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Identification of the subunits of bovine heart mitochondrial NADH dehydrogenase that are exposed to the phospholipid bilayer by photolabelling with 5-iodonaphth-1-yl azide

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Mitochondrial NADH dehydrogenase may be isolated from bovine heart as a lipoprotein complex (Complex I or NADH–ubiquinone oxidoreductase). Polypeptide subunits that are exposed to the hydrophobic region of the phospholipid bilayer were identified by photolabelling with the hydrophobic probe, 5-[^{125}I]iodonaphth-1-yl azide. Chaotropic resolution of the labelled enzyme showed that the hydrophilic flavoprotein and iron–protein fragments of the enzyme were not in contact with the phospholipid bilayer. When Complex I that had been partially depleted of phospholipids was photolabelled, incorporation of radioactivity into certain polypeptides was increased, indicating either conformational changes in the protein or preferential association of these polypeptides with residual cardiolipin. A model of NADH dehydrogenase structure is proposed on the basis of these results and those obtained with hydrophilic probes by Smith & Ragan [(1980) *Biochem. J.* **185**, 315–326].

Mitochondrial NADH dehydrogenase is an enzyme of enormous complexity. It may be isolated from bovine heart mitochondria as a detergent-soluble lipoprotein that catalyses the reduction of ubiquinone analogues by NADH (Complex I in the nomenclature of Hatefi *et al.*, 1962*a*). This preparation consists of at least 26 different polypeptides (Heron *et al.*, 1979*b*). When treated with chaotropic agents such as $NaClO_4$ the enzyme may be resolved into three fractions: an insoluble residue, a water-soluble iron–sulphur flavoprotein (referred to below as the 'flavoprotein fragment') and a water-soluble iron–sulphur protein (referred to below as the 'iron–protein fragment') (Hatefi & Stempel, 1967). The flavoprotein fragment comprises three polypeptides in 1:1:1 molar proportions and contains all the FMN of the original enzyme (Galante & Hatefi, 1979). The iron–protein fragment consists of eight polypeptides (Heron *et al.*, 1979*b*) and contains no FMN. The structural organization of the enzyme both in isolation and in the mitochondrial inner membrane has been studied by using two hydrophilic membrane-impermeable protein labels, diazobenzene[^{35}S]sulphonate and the lactoperoxidase-catalysed incorporation of ^{125}I (Ragan, 1976; Smith & Ragan, 1980). On the basis of accessibility to these two labels, the subunits of NADH dehydrogenase were divided into five groups: (*a*) those that are buried in the interior of the enzyme; (*b*) those that are accessible to labelling in the isolated enzyme but not when the enzyme is in the mitochondrial inner membrane; (*c*) those that are exposed on the matrix side of the membrane; (*d*) those that are exposed on the cytoplasmic side of the membrane; (*e*) those that are transmembranous. The subunits of the flavoprotein fragment and all but three subunits of the iron–protein fragment belong to the first group, since they are inaccessible to both labels. Two subunits of the iron–protein fragment are transmembranous. Many subunits fall into the second group, i.e. they can only be labelled in the isolated enzyme. This suggests (Smith & Ragan, 1980) that, in the membrane, these polypeptides are adjacent to the hydrophobic region of the phospholipid bilayer. In the isolated enzyme, which has perhaps only a single shell of phospholipid around it, these subunits are more exposed to the aqueous phase and become accessible to membrane-impermeable reagents. This group of subunits are all found in the insoluble residue from chaotropic resolution and appear to be very hydrophobic, consistent with their proposed location adjacent to the fatty acid region of the bilayer.

In the present paper, evidence has been sought in

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support of these proposals. We have used as a probe 5-[^{125}I]iodonaphth-1-yl azide, which is reputed to label protein subunits embedded in the hydrophobic region of the phospholipid bilayer (Bercovici & Gitler, 1978). This compound partitions preferentially into the hydrophobic region of membranes, and on illumination it gives a highly reactive nitrene, which inserts into adjacent protein or lipid molecules. Bayley & Knowles (1978) have objected to the use of nitrenes for this kind of study on the grounds that the nitrene is more polar than the azide and could, within the lifetime of the molecule, migrate to the polar surface of the membrane before reaction. Thus it would not act as a probe for hydrophobic regions of the membrane. It is perhaps unfortunate that the model compound used by these authors, phenyl azide, is at least two orders of magnitude more soluble in aqueous solutions than is 5-iodonaphth-1-yl azide. Moreover, the results obtained by Bercovici & Gitler (1978) with sarcoplasmic reticulum and erythrocyte membranes, the work of Cerletti & Schatz (1979) with cytochrome oxidase and the results described in the present paper are consistent with the idea that 5-iodonaphth-1-yl azide is a hydrophobic label.

Materials and methods

Biological preparations

Complex I (EC 1.6.99.3) was purified from bovine heart mitochondria by the method of Hatefi & Rieske (1967). Chaotropic resolution of Complex I was performed with $NaClO_4$ as described by Smith & Ragan (1980). The flavoprotein and iron–protein fragments were purified by $(NH_4)_2SO_4$ fractionation as described by Hatefi & Stempel (1969).

Complex I was depleted of phospholipids by cholate and $(NH_4)_2SO_4$ treatment by the method of Heron *et al.* (1977). This treatment decreased the phospholipid content from 0.20–0.23 to 0.04–0.05 μmol of phospholipid P/mg of protein. The residual lipid was nearly all cardiolipin (Heron *et al.*, 1977). Phospholipid-enriched Complex I was prepared by addition of purified soya-bean phosphatidylcholine (Ragan & Racker, 1973). The phosphatidylcholine was dispersed by ultrasonic irradiation in 20mM-potassium phosphate buffer, pH 8, containing 2% (w/v) sodium cholate to give a final concentration of 100mM-phospholipid P. Complex I dissolved in 0.67M-sucrose/50mM-Tris/HCl buffer, pH 8, at a concentration of 20mg of protein/ml was mixed with the phosphatidylcholine dispersion to raise the phospholipid content from 0.23 to 0.69 μmol of phospholipid P/mg of protein.

Endogenous phosphatidylcholine and phosphatidylethanolamine in Complex I were replaced by 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine as described by Heron *et al.* (1979*a*). Cholate was removed from the lipid-replaced enzyme by dialysis overnight against several hundred volumes of 0.67M-sucrose/50mM-Tris/HCl buffer, pH 8.0, at 4°C.

Synthesis of 5-[^{125}I]iodonaphth-1-yl azide

5-[^{125}I]Iodonaphth-1-yl azide was synthesized by method 2 of Bercovici & Gitler (1978) as modified by Cerletti & Schatz (1979). Carrier-free Na^{125}I from The Radiochemical Centre (Amersham, Bucks., U.K.) was diluted with NaI to a specific radioactivity of 8 Ci/mmol before use.

Photolabelling of Complex I

Complex I (4mg of protein/ml of 0.67M-sucrose/50mM-Tris/HCl buffer, pH 8.0) was mixed with 5-[^{125}I]iodonaphth-1-yl azide in 1.5ml silica spectrophotometer cells of 1cm light-path. The radioactive label was added as an ethanolic solution such that the final ethanol concentration did not exceed 1% (v/v). The amounts of radioactivity added are given in the Figure legends. After incubation for 10min in the dark at room temperature, the sample was exposed to a 125W high-pressure mercury-vapour lamp (Thorn Electrical, Southampton, U.K.) at a distance of 30cm for 80s. The sample was stirred every few seconds during exposure.

Determination of protein-bound radioactivity

After the photolabelling, portions (20 μl) containing 80 μg of protein were mixed with 0.4ml of ice-cold 10% (w/v) trichloroacetic acid. Precipitated protein was collected on glass-fibre filters (Whatman GF/C) by suction and washed successively with 2ml volumes of 5% (w/v) trichloroacetic acid, acetone, ethanol/ether (1:1, v/v) and ether. After the filters had dried, their radioactivities were counted in a Beckman Biogamma counter.

Polyacrylamide-gel electrophoresis

After the photolabelling, portions (0.5ml) containing 2mg of protein were mixed with 4.5ml of ice-cold acetone, and the precipitated protein was collected by centrifugation in a bench centrifuge. The pellets were dried and redissolved in 20mM-sodium phosphate buffer, pH 8.0, containing 2% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol by heating at 100°C for 2min. Acetone-precipitation largely removed a band of highly radioactive material that ran with the tracking dye on electrophoresis. This material was probably photolabelled phospholipids. The dissociated protein samples (100 μg per gel) were analysed by the method of Weber & Osborn (1969) on cylindrical polyacrylamide gels (12cm long × 0.6cm internal diam.) containing 12.5% (w/v) acrylamide and 0.34% bisacrylamide. Samples were also analysed by discontinuous slab-gel electrophoresis after

dialysis overnight against 100vol. of 25mM-Tris/0.192M-glycine containing 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. Running gels (12cm long × 1mm thick) of the above composition were overlaid by a stacking gel (1cm long × 1mm thick) containing 3% (w/v) acrylamide and 0.24% bisacrylamide. Samples containing 50μg of protein were applied to each track. The buffer system used was that of Laemmli (1970).

Gels were stained with Brilliant Blue R (Sigma, Poole, Dorset, U.K.) and destained as described by Weber & Osborn (1969).

Determination of radioactivity in gels

Cylindrical gels were sliced into 1mm-thick sections with a Mickle gel slicer (Mickle Engineering Co., Gomshall, Surrey, U.K.). The radioactivities of the slices were then counted directly in a Beckman Biogamma counter.

Slab gels were dried down on to filter paper for radioautography. Kodak X-OMAT H film was exposed to the dried gel at −20°C for 7 days and developed in accordance with the manufacturers' instructions.

Assays

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V from Sigma) as a standard. NADH–ubiquinone-1 oxidoreductase was assayed as described by Ragan (1976).

Results

Time course of incorporation of 5-iodonaphth-1-yl azide

Under the conditions of illumination described in the Materials and methods section, photolabelling of Complex I was nearly maximal after 2min (Fig. 1), at which time approx. 9% of the added radioactivity had been incorporated into protein. Also shown in Fig. 1 is the inactivation of the enzyme by irradiation. The extent of inactivation was the same in the presence and in the absence of 5-iodonaphth-1-yl azide. To minimize labelling of denatured enzyme, which might have had an altered conformation, we adopted a standard illumination period of 80s.

The experiment of Fig. 1 was performed after a preincubation period of 10min in the dark to allow the reagent to equilibrate with the enzyme. A similar procedure was adopted by Bercovici & Gitler (1978) and Cerletti & Schatz (1979). In practice, we found that equilibration was very rapid, and incorporation by a 10s illumination period was the same for preincubation periods ranging from 15s to 5min (results not shown).

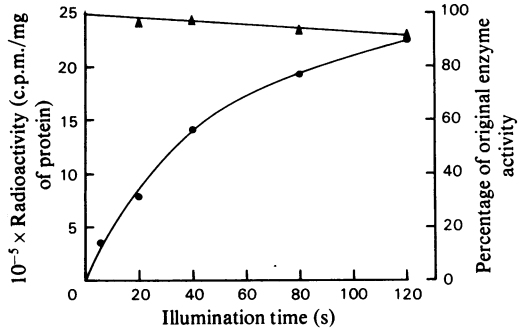

Fig. 1. *Time course of photolabelling of Complex I*
Complex I was photolabelled with 5-[^{125}I]iodonaphth-1-yl azide (2.6×10^7c.p.m./mg of protein) as described in the Materials and methods section. Portions were removed at intervals for measurement of protein-bound radioactivity and NADH–ubiquinone-1 oxidoreductase. No radioactivity was found in a parallel sample incubated in the dark. ●, Radioactivity; ▲, enzyme activity.

Labelling of NADH dehydrogenase polypeptides

Fig. 2(*b*) shows the distribution of radioactivity between Complex-I polypeptides (Fig. 2*a*) after photolabelling by 5-iodonaphth-1-yl azide. Most of the labelling occurred in the smaller polypeptides of mol.wt. less than 30000. Clearly the labelling is quite selective. This selectivity was largely abolished when the enzyme was dissociated with sodium dodecyl sulphate before the photolabelling (Fig. 2*c*). Thus, when each subunit is surrounded by a hydrophobic layer of sodium dodecyl sulphate molecules, it can be readily labelled with 5-iodonaphth-1-yl azide. Similar findings were reported by Cerletti & Schatz (1979) for cytochrome oxidase.

As isolated, Complex I forms optically clear solutions at high concentration because of cholate and $(NH_4)_2SO_4$ present from the last step of purification. On dilution into detergent-free media, the enzyme becomes insoluble and forms membranous aggregates. The photolabelling experiment of Fig. 2 was performed at a protein concentration of 4mg/ml, which is high enough to keep a considerable proportion of the enzyme in monomeric form. To test whether the labelling pattern was dependent on the state of aggregation of the enzyme or the presence of detergent, cholate and $(NH_4)_2SO_4$ were removed by dialysis before the photolabelling. Small effects were observed in the high-molecular-weight region of the gel (Fig. 3), where the degree of labelling of the subunits of mol.wts. 75000 to 39000 was increased. Since these subunits were labelled to only a small extent anyway, it may be that their degree of labelling was more sensitive to variations in the partition of 5-iodonaphth-1-yl azide between the lipoprotein and cholate micelles. In the experi-

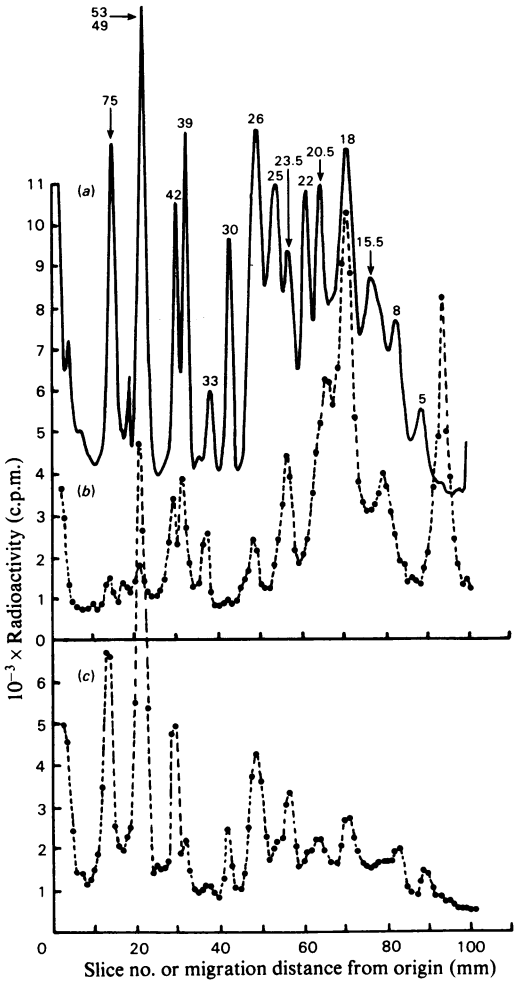

Fig. 2. *Labelling of Complex-I subunits by 5-[^{125}I]iodonaphth-1-yl azide*

Complex I was photolabelled with 5-[^{125}I]iodonaphth-1-yl azide (2.6×10^7 c.p.m./mg of protein) as described in the Materials and methods section, and analysed by polyacrylamide-gel electrophoresis with the buffer system of Weber & Osborn (1969). Radioactivity was measured in 1 mm-thick slices of the gel. (*a*) shows the densitometer scan of the stained gel; (*b*) gives the radioactivity of the gel slices and (*c*) the radioactivity of a sample of Complex I that was labelled in the presence of 2% (w/v) sodium dodecyl sulphate. The polypeptides are indicated by their molecular weights (in thousands).

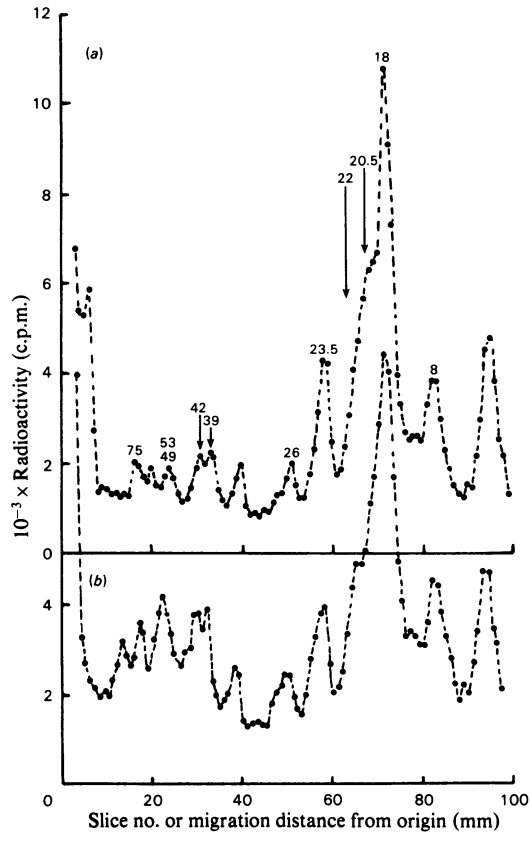

Fig. 3. *Effects of dialysis on the labelling profile*

Complex I (*a*) or Complex I dialysed for 16 h against 500 vol. of 0.67 M-sucrose/50 mM-Tris/HCl buffer, pH 8.0, at 4°C (*b*) was labelled with 5-[^{125}I]iodonaphth-1-yl azide (2.4×10^7 c.p.m./mg of protein) and analysed by the Weber & Osborn (1969) electrophoretic procedure. The positions of the polypeptides are indicated by the molecular weights (in thousands).

ments of Figs. 2 and 3, a peak of radioactivity was found at the dye front in a region of the gel that contained no stained protein bands. Since photolysis products of the reaction are removed by the gel staining and destaining procedure, it is likely that this peak is due to labelled phospholipids, which would not be completely extracted by 90% (v/v) acetone (Bercovici & Gitler, 1978).

Distribution of label between the products of chaotropic resolution

In the experiment of Fig. 4, photolabelled Complex I was treated with $NaClO_4$, and the iron–protein and flavoprotein fragments were isolated by $(NH_4)_2SO_4$ fractionation. None of the constituent polypeptides of either of these fragments was labelled, and all radioactivity remained in the insoluble hydrophobic residue.

Effects of hydrophobic ligands of Complex I on the labelling pattern

Inhibitors of NADH–ubiquinone oxidoreductase activity that probably bind to hydrophobic regions

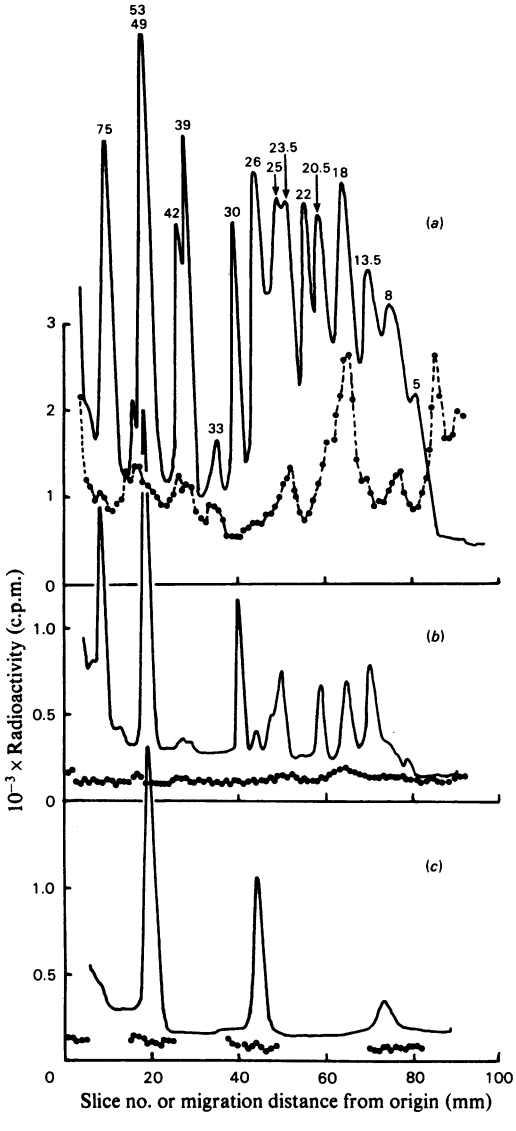

Fig. 4. *Chaotropic resolution of photolabelled Complex I*

Complex I (20mg of protein in 2ml of 0.67M-sucrose/50mM-Tris/HCl buffer, pH8.0) was illuminated for 80s in the presence of 5-[^{125}I]-iodonaphth-1-yl azide (1.27×10^8c.p.m.) as otherwise described in the Materials and methods section. A portion (100μl) was removed for analysis, and to the rest 4M-$NaClO_4$ was added to give a final concentration of 0.5M. Resolution was carried out at 35°C for 10min as described by Smith & Ragan (1980). The iron–protein and flavoprotein fragments were purified from the supernatant obtained by centrifugation of the resolved Complex I as described by Hatefi & Stempel (1969). Samples were analysed by polyacrylamide-gel electrophoresis with the Weber & Osborn (1969) buffer system. (*a*) Complex I (100μg of protein); (*b*) iron–protein fragment (derived from 100μg of Complex-I protein); (*c*) flavoprotein fragment (derived from 200μg of Complex-I protein). ——, Densitometer scans of the stained gels; ●--●, radioactivity. The radioactivity scale has been increased for the fragments. The polypeptides are indicated by their molecular weights (in thousands).

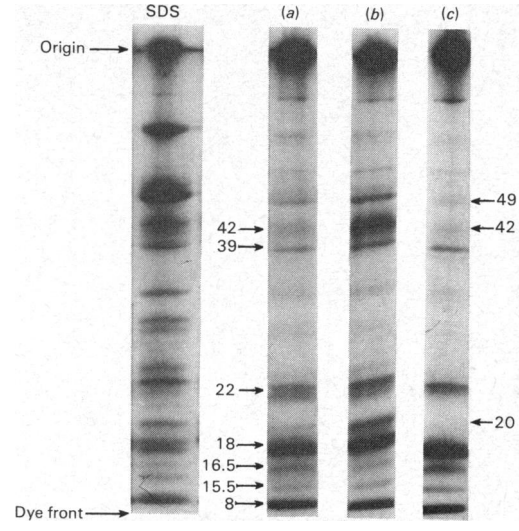

Fig. 5. *Effects of lipid/protein ratio on the pattern of labelling*

Complex I was depleted of lipids or supplemented with lipid as described in the Materials and methods section. All samples were dialysed before photolabelling with 5-[^{125}I]iodonaphth-1-yl azide (2.5×10^7c.p.m./mg of protein). Labelled protein was analysed by discontinuous polyacrylamide-gel electrophoresis and radioautography. The Figure shows radioautography of (*a*) Complex I, (*b*) lipid-depleted Complex I and (*c*) lipid-supplemented Complex I. A sample of Complex I that had been treated with sodium dodecyl sulphate before the labelling is included as a marker (SDS). The major labelled polypeptides in native Complex I are indicated by their molecular weights (in thousands). Those polypeptides whose labelling was increased in lipid-depleted Complex I are indicated on the right of track (*c*). Molecular weights were taken from Crowder & Ragan (1977).

of the enzyme are rotenone (Lindahl & Öberg, 1961) and diphenyleneiodonium (Ragan & Bloxham, 1977). The distribution of radioactivity after the photolabelling was unaffected by the presence of either of these compounds at concentrations of 1nmol/mg of protein and 100nmol/mg of protein respectively (results not shown).

Effects of varying the lipid/protein ratio on the distribution of label

Complex I contains phosphatidylcholine, phosphatidylethanolamine and cardiolipin. The first

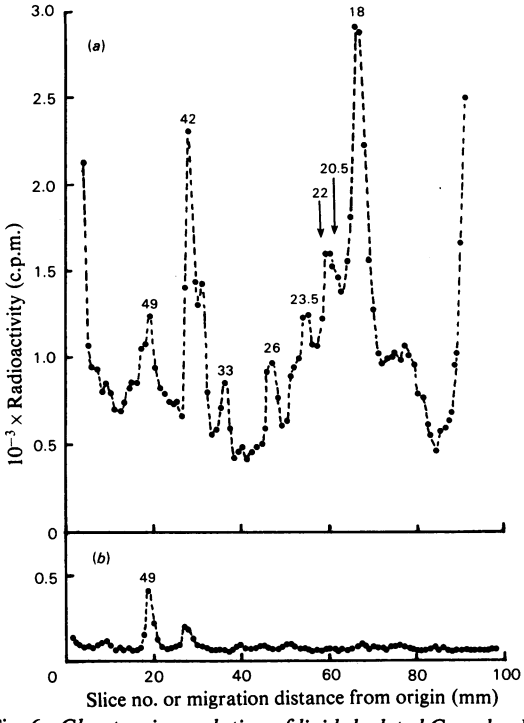

Fig. 6. *Chaotropic resolution of lipid-depleted Complex I*
Dialysed lipid-depleted Complex I (5.5 mg of protein in 0.55 ml of 0.67 M-sucrose/50 mM-Tris/HCl buffer, pH 8.0) was photolabelled with 5-[^{125}I]iodonaphth-1-yl azide (1.27×10^8 c.p.m.). The enzyme was resolved with 0.5 M-$NaClO_4$ and centrifuged to separate the soluble and insoluble fractions. (*a*) Insoluble fraction (derived from 100 μg of Complex-I protein); (*b*) soluble fraction (derived from 100 μg of Complex-I protein). Analysis was by polyacrylamide-gel elctrophoresis with the Weber & Osborn (1969) system. The positions of the polypeptides are indicated by the molecular weights (in thousands).

two phospholipids can be almost completely removed by $(NH_4)_2SO_4$ precipitation of the enzyme in the presence of high concentrations of cholate (Heron *et al.*, 1977). The cardiolipin content (approx. 20% of the total phospholipid P) is unaffected.

When lipid-depleted Complex I was photolabelled, several differences in the labelling pattern were apparent (Fig. 5). Most polypeptides were labelled to exactly the same extent as they were in undepleted Complex I (Fig. 5*a*). However, three subunits of mol.wts. 49000, 42000 and 20000 were labelled to a much greater extent in lipid-depleted Complex I (Fig. 5*b*). The converse experiment, in which the lipid content of Complex I was increased, revealed no changes in the labelling pattern (Fig. 5*c*).

The increased labelling of the 49000-mol.wt. polypeptide prompted us to repeat the chaotropic resolution experiment. As shown in Fig. 6, some of the radioactive label in this position is solubilized by $NaClO_4$, and therefore is associated with the 49000-mol.wt. polypeptide of the iron–protein fraction. The remaining label in this position, which is not solubilized, is probably associated with one of the high-molecular-weight subunits of ubiquinol–cytochrome *c* oxidoreductase (Complex III; Hatefi *et al.*, 1962*b*). This enzyme is always present in small amounts in Complex-I preparations, and its largest subunit, of mol.wt. 50000 (Gellefors & Nelson, 1975), is a major identifiable contaminent in Complex I (Heron *et al.*, 1979*b*).

The effects on the labelling pattern of lipid-depletion are rather hard to interpret, since it is not certain how 5-iodonaphth-1-yl azide acts. The observation that the degree of labelling of many of the subunits is unaltered could be explained if the reagent binds directly to hydrophobic regions of the protein whether they are in contact with the lipid bilayer or not. Bercovici & Gitler (1978) showed that bovine serum albumin can be photolabelled in this way. However, they regard this as an unlikely mechanism for labelling of membrane proteins, because the rapid and near complete partition of the compound into the lipid phase would leave very little over to bind from the aqueous phase to exposed hydrophobic regions of the protein. We could not test this directly by removing all phospholipids from Complex I, because this cannot be done in a non-destructive manner. Assuming that labelling does occur exclusively from the lipid phase, then decreasing the lipid/protein ratio would merely serve to increase the concentration of 5-iodonaphth-1-yl azide in the lipid that remained. If this lipid were randomly distributed over the hydrophobic surface of the enzyme, no change in the labelling pattern would be expected. The increased labelling of certain polypeptides in the depleted enzyme can be explained in two ways. Firstly, conformational changes in the protein could bring these polypeptides into closer or more extensive contact with the residual lipid. Alternatively, the distribution of the remaining cardiolipin might not be completely random. Those proteins associated with this lipid would therefore become more heavily labelled because of the higher concentration of 5-iodonaphth-1-yl azide in the vicinity. At present we cannot distinguish between these possibilities.

An attempt was made to provide evidence that 5-iodonaphth-1-yl azide does label proteins from the lipid phase by using Complex I whose endogenous phosphatidylcholine and phosphatidylethanolamine had been replaced with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (Heron *et al.*, 1979*a*). This lipid in the pure state undergoes a phase transition between the liquid-crystalline and gel states at 24°C (Hinz & Sturtevant, 1972). It was

hoped that at low temperatures lipid-replaced Complex I might show decreased photolabelling, because partition into the lipid phase would be slower and less complete. In addition, if protein labelling occurred after migration of the nitrene to the lipid/water interface (Bayley & Knowles, 1978), this might be prevented in gel-phase lipid. Unfortunately, we were not able to show any temperature-dependent effects of this type. The pattern of labelling of lipid-replaced Complex I (under any conditions) was very similar to that of native Complex I, except that there was increased labelling of certain high-molecular-weight polypeptides, reminiscent of the results obtained with lipid-depleted Complex I. Since lipid-replacement always results in some denaturation of the enzyme, this finding supports the possibility discussed above that the increased labelling of certain polypeptides in lipid-depleted Complex I is a consequence of conformational changes in the protein.

Discussion

The inability of 5-iodonaphth-1-yl azide to label the subunits of the iron–protein and flavoprotein fragments of Complex I is consistent with the idea that these components are mostly buried within the enzyme. The two largest iron–protein subunits, of mol.wts. 75000 and 49000, are exposed to the aqueous phase on either side of the mitochondrial inner membrane (Smith & Ragan, 1980). Their inaccessibility to photolabelling by 5-iodonaphth-1-yl azide suggests that they do not come into contact with the lipid bilayer, but are surrounded by other polypeptides except where they make contact with the aqueous phases. The labelling of these two polypeptides clearly distinguishes the mode of action of 5-iodonaphth-1-yl azide from that of hydrophilic probes such as diazobenzenesulphonate.

The polypeptides that are most heavily labelled in Complex I are those of mol.wts. 42000, 39000, 22000 (more than one of very similar mol.wts.), 18000, 16500, 15500 and 8000. Some of these, and possibly all, are subunits that are inaccessible to hydrophilic labels such as diazobenzene[^{35}S]-sulphonate when Complex I is in the membrane, but accessible when the enzyme is in isolation (Smith & Ragan, 1980). Exact identification is impossible in some instances because of the multiplicity of subunits of similar molecular weights. The results in the present paper therefore support the conclusion that these subunits are in contact with the hydrophobic region of the bilayer and surround the more hydrophilic flavoprotein and iron–protein fragments.

Since NADH dehydrogenase contains far more polypeptides than redox centres, most of the subunits must be catalytically inactive. Of the six redox centres in the enzyme (Ohnishi, 1979), three are probably located in the flavoprotein fragment (Galante & Hatefi, 1979), and at least one more in the iron–protein fragment. Thus most of the catalytic machinery is associated with the fragments that are solubilized by chaotropic agents and very little with the large number of more hydrophobic polypeptides that are not solubilized by chaotropic agents. The flavoprotein fragment retains the ability to oxidize NADH in the presence of artificial electron acceptors (Hatefi & Stempel, 1969). Kinetic analysis of this process both in the flavoprotein fragment and in Complex I showed that the interaction of the protein with NADH was very similar in both preparations (Dooijewaard & Slater, 1976*a*,*b*). Any similarity is remarkable, since the flavoprotein fragment is a truly water-soluble protein, whereas the native enzyme is an insoluble membrane-associated protein. The likely explanation is that the environment of the flavoprotein fragment is very similar in both the soluble and bound states, except that the electron acceptor in the latter condition is a protein-bound redox centre. This explanation now provides a role for the catalytically inactive subunits that surround the flavoprotein fragment in NADH dehydrogenase. Their function is to provide a hydrophilic environment for the flavoprotein fragment within the membrane. The large number of such subunits can be predicted from simple geometrical considerations. The flavoprotein fragment has a mol.wt. of 70000 (Galante & Hatefi, 1979). To cover a globular protein of this size completely with globular proteins of mol.wt. 25000 requires about 20 such proteins. There are in NADH dehydrogenase at least 23 subunits other than those of the flavoprotein fragment, and these have an average mol.wt. of 25000. The expected number of subunits would be increased if the iron–protein fragment is included with the flavoprotein fragment

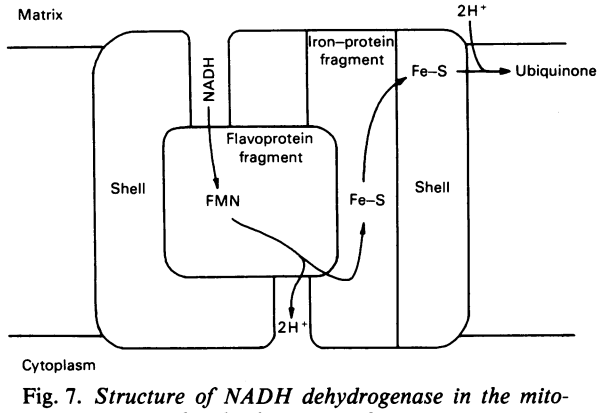

Fig. 7. *Structure of NADH dehydrogenase in the mitochondrial inner membrane*

in the central core of the enzyme and decreased if those subunits of these fragments that are exposed to the aqueous phases (e.g. the two largest subunits of the iron-protein fragment) are excluded.

Fig. 7 summarizes our conclusions and also shows how NADH dehydrogenase might act as a proton-translocator. The flavoprotein fragment is shown completely buried within a shell of other proteins except that access for NADH has to be provided by a channel in the shell. This channel does not allow access for hydrophilic or hydrophobic probes (Smith & Ragan, 1980, and the present paper). Electron transfer to the transmembranous iron–protein fragment is accompanied by loss of two protons per pair of electrons to the cytoplasmic phase. The iron–protein fragment is also excluded from direct interaction with the lipid phase of the membrane by the shell, but is exposed to the aqueous phases on both sides of the membrane (Smith & Ragan, 1980). This fragment catalyses transfer of electrons across the membrane and eventually to a further iron–sulphur centre in the shell. Reduction of ubiquinone in the membrane then takes place with the uptake of protons from the matrix. Smith & Ragan (1980) showed that there were several polypeptides other than the iron–protein subunits that were exposed on the matrix side of the membrane and could fulfil this last step.

The ordering of the electron-transfer sequence as a 'loop' (Mitchell, 1968) is essentially the same as that proposed by Lawford & Garland (1972) for NADH dehydrogenase. Although this mechanism is adequate to explain a proton/electron stoicheiometry of 1:1, it would require revision such as the addition of a separate proton pump to account for other proposed stoicheiometries such as 1.5 or 2 protons per electron.

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