Biochem. J. (1980) **185**, 315–326
Printed in Great Britain

The Organization of NADH Dehydrogenase Polypeptides in the Inner Mitochondrial Membrane

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(*Received* 15 *June* 1979)

The organization of the constituent polypeptides of mitochondrial NADH dehydrogenase was studied by using two membrane-impermeable probes, diazobenzene-[^{35}S]sulphonate and lactoperoxidase-catalysed radioiodination. The incorporation of label into the subunits of the isolated enzyme was compared with that obtained with enzyme immunoprecipitated from labelled mitochondria or inverted submitochondrial particles. On the basis of accessibility to these two labels, we divide the polypeptides of Complex I into five groups: those that are apparently buried in the enzyme, those that are accessible to labelling in the isolated enzyme but not in the membrane, those that are exposed on the cytoplasmic face of the membrane, those that are exposed on the matrix face and finally those that are exposed on both faces and are therefore transmembranous. We conclude that NADH dehydrogenase is asymmetrically organized across the inner mitochondrial membrane.

Mitochondrial NADH dehydrogenase catalyses proton translocation coupled to the oxidation of NADH by ubiquinone (Lawford & Garland, 1972; Ragan & Hinkle, 1975). It has been proposed that transmembrane movement of reducing equivalents (H one way and electrons the other) is responsible for proton translocation (Mitchell, 1966; Lawford & Garland, 1972), i.e. that the electron (or H)-transfer pathway is 'looped' across the membrane. In the present paper, we show that the NADH dehydrogenase protein is transmembranous and that the distribution of its constituent polypeptides across the inner mitochondrial membrane is consistent with a 'loop' mechanism. We have used a similar approach to that used by others to study the topography of cytochrome oxidase (Eytan *et al.*, 1975) and the cytochrome b-c_1 segment (Mendel-Hartvig & Nelson, 1978). Briefly, polypeptides exposed on the external surface of both mitochondria and inverted submitochondrial particles were radiochemically labelled with reagents that do not penetrate the membrane (diazobenzene[^{35}S]sulphonate and the lactoperoxidase-catalysed incorporation of ^{125}I). The labelled membranes were solubilized with detergents, and NADH dehydrogenase was separated by specific immunoprecipitation with antibodies raised against purified NADH–ubiquinone oxidoreductase (Complex I of Hatefi *et al.*, 1962). The distribution of radioactivity beween the constituent polypeptides of the enzyme was then analysed by SDS/polyacrylamide-gel electrophoresis. A preliminary report of some of this work has been published (Smith & Ragan, 1978).

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and Methods

Biological preparations

Mitochondria from bovine heart were prepared as described by Crane *et al.* (1956), and separated into heavy-layer and light-layer fractions as described by Blair (1967). Submitochondrial particles were prepared by ultrasonic disruption of mitochondria by the method of Racker (1962) or by alkali treatment of mitochondria as described by Ringler *et al.* (1963). Purification of submitochondrial particles by affinity chromatography on cytochrome c–a agarose columns was exactly as described by Lötscher *et al.* (1979). Complex I (EC 1.6.99.3) was purified by the method of Hatefi & Rieske (1967). F_1-ATPase was solubilized from submitochondrial particles by treatment with chloroform as described by Beechey *et al.* (1975).

Preparation of antisera

Rabbits were immunized with Complex I as described by Heron *et al.* (1979). Serum samples were clarified by centrifugation at 100000**g** for 10min in the 10ml × 10ml rotor of an MSE 65 centrifuge and stored frozen at −20°C.

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Preparation of immunoprecipitates from solubilized mitochondria and submitochondrial particles

Mitochondria or submitochondrial particles were diluted to 1 mg of protein/ml in a solution containing 0.25 M-sucrose, 50 mM-Tris/HCl, pH 8.0, 0.5% (w/v) sodium deoxycholate and 0.5 M-KCl. After centrifugation in the 10 × 10 ml rotor of an MSE 65 centrifuge at 25000 rev./min for 40 min the supernatant was made 1% (w/v) in Triton X-100 and left overnight at 4°C. The solution was then recentrifuged as above. Neither of these centrifugation steps sedimented any NADH–$K_3Fe(CN)_6$ oxidoreductase activity. Per mg of initial protein, 0.45 ml of anti-(Complex I) serum was added, together with Triton X-100 to maintain the latter concentration at 1% (w/v). After incubation overnight at 4°C, the immunoprecipitate was collected by centrifugation at 20000 rev./min for 20 min. The pellet was washed twice by homogenization and recentrifugation in a solution containing 1% (w/v) Triton X-100 and 0.9% (w/v) NaCl. Variations in this procedure are given in the Figure legends.

Chaotropic resolution of Complex I

Complex I (7 mg of protein/ml of 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0) was incubated with 0.5 M-$NaClO_4$ at 35°C for 10 min. The flavoprotein and iron–protein fractions were purified from the supernatant by $(NH_4)_2SO_4$ fractionation as described by Hatefi & Stempel (1969).

Labelling with diazobenzene[^{35}S]sulphonate

[^{35}S]Sulphanilic acid (8 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive sulphanilic acid was added to give an original specific radioactivity of 100 mCi/mmol. Diazobenzene[^{35}S]sulphonate was synthesized from 6 μmol of [^{35}S]sulphanilic acid as described by Dilley *et al.* (1972), and diluted to a final volume of 0.3 ml. The nominal concentration of diazobenzene[^{35}S]sulphonate was therefore 20 mM. Freshly prepared solutions were used for labelling experiments, although we have found that diazobenzene[^{35}S]sulphonate is quite stable when stored at −20°C in neutral solution.

Complex-I solutions were dialysed extensively against 0.25 M-sucrose/50 mM-sodium phosphate, pH 7.8 at 4°C, to remove Tris buffer, and diluted with the same buffer to a final concentration of 3 mg of protein/ml. Diazobenzene[^{35}S]sulphonate was added to a final concentration of 2 mM and the reaction was allowed to proceed for 30 min at 4°C. Further reaction was prevented by adding 0.5 M-Tris/HCl, pH 7.8, to a final concentration of 100 mM.

Heavy-layer mitochondria (freshly prepared) were washed twice by centrifugation at 17000 rev./min for 20 min in the 8 × 50 ml rotor of an MSE 18 centrifuge and resuspension in 0.25 M-sucrose/10 mM-sodium phosphate, pH 7.8 at 4°C. The mitochondria were finally resuspended at a concentration of 10 mg of protein/ml and allowed to react with 2 mM-diazobenzene[^{35}S]sulphonate for 30 min at 4°C. The reaction was stopped by addition of 0.5 M-Tris/HCl, pH 8.0, to a final concentration of 0.05 M, and the mitochondria were washed twice by centrifugation at 30000 rev./min for 20 min in the 10 × 10 ml rotor of an MSE 65 centrifuge and resuspension in 0.25 M-sucrose/50 mM-Tris/HCl, pH 8.0 at 4°C.

Submitochondrial particles (prepared in sucrose solution) were suspended by homogenization in 0.25 M-sucrose/50 mM-sodium phosphate, pH 7.8 at 4°C, at a final protein concentration of 10 mg of protein/ml. Labelling was then conducted as described for mitochondria. After the reaction had been stopped with Tris, the submitochondrial particles were washed twice by centrifugation at 35000 rev./min for 30 min in the 10 × 10 ml rotor of an MSE 65 centrifuge and resuspension in 0.25 M-sucrose/50 mM-Tris/HCl, pH 8.0.

Labelling with ^{125}I

The procedure was based on that described by Phillips & Morrison (1971). Carrier-free $Na^{125}I$ was obtained from The Radiochemical Centre, and lactoperoxidase (EC 1.11.1.7) from Boehringer Corp. (London) Ltd.

Complex I (1 mg of protein/ml of 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0) was incubated at room temperature with lactoperoxidase (40 μg of protein/ml) and carrier-free $Na^{125}I$ (40 μCi/ml). Additions of 5 mM-H_2O_2 to final concentrations of 20 μM were made at 1 min intervals for a total of 10 min.

After 15 min at room temperature, any further reaction was prevented by addition of 0.4 vol. of 0.1 M-EDTA, pH 8.0. The iodinated Complex I was collected by centrifugation at 35000 rev./min for 30 min in the 10 × 10 ml rotor of an MSE 65 centrifuge.

Heavy-layer mitochondria were suspended in 0.25 M-sucrose/50 mM-Tris/HCl, pH 8.0, to a final concentration of 5 mg of protein/ml and incubated at room temperature with lactoperoxidase (40 μg/ml) and carrier-free $Na^{125}I$ (50 μCi/ml). Additions of 50 mM-H_2O_2 to final concentrations of 2.5 μM were made at 30 s intervals for 15 min. After a further 15 min at room temperature, EDTA (2.5 mM final concentration) was added and the mitochondria were washed by centrifugation as described above for diazobenzenesulphonate labelling.

Submitochondrial particles suspended in sucrose/Tris buffer to a final protein concentration of 5 mg/ml were iodinated as otherwise described for mitochondria. After the reaction had been stopped with EDTA, the particles were washed by centri-

fugation as described for diazobenzenesulphonate labelling.

SDS/polyacrylamide-gel electrophoresis

For most analyses, we used the discontinuous system J4179 of Jovin *et al.* (1971), as described by Neville & Glossman (1974). The stacking gel (1 cm long × 0.6 cm internal diam.) contained 3% (w/v) acrylamide and 0.24% bisacrylamide. The running gel (10.5 cm long × 0.6 cm internal diam.) contained 12.5% (w/v) acrylamide and 0.34% bisacrylamide. Samples (1–2 mg of protein/ml) were dissociated by heating to 100°C for 2 min in a solution containing 1% SDS and 1% 2-mercaptoethanol. Occasionally, we used the Weber & Osborn (1969) continuous buffer system, dispensing with the stacking gel. The amounts of protein applied to each gel are given in the respective Figure legends. After electrophoresis, protein bands were stained with either Coomassie Blue or Brilliant Blue R (Sigma, Poole, Dorset, U.K.) as described by Weber & Osborn (1969). Molecular weights of Complex-I subunits were taken from Crowder & Ragan (1977).

Determination of radioactivity in gel slices

After staining for protein, polyacrylamide gels were fractionated into 1 mm slices with a Mickle gel slicer (Mickle Engineering Co., Gomshall, Surrey, U.K.). For ^{35}S determinations, each slice was incubated with 0.3 ml of NCS Solubilizer (from Hopkin and Williams, Chadwell Heath, Essex, U.K.) at 50°C for 12 h or until the slice had extensively swollen. To each slice, 10 ml of a solution containing 6 g of 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/litre of toluene was added. Samples were counted for radioactivity in an Intertechnique SL33 liquid-scintillation counter with 90% efficiency. A quench correction was applied, and all ^{35}S determinations are given in d.p.m. For ^{125}I determinations, 1 mm slices were counted directly in a Beckman Biogamma counter.

Assays

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Fraction V from Sigma) as a standard. NADH–$K_3Fe(CN)_6$ oxidoreductase activity was assayed as described previously (Crowder & Ragan, 1977).

Results

The sidedness of the mitochondria

For all the experiments described in the present paper, freshly prepared heavy-layer mitochondria were used. The intactness of the preparations was checked by the accessibility of F_1-ATPase to labelling by diazobenzene[^{35}S]sulphonate and ^{125}I. Since F_1-ATPase is known to be located on the inside face of the inner mitochondrial membrane, any label taken up by this protein could be ascribed to the presence of broken or inverted membranes in the mitochondrial preparations. In Fig. 1(*a*), the distribution of radioactivity between the polypeptides of intact mitochondria labelled with diazobenzene-[^{35}S]sulphonate was analysed by SDS/polyacrylamide-gel electrophoresis. The labelling pattern observed was similar to that reported by others (Tinberg *et al.*, 1974; Mendel-Hartvig & Nelson, 1978), with most of the radioactivity confined to a polypeptide of mol.wt. 30000. Very little radioactivity was found in the region of the gel containing the major subunits of the F_1-ATPase. A portion of the labelled mitochondria was ultrasonically disrupted, and F_1-ATPase purified from the membrane fraction by treatment with chloroform as described by Beechey *et al.* (1975). As shown in Fig. 1(*b*),

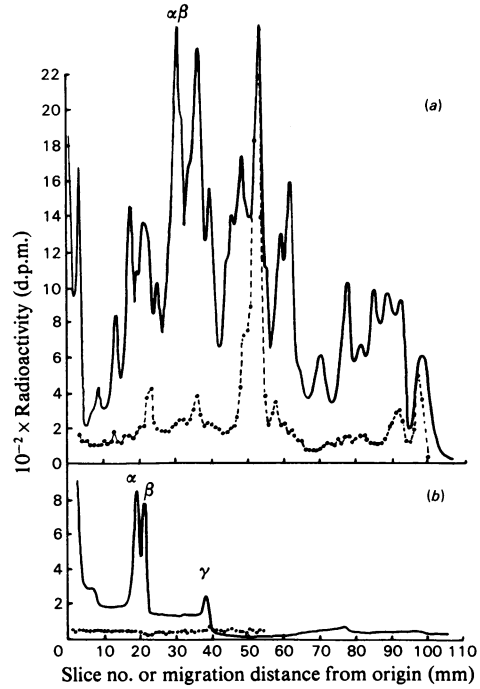

Fig. 1. *Labelling of intact mitochondria with diazobenzene*[^{35}S]*sulphonate*

Mitochondria were labelled as described in the Materials and Methods section with 2 mM-diazobenzene[^{35}S]sulphonate (35 Ci/mol). A portion (50 mg of protein) was used for purification of F_1-ATPase. (*a*) Mitochondrial protein (100 μg) was analysed by discontinuous SDS/polyacrylamide-gel electrophoresis; (*b*) F_1-ATPase from 100 μg of mitochondrial protein was analysed by the Weber & Osborn (1969) electrophoretic procedure. ——, Densitometer scans of the stained gels; – – – –, radioactivity. α, β and γ show the positions of the three largest F_1-ATPase subunits.

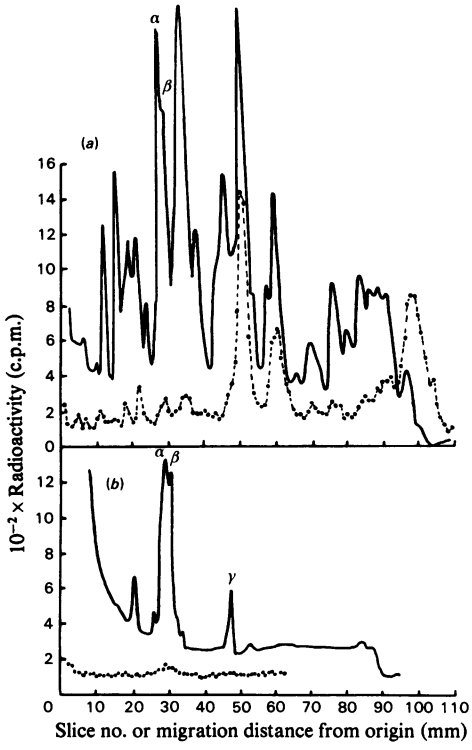

Fig. 2. *Labelling of intact mitochondria with* ^{125}I
Mitochondria were labelled as described in the Materials and Methods section. A portion (50 mg of protein) was used for purification of F_1-ATPase. (*a*) Mitochondrial protein (100 μg) and (*b*) F_1-ATPase from 100 μg of mitochondrial protein were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis. ——, Densitometer scans; ----, radioactivity.

almost no radioactivity was associated with the F_1-ATPase polypeptides, in contrast with the results obtained with submitochondrial particles, which are described below.

Comparable results were obtained when lactoperoxidase-catalysed radioiodination was used to label the mitochondria (Fig. 2*a*). The labelling pattern was very similar to that found by Boxer (1975), but not quite the same as that obtained with diazobenzene[^{35}S]sulphonate. Again, the F_1-ATPase polypeptides were not labelled (Fig. 2*b*). Our preparations of mitochondria therefore appeared to be largely intact and non-inverted.

The sidedness of the submitochondrial particles

It is widely accepted that submitochondrial particles are largely inverted with respect to mitochondria. However, production of a homogeneous population of inverted vesicular membranes is not easy (e.g. Harmon *et al.*, 1974). Besides potentially consisting of a mixture of inverted and non-inverted membrane vesicles, there is also the possibility of so-called 'scrambling' of the membrane in which the relative orientation of protein complexes is altered. Finally, membrane fragments in which both surfaces are accessible to the external medium could also be present. We have applied a number of tests designed to show the degree of inhomogeneity of our submitochondrial-particle preparations.

A simple test is the degree of stimulation of NADH oxidation by added cytochrome *c*. In a population of fully inverted closed inner-membrane vesicles, oxidation of NADH should proceed via internal cytochrome *c*. Stimulation of activity by added cytochrome *c* therefore indicates 'scrambling' or the presence of membrane fragments. According to Crane *et al.* (1956) and Harmon *et al.* (1974), some stimulation is usually found with particles prepared by ultrasonic disruption, but this is minimized if the submitochondrial particles are prepared by alkali treatment (Ringler *et al.*, 1963). In our hands, submitochondrial particles prepared by either method showed less than 5% stimulation of succinate or NADH oxidation by added cytochrome *c*. We take this as evidence that the NADH dehydrogenase segment was not 'scrambled' with respect to the cytochrome *c*-binding site and that non-vesicular membrane fragments were not present.

We also used ascorbate as a membrane-impermeable reductant for cytochrome *c* to check on the location of cytochrome *c* in the particles (Quintanilha & Packer, 1977). As shown in Fig. 3(*a*), addition of ascorbate to cyanide-inhibited particles resulted in a very slow and incomplete reduction of endogenous cytochrome *c*, which contrasts with the rapid reduction obtained with dithionite (Fig. 3*b*) and the reduction of exogenous cytochrome *c* by ascorbate (Fig. 3*c*). The cytochrome *c* of intact mitochondria was reduced rapidly by ascorbate (Fig. 3*d*). These experiments establish that all the cytochrome *c* in the preparations of submitochondrial particles was internal, but it is possible that cytochrome *c* had been lost from external binding sites (i.e. from membrane vesicles with a non-inverted orientation). To check on this, we used affinity chromatography on a cytochrome *c*–agarose column (Gautheron *et al.*, 1977). Inverted particles with no external cytochrome *c*-binding sites can be eluted from such columns at low ionic strength, while non-inverted particles are retained by the column and may be eluted by 1 M-KCl (Lötscher *et al.*, 1979). In several experiments we found that the total yield of submitochondrial-particle protein off the column was rather variable and depended on the initial loading. However, the proportion of protein eluted at low ionic strength was far greater than that eluted at high ionic strength. The former material was collected by centrifugation and used for labelling studies as described below. Since we had

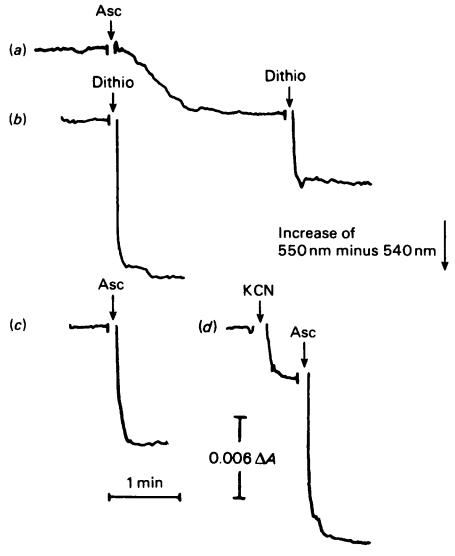

Fig. 3. *Reduction of cytochrome c in mitochondria and submitochondrial particles*

Cytochrome *c* reduction was measured at 550nm minus 540nm. (*a* and *b*) Submitochondrial particles (4.8mg of protein) were incubated at room temperature in a final volume of 3ml containing 0.25M-sucrose/50mM-sodium phosphate, pH7.6, and 1mM-KCN; (*c*) 0.47μM-cytochrome *c* replaced the submitochondrial particles; (*d*) mitochondria (4.6mg of protein) and antimycin A (5μg) replaced the submitochondrial particles and KCN. Additions as indicated were 5μl of 0.2M-ascorbate (Asc), 0.1mg of solid sodium dithionite (Dithio) and 20μl of 0.5M-KCN(KCN).

already found that NADH dehydrogenase was not 'scrambled' with respect to the cytochrome *c*-binding site, the column-purified particles should have contained NADH dehydrogenase with an orientation opposite to that of intact mitochondria.

Eytan *et al.* (1975) used a very direct approach to the sidedness of the submitochondrial particles used in their studies of cytochrome oxidase topography. They found that antibody to cytochrome oxidase only reacted with that portion of the enzyme exposed on the cytoplasmic side of the membrane. Thus they could immunoprecipitate 'scrambled', broken or non-inverted membranes from their submitochondrial-particle preparations. Unfortunately, we were not able to use the same approach, as our anti-(complex I) serum agglutinated inverted membranes, as shown by the complete immunoprecipitation of NADH–$K_3Fe(CN)_6$ oxidoreductase activity. Even if the immunoprecipitated material had consisted of pure inverted membranes, the binding of antibodies to the Complex-I subunits would have interfered with subsequent solubilization and purification of the enzyme.

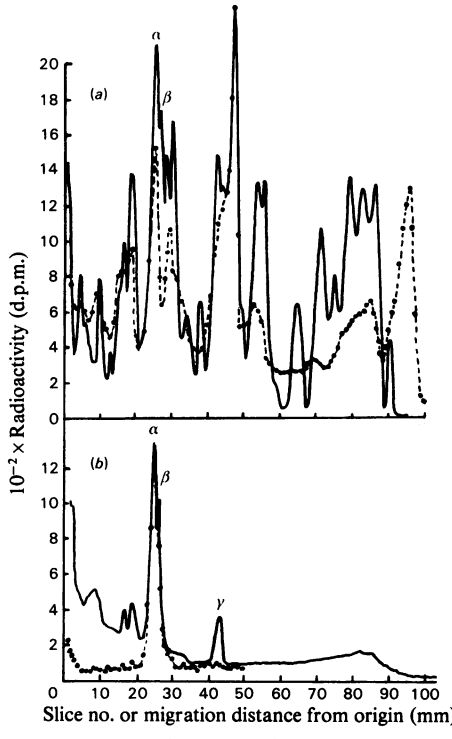

Fig. 4. *Labelling of submitochondrial particles with diazobenzene[^{35}S]sulphonate*

Submitochondrial particles prepared by ultrasonic disruption of mitochondria were labelled with 2mM-diazobenzene[^{35}S]sulphonate (28Ci/mol) as described in the Materials and Methods section. Extraction of F_1-ATPase and discontinuous SDS/polyacrylamide-gel electrophoresis were performed exactly as in Figs. 1 and 2. (*a*) Submitochondrial particles; (*b*) F_1-ATPase. ——, Densitometer scans; – – – –, radioactivity.

Figs. 4 and 5 show experiments similar to those of Figs. 1 and 2, except that submitochondrial particles were labelled. As in Figs. 1 and 2, the labelling patterns obtained with diazobenzene[^{35}S]sulphonate (Fig. 4*a*) and ^{125}I (Fig. 5*a*) were not identical. However, in each case F_1-ATPase was heavily labelled (Figs. 4*b* and 5*b*). Results obtained with submitochondrial particles prepared by any method were much the same.

Immunoprecipitation of Complex I from mitochondria and submitochondrial particles

Antiserum raised against Complex I failed to inhibit either the NADH–ubiquinone or NADH–$K_3Fe(CN)_6$ oxidoreductase activities of the isolated enzyme or of submitochondrial particles. However, the isolated enzyme was quantitatively immunoprecipitated. Similar results were obtained when anti-

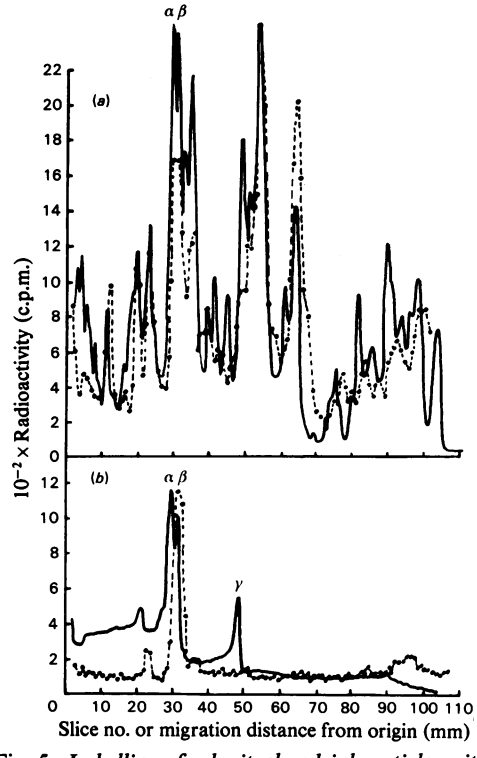

Fig. 5. *Labelling of submitochondrial particles with* ^{125}I

All conditions and data presentation were as described in the Materials and Methods section or as in previous Figure legends.

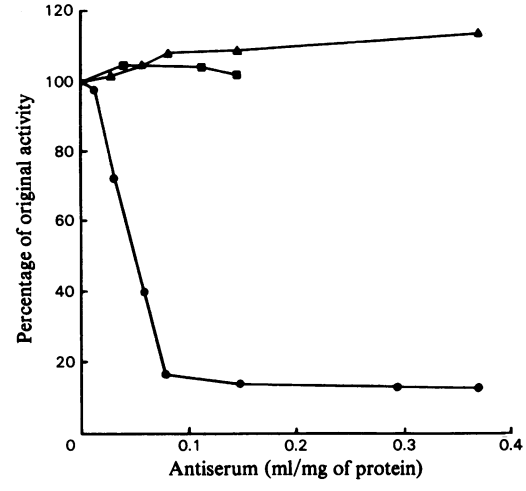

Fig. 6. *Immunoprecipitation of Complex I from solubilized submitochondrial particles*

Submitochondrial particles were solubilized with deoxycholate as described in the Materials and Methods section. Portions (50μg of protein in 50μl) were diluted to 0.5 ml with 0.25 M-sucrose/50 mM-sodium phosphate, pH 7.6, the indicated volume of control serum or antiserum and 10% (w/v) Triton X-100 to a final concentration of 1% (w/v). After 1 h at 4°C, samples were assayed for NADH–$K_3Fe(CN)_6$ oxidoreductase activity before (▲) and after (●, ■) centrifugation at 25000 rev./min for 40 min in the 10 × 10 ml rotor of an MSE 65 centrifuge. ●, ▲, Antiserum; ■, control serum.

(Complex I) serum was allowed to react with detergent-solubilized mitochondria or submitochondrial particles. Fig. 6 shows a titration of deoxycholate- and Triton X-100-treated submitochondrial particles with anti-(Complex I) serum. The NADH–$K_3Fe(CN)_6$ oxidoreductase activity of the particles was unaffected by either antiserum or control serum, but the former caused precipitation of approx. 85% of the activity. The activity not precipitated by antiserum may be due to another enzyme with diaphorase activity, such as lipoamide dehydrogenase. Interestingly, antibody excess did not lead to a reduction in the amount of activity precipitated. This is presumably because the antigen is not truly water-soluble, and any antibody–antigen complex (whatever the ratio of the two constituents) is insoluble even in the presence of detergents. We have also found that the end point of titrations such as those of Fig. 6 cannot be used to estimate the concentration of antigen in crude preparations such as mitochondria, since the end point was not greatly different when the mitochondrial preparation was supplemented with purified Complex I.

We initially chose to solubilize the submitochondrial particles and mitochondria with deoxycholate because this detergent is used in the initial stages of Complex-I purification. However, as detailed below, the preparation of immunoprecipitates uncontaminated with other proteins required the further use of Triton X-100. Unfortunately, this detergent destroys the NADH–ubiquinone oxidoreductase activity of Complex I probably by displacement of essential phospholipids (Heron *et al.*, 1977).

Preparation of pure immunoprecipitates

Complex I is a single enzyme consisting of at least 26 polypeptides (Heron *et al.*, 1979). This means that any single polypeptide accounts for only 4% of the total protein on average. It is therefore extremely important that the immunoprecipitates should be free from contamination by even small amounts of other proteins. This is not readily achieved for membrane-bound antigens, as is clearly shown in the literature (e.g. Eytan *et al.*, 1975; Lin *et al.*, 1978). However, the simpler protein composition of antigens such as cytochrome oxidase or cytochrome *b* enables the constituent polypeptides to be easily seen

on SDS/polyacrylamide gels over the background contamination.

Immunoprecipitates can be contaminated by other serum proteins or other mitochondrial proteins. The former can be eliminated by purifying the immunoglobulin G fraction from whole serum, but since serum proteins would not be labelled in the experiments to be described this was not done. Contaminating serum proteins appear on SDS/polyacrylamide gels as several high-molecular-weight polypeptides. These proteins are not of mitochondrial origin, since they are also seen in immunoprecipitates obtained by allowing purified Complex I to react with antiserum. Contamination by other mitochondrial proteins presents a serious problem, since these proteins may be radioactively labelled and co-migrate with Complex-I polypeptides. Such contaminating proteins could be seen in precipitates produced by incubation of mitochondrial extracts with control serum. Fig. 7 shows the evolution of our current procedure for eliminating such contaminants and producing pure immunoprecipitates. Incubation with antiserum for 16h was used to produce bulky precipitates which could be sedimented easily. To ensure dispersal of the mitochondrial protein complexes, high concentrations of Triton X-100 were included in all incubations. Despite this, precipitates were always obtained with control serum (Fig. 7*a*). The major constituent of such precipitates had a mol.wt. of 30000 and might well be a portion of the adenine nucleotide translocase, which is the most abundant protein of the inner mitochondrial membrane (Riccio *et al.*, 1975). This polypeptide and some of the others of Fig. 7(*a*) were found to be radioactively labelled when derived from mitochondria or submitochondrial particles that had been labelled with diazobenzene[^{35}S]sulphonate or ^{125}I. These polypeptides were also precipitated gradually with time in the absence of serum. Therefore the mitochondrial extracts were incubated with Triton X-100 for 24h and centrifuged before treatment with control serum (Fig. 7*b*) or antiserum (Fig. 7*d*). The precipitates thus obtained with control serum (Fig. 7*b*) were free from all but a few high-molecular-weight polypeptides probably derived from the serum. Whatever their source, they were not radioactively labelled. A second refinement was to preincubate the mitochondrial extracts firstly with Triton X-100 and secondly with control serum. After centrifugation, a further incubation with either control serum or antiserum was performed. No detectable precipitate was formed with control serum (Fig. 7*c*). Because of the length of time involved in this procedure, immunoprecipitates were routinely obtained as in Fig. 7(*d*), i.e. antiserum was added after preincubation of the mitochondrial extract with Triton X-100. The polypeptide profile obtained is identical with that of purified Complex I, except that

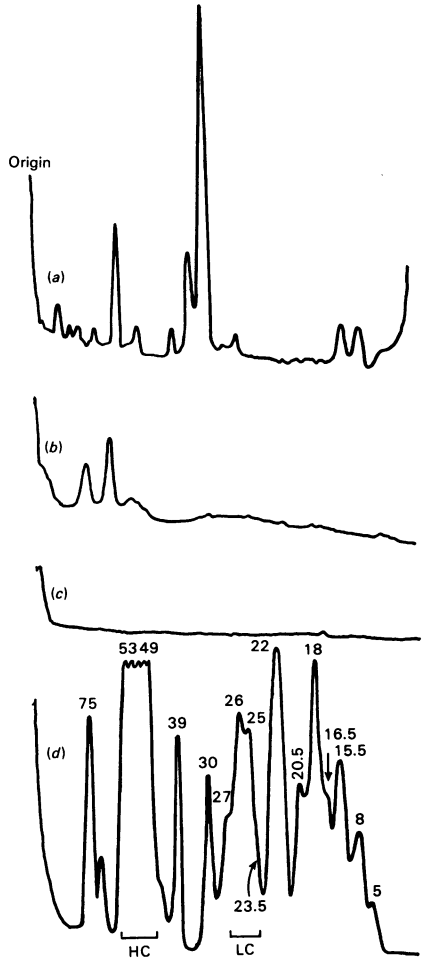

Fig. 7. *Polypeptide composition of precipitates formed by allowing solubilized submitochondrial particles to react with control serum and antiserum*

Submitochondrial particles prepared by ultrasonic disruption of mitochondria were solubilized as described in the Materials and Methods section. Precipitates were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis and stained for protein. The profiles (*b*) and (*d*) were obtained from precipitates prepared exactly as described in the Materials and Methods section from 2mg of submitochondrial particle protein allowed to react with control serum or antiserum respectively. For (*a*), the precipitate was obtained by allowing 2mg of submitochondrial-particle protein to react directly with control serum in 1% (w/v) Triton X-100, omitting the incubation with Triton X-100 alone. For (*c*), the procedure was the same as in (*b*), except that a second incubation with control serum in Triton X-100 was performed. The precipitate resulting from the second treatment was analysed. Molecular weights (in thousands) are indicated in (*d*). HC, immunoglobulin heavy chains; LC, immunoglobulin light chains.

the 42000-mol.wt. polypeptide is missing, as previously reported (Smith & Ragan, 1978; Heron *et al.*, 1979). In addition, the heavy and light chains of immunoglobulin G are also present and obscure certain portions of the gel. Further separation of Complex-I subunits and removal of interference by the heavy and light chains has been achieved by two-dimensional analysis combining isoelectric focusing and SDS/polyacrylamide-gel electrophoresis (Heron *et al.*, 1979). However, since the labelling procedures used in the present paper produce changes in the isoelectric points of the modified proteins, we have not routinely extended our analyses with this refined separation method.

Labelling of isolated and membrane-bound Complex I with diazobenzene[^{35}S]sulphonate

When isolated Complex I was labelled with diazobenzene[^{35}S]sulphonate, radioactivity was found to be associated with most of the polypeptides (Fig. 8*a*). Treatment of Complex I with urea, $NaClO_4$ or other chaotropic agents solubilizes an iron–protein fraction and a flavoprotein fraction (Hatefi & Stempel, 1969). The latter consists of three polypeptides of mol.wts. 53000, 27000 and 15500 (Ragan, 1976; Dooijewaard *et al.*, 1978; Galante & Hatefi, 1979; Heron *et al.*, 1979). When isolated from labelled Complex I, no radioactivity was found to be associated with any of the constituent polypeptides (Fig. 8*c*). The iron–protein fraction consists of eight polypeptides (Ragan, 1976; Heron *et al.*, 1979), of which only those of mol.wts. 75000 and 49000 are labelled to any extent (Fig. 8*b*). Thus, in gels of immunoprecipitates, where the polypeptides of mol.wts. 53000 and 49000 are obscured by the heavy chains of immunoglobulin G, any label under the peak can clearly be ascribed to the 49000-mol.wt. polypeptide only. This was confirmed directly by two-dimensional analysis (results not shown).

Fig. 9 shows the labelling of the subunits of Complex I immunoprecipitated from mitochondria (Fig. 9*a*), and submitochondrial particles prepared by ultrasonic disruption (Fig. 9*b*), alkali treatment (Fig. 9*c*) or purified by affinity chromatography (Fig. 9*d*). The subunits heavily labelled in mitochondria had mol.wts. of 75000, 49000 and 30000. With the discontinuous buffer system used for the gels of Fig. 9, two polypeptides co-migrate with an apparent mol.wt. of 30000 (Crowder & Ragan, 1977). These may be separated by electrophoreses in the buffer system described by Weber & Osborn (1969), where they have apparent mol.wts. of 30000 and 33000. The former is a component of the iron–protein fraction, and the latter is not solubilized by chaotropic agents. Only the 33000-mol.wt. polypeptide was labelled in the experiments of Figs. 8 and 9. Polypeptides

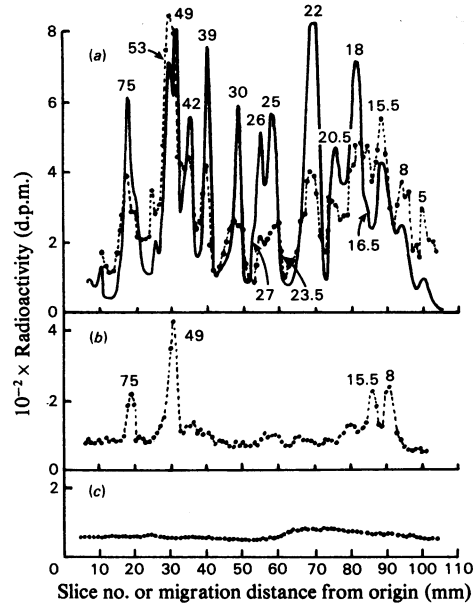

Fig. 8. *Chaotropic resolution of Complex I labelled with diazobenzene[^{35}S]sulphonate*

Complex I (approx. 20mg of protein) was labelled with diazobenzene[^{35}S]sulphonate (46Ci/mol) as described in the Materials and Methods section and washed thrice by centrifugation at 40000rev./min for 30min in the 10ml × 10ml rotor of an MSE 65 centrifuge and resuspension in 0.67M-sucrose/50mM-Tris/HCl, pH8.0 at 4°C. The pellet was finally resuspended to a final volume of 2ml in sucrose/Tris buffer (the protein concentration was 7mg/ml). Resolution with $NaClO_4$ was carried out as described in the Materials and Methods section. Fractions were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis. (*a*) Unresolved Complex I (100μg of protein); (*b*) iron–protein fraction (derived from 100μg of Complex-I protein); (*c*) flavoprotein fraction (derived from 100μg of Complex-I protein). Molecular weights (in thousands) are indicated. ——, Densitometer scans; – – – –, radioactivity.

labelled to a lesser extent in Fig. 9(*a*) had mol.wts. of 22000, 15500 and 8000. The labelling patterns of Complex I precipitated from submitochondrial particles prepared by ultrasonic disruption (Fig. 9*b*) or alkali treatment (Fig. 9*c*) were identical. Again, polypeptides of mol.wts. 75000, 49000 and 30000 (33000) were heavily labelled, but the relative incorporation of label into these proteins was quite different from that of mitochondria. Several lower-molecular-weight polypeptides were also labelled, namely those of mol.wts. 22000, 15500 and 8000. However, the 22000-mol.wt. polypeptide was labelled very much less when submitochondrial particles purified by affinity chromatography were used (Fig.

9*d*). The other polypeptides were labelled to comparable extents in Figs. 9(*b*), 9(*c*) and 9(*d*).

Deciding which polypeptides are 'significantly' labelled in mitochondria or submitochondrial particles is difficult. If we assume that 2mM-diazobenzene[^{35}S]sulphonate is sufficient to label all available sites to the same extent, we can compare the degree of labelling of isolated Complex I (Fig. 8) with the profiles of Fig. 9. On the basis of the Coomassie Blue staining, approximately the same amount of Complex-I protein was present on all the gels (handling losses during washing of the immunoprecipitates make the amount on each gel somewhat variable). Taking account of the differences in specific radioactivity, the amount of diazobenzenesulphonate incorporated into the numbered polypeptides of Fig. 9 is of the order of magnitude expected from Fig. 8.

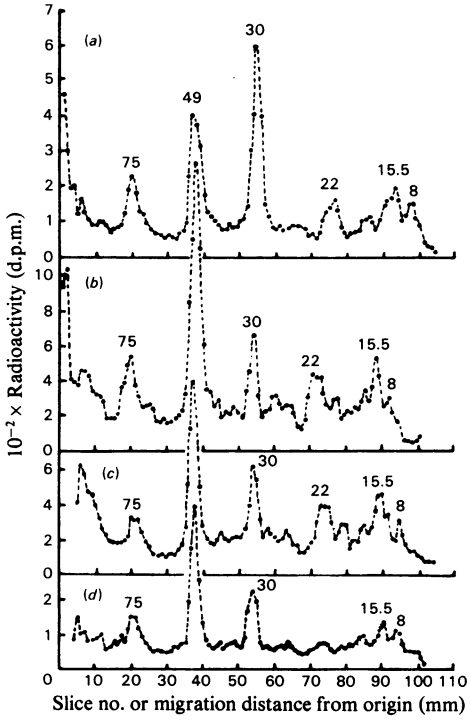

Fig. 9. *Labelling of Complex I isolated from mitochondria and submitochondrial particles labelled with diazobenzene*[^{35}*S*]*sulphonate*

Labelling and immunoprecipitation were carried out as described in the Materials and Methods section. Immunoprecipitates were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis. (*a*) Complex I immunoprecipitated from mitochondria (3mg of protein). Specific radioactivity was 31Ci/mol. (*b*) Complex I immunoprecipitated from submitochondrial particles (2mg of protein) prepared by ultrasonic disruption of mitochondria. Specific radioactivity was 95Ci/mol. (*c*) As (*b*), but using particles prepared by alkali treatment of mitochondria. Specific radioactivity was 83Ci/mol. (*d*) As (*b*), but using particles further purified by affinity chromatography. Specific radioactivity was 26Ci/mol. Molecular weights (in thousands) are indicated.

Labelling of isolated and membrane-bound Complex I with ^{125}*I*

Iodination of isolated Complex I labels most of the subunits (Ragan, 1976). The profile of Fig. 10 is similar to that obtained with diazobenzene[^{35}S]-sulphonate labelling, as are the labelling profiles of the fragments obtained by chaotropic resolution of iodinated Complex I. Thus the subunits of the flavoprotein fraction are unlabelled and the iron–protein fraction is mainly labelled in the 75000- and 49000-mol.wt. polypeptides (Ragan, 1976) similar to Fig. 8. However, the labelling of Complex I immunoprecipitated from labelled mitochondria and submitochondrial particles showed clear differences from labelling with diazobenzene[^{35}S]sulphonate. Thus, in mitochondria (Fig. 11*a*), besides subunits of mol.wts. 75000, 49000, 30000 and 8000, a 22000-mol.wt. polypeptide was heavily labelled with ^{125}I, whereas there was little or no label in the 15500-mol.wt. polypeptide. These differences can be attributed to the different chemical specificities of the two labels (Higgins & Harrington, 1959; Phillips & Morrison, 1971). These differences were not apparent in the labelling patterns of isolated Complex I (Figs. 8 and 10). This is explained by the finding that there are three subunits of mol.wt. 22000 and four of mol.wt. 15500 in Complex I (Heron *et al.*, 1979), any or all of which might be labelled

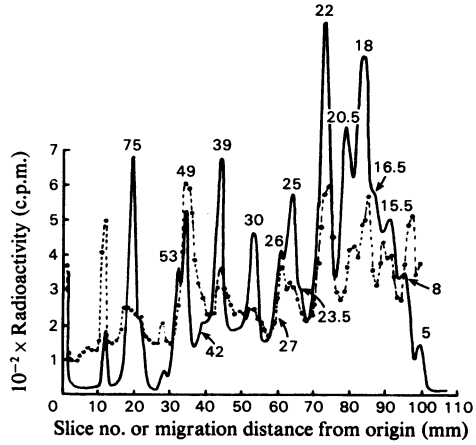

Fig. 10. *Labelling of Complex I with* ^{125}*I*

Complex I was labelled as described in the Materials and Methods section, and 100μg of protein was analysed by discontinuous SDS/polyacrylamide-gel electrophoresis. ——, Densitometer scan; – – – –, radioactivity. Molecular weights (in thousands) are indicated.

in the isolated enzyme. Thus variation in the reactivity of an individual polypeptide with one or other of the labels used might well be masked if several co-migrating polypeptides were labelled.

Further differences appeared when the labelling patterns of submitochondrial particles were compared (Figs. 11*b* and 11*c*). In particles prepared either by ultrasonic disruption of mitochondria (Fig. 11*b*) or further purified by affinity chromatography (Fig. 11*c*), the polypeptides of mol.wts. 75000, 49000, 30000 and 8000 were labelled, similar to the results shown in Fig. 9. However, an additional polypeptide of mol.wt. 26000 was consistently and heavily labelled by ^{125}I. A 22000-mol.wt. polypeptide was heavily labelled in unpurified submitochondrial particles (Fig. 11*b*), but scarcely at all in purified particles (Fig. 11*c*). This result is exactly the same as was encountered with diazobenzene[^{35}S]-sulphonate as label. The labelling of this protein in crude submitochondrial particles is probably due to the presence of non-inverted or dislocated NADH dehydrogenase in the membrane preparation. The same seems to be true of a 15500-mol.wt. polypeptide which is labelled in Fig. 11(*b*), but not in Fig. 11(*c*). Labelling by ^{125}I offers a clearer distinction between mitochondria and submitochondrial particles than labelling by diazobenzene[^{35}S]-sulphonate, because of the unique labelling of the 26000- and 22000-mol.wt. polypeptides from different faces of the membrane.

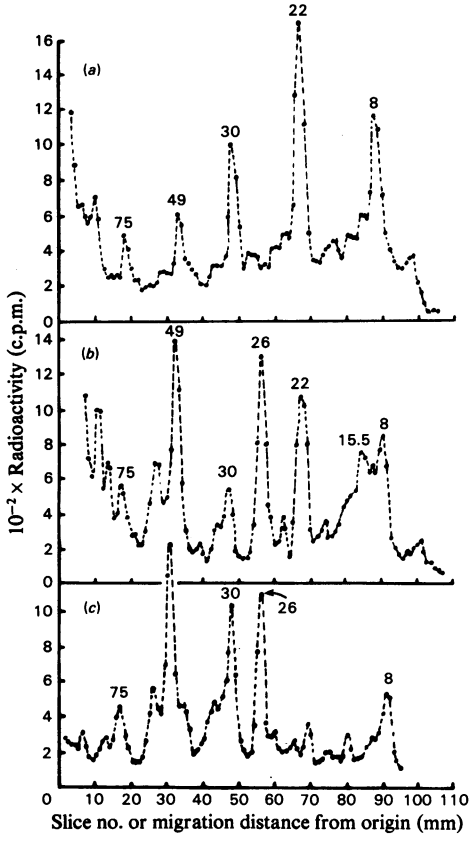

Fig. 11. *Labelling of Complex I isolated from mitochondria and submitochondrial particles labelled with* ^{125}I

Labelling and immunoprecipitation were carried out as described in the Materials and Methods section. Immunoprecipitates were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis. (*a*) Complex I immunoprecipitated from mitochondria (3mg of protein); (*b*) Complex I immunoprecipitated from submitochondrial particles (2mg of protein) prepared by ultrasonic disruption of mitochondria; (*c*) as (*b*), but using particles further purified by affinity chromatography. Molecular weights (in thousands) are indicated.

Comparison of the degree of radioiodination of Complex I in isolation, in submitochondrial particles and in mitochondria, is complicated by the presence in the latter of catalase which removes H_2O_2. Although we have made no direct attempts to label different preparations to comparable extents, Figs. 2 and 5 show that the extent of labelling of the 30000-mol.wt. polypeptide (the adenine nucleotide translocase) was similar in both mitochondria and submitochondrial particles. Thus one transmembranous protein was labelled approximately equally from either side of the membrane under our conditions. We would therefore expect that Complex I polypeptides exposed on either side of the membrane would be labelled to comparable extents. As shown in Fig. 11, this was found to be so. We conclude that it is most unlikely that the labelling seen with submitochondrial particle preparations was due to the presence of a small proportion of mitochondria (or vice versa).

Discussion

The results presented above clearly demonstrate that some subunits of Complex I are exposed on the cytoplasmic face or 'C side' of the inner mitochondrial membrane. The striking similarity between those proteins exposed on the matrix face or 'M side' of the membrane might suggest that our inverted submitochondrial particles were in fact 'scrambled' or contained a substantial proportion of non-inverted membranes. We have presented some evidence to show that this was not the case and further support comes from the experience of other workers in related experiments. Eytan *et al.* (1975) used an immunological method to purify vesicles containing cytochrome oxidase with an inverted orientation. However, the cytochrome oxidase labelling pattern obtained with these was not greatly different from that obtained with crude submitochondrial particles prepared by ultrasonic disrup-

tion. Moreover, the pattern obtained with the latter preparation was clearly different from that obtained with mitochondria. Similar results were obtained by Mendel-Hartvig & Nelson (1978) in their studies of ubiquinol–cytochrome *c* oxidoreductase topography. They found quite dissimilar labelling patterns in mitochondria and unpurified submitochondrial particles. When the patterns are quite different, of course, there is little need to prove the homogeneity of the preparations used. In the present paper, the similarity between the labelling patterns obtained with mitochondria and submitochondrial particles required us to investigate the homogeneity of our preparations in greater detail.

Fig. 12 summarizes our conclusions. The three subunits of the flavoprotein fraction (of mol.wts 53000, 27000 and 15000) are inaccessible to labelling even in isolated Complex I. However, since this fraction contains the NADH-binding site, some part of it must be located close to the 'M side' of the membrane for NADH oxidation to occur in the matrix. Polypeptides of mol.wts. 75000, 49000 and 30000 appear to be transmembranous, since they are labelled from either side of the membrane, and Complex I contains only one molecule of each per molecule of FMN (Ragan, 1976). The latter polypeptide has been shown in Fig. 12 with a mol.wt. of 33000 [as determined in the Weber & Osborn (1969) system]. This is to distinguish it from the 30000-mol.wt. polypeptide of the iron–protein fraction, which, like the 23500-mol.wt. polypeptide, is not labelled, even in isolated Complex I. Polypeptides of mol.wts. 15500 and 8000 appear to be labelled from either side of the membrane. However, since there are more than one non-identical polypeptides of these molecular weights in Complex I (Heron *et al.*, 1979), we cannot conclude that they are transmembranous. Thus Fig. 12 shows different subunits of these molecular weights on either side of the membrane.

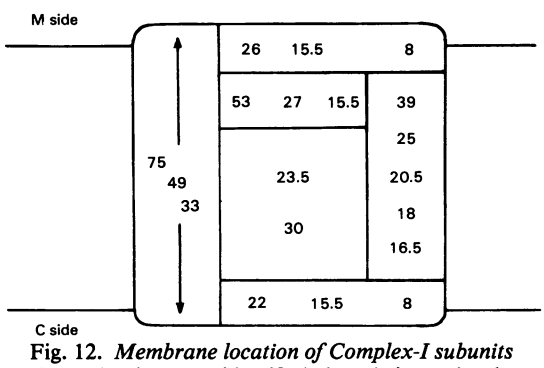

Fig. 12. *Membrane location of Complex-I subunits*
The subunits are identified by their molecular weights (in thousands). The diagram is not intended to show the relative positions of the various subunits in the overall structure, but merely groups them according to their accessibility to the membrane probes used, as described in the text.

There are several polypeptides that can be labelled in the isolated enzyme but are not exposed on either side of the mitochondrial membrane. For example, the 39000-mol.wt. polypeptide of isolated Complex I is clearly labelled by either diazobenzene[^{35}S]-sulphonate (Fig. 8) or ^{125}I (Fig. 10), but is not labelled in the membrane (Figs. 9 and 11). Assuming that Complex I does not undergo gross conformational changes on isolation, polypeptides such as this one are probably located on the outside of the protein structure, but face the hydrophobic interior of the membrane, as shown in Fig. 12. In the isolated enzyme, which appears to carry with it only a single annulus of phospholipid (Heron *et al.*, 1977), such polypeptides could be labelled by reagents with only a limited ability to penetrate the hydrophobic regions of the membrane. Indeed, the different patterns of labelling obtained with either diazobenzene[^{35}S]sulphonate or ^{125}I may be a function of different degrees of membrane penetration as well as different chemical specificities. In addition to the 39000-mol.wt. polypeptide it is possible to identify several others in the same category, namely those of mol.wts. 25000, 20500, 18000 and 16500.

Although little is known about the function of individual polypeptides in Complex-I activity, the results presented here are consistent with a 'loop' mechanism for the transfer of hydrogen atoms from NADH to ubiquinone, as depicted by Lawford & Garland (1972). Thus the H-carrying arm could consist of the FMN prosthetic group associated with the three flavoprotein subunits, and transmembrane electron transfer could be catalysed by iron–sulphur centres associated with the transmembranous polypeptides of the iron–protein fraction.

This research was supported by a Science Research Council grant to C.I.R. We are grateful to Dr. H. Hauser, Laboratorium für Biochimie, Eidgenössische Technische Hochschule, Zürich, Switzerland, for information on the purification of submitochondrial particles by affinity chromatography.

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