.0, 5μg of lactoperoxidase and 0.5μmol of KI; 20 portions (4μl each) of 10mM-H_2O_2 were added at 30s intervals. The final volume was therefore 2.0ml. ——, Untreated Complex I (2.0mg of protein/ml) in both cuvettes, test side reduced with 2μl of 20mM-NADH. ----, Iodinated Complex I (2.0mg of protein/ml) in both cuvettes, test side reduced with 2μl of 20mM-NADH. Light-path was 1cm.

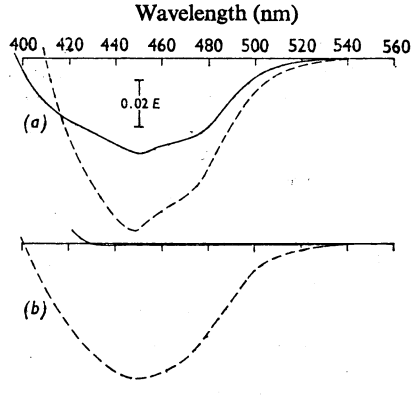

Fig. 11. *Reduced-minus-oxidized difference spectra of the flavoprotein fraction*

Flavoprotein was prepared from Complex I by the method of Hatefi & Stempel (1967). (*a*) Flavoprotein (0.47mg of protein/ml in 50mM-Tris/HCl, pH8.0) in both cuvettes: ——, 10μl of 20mM-NADH to test side; ----, dithionite to test side. (*b*) Flavoprotein (0.9mg of protein) was incubated in a final volume of 1.94ml at room temperature containing 50mM-Tris/HCl, pH8.0, 5μg of lactoperoxidase and 0.4μmol of KI, and 15 portions (4μl each) of 10mM-H_2O_2 were added at 30s intervals. Final protein concentration was therefore 0.45mg/ml. The sample was divided equally into two cuvettes: ——, 10μl of 20mM-NADH to test side; ----, dithionite to test side. Light-path was 1cm.

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.

Stoicheiometry of the subunits of Complex I

On the basis of the FMN content of Complex I (1.2–1.3nmol/mg of protein) or of the soluble high-molecular-weight NADH dehydrogenase (1.1nmol/mg of protein; Baugh & King, 1972) the minimum protein molecular weight of the NADH dehydrogenase region, assuming 100% purity, is of the order of 850000. 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 26K-mol.wt. band and one of the 53K-mol.wt.

Table 3. *Molar proportions of Complex I subunits*

Complex I was electrophoresed as described in the Materials and Methods section except that a 6h 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 850000 for Complex I. The data are averages from three separate determinations.

Mol.wt. of polypeptide	Concentration (% of total protein)	Molar proportion
75000	8.35±0.3	0.95±0.04
53000	14.0 ±0.2	2.24±0.04
42000*	10.1 ±0.7	2.12±0.15
39000*		
33000	3.45±0.2	0.90±0.05
29000	3.10±0.1	0.91±0.03
26000†	2.65	1.0

* 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. 26K and one subunit of mol.wt. 53K. However, Table 3 indicates that Complex I contained two molecules of the 53K-mol.wt. polypeptide. Since it was shown above that the 53K-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 75K- and 29K-mol.wt. bands strongly suggests that these (together with a 53K-mol.wt. band) are integral components of the iron–protein. One molecule of the 33K-mol.wt. component is present per molecule of Complex I, strengthening the conclusion that it is a true constituent. The incompletely resolved 39K- and 42K-mol.wt. bands are present in a total concentration of 2mol/mol of Complex I. The 42K-mol.wt. band appears to be present in lower concentrations than the 39K-mol.wt. band (Fig. 1), but it is possible that spreading of the former band during electrophoresis is greater than that of the 39K-mol.wt. band and that each is present at the same concentration, i.e. 1mol/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 $28.1 \pm 1.5\%$, 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 F_1 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 multienzyme 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 stoicheiometric 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-1 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 molar concentration than the F_1 ATPase (Capaldi *et al.*, 1973). Oligomycin-sensitive ATPase exhibiting $^{32}P_i$–ATP exchange activity has been isolated by Hatefi *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 F_1 ATPase from the membrane sector components. In view of this, and the absence of F_1 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 (Rydström, 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-1 was kindly given by Professor K. Folkers, Institute for Biomedical Research, The University of Texas at Austin, Austin, Tex. 78712, U.S.A.

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