

# Structural studies on mitochondrial NADH dehydrogenase using chemical cross-linking

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The structure of bovine heart mitochondrial NADH dehydrogenase was investigated by cross-linking constituent subunits with disuccinimidyl tartrate, (ethylene glycol)yl bis(succinimidyl succinate) and dimethyl suberimidate. Cross-linked products were identified by Western blotting with monospecific antisera to nine subunits of the enzyme. Cross-links between subunits within the flavoprotein, iron-protein and hydrophobic domains of the enzyme were identified. Cross-linking between the 75 kDa iron-protein-domain subunit and the 51 kDa flavoprotein-domain subunit was modulated by the substrate NADH. Cross-linking of subunits of the iron-protein and flavoprotein domains to constituents of the hydrophobic domain was also found. This was further substantiated by photolabelling subunits of the latter region, which were in contact with the membrane lipid, with 3-(trifluoromethyl)-3-(*m*-[<sup>125</sup>I]iodophenyl)diazirine. One such subunit of  $M_r$  19000 could be cross-linked to components of the iron-protein domain.

## INTRODUCTION

The mitochondrial NADH dehydrogenase complex (Complex I) consists of more than 20 different polypeptide subunits [1] and contains FMN and several iron-sulphur clusters [1–4]. Fragmentation of the enzyme with chaotropic agents [5] and detergents has led to the identification of several of the subunits which carry redox groups [6,7] and photoaffinity labelling has revealed the polypeptides responsible for binding NADH [8] and the inhibitor of ubiquinone reduction, rotenone [9,10]. Analysis of the structure of such a complicated enzyme is necessarily at a primitive stage. The overall shape and dimensions of the *Neurospora crassa* enzyme are known from analysis of two-dimensional crystals [11], and a fragment of the bovine heart enzyme has been studied in the same way [12,13]. At the present level of resolution, individual subunits cannot be distinguished, and structure prediction based on primary sequence data, even where available [1], is of limited value in an oligomeric enzyme of such complexity.

Traditional techniques of chemical modification are therefore still of value for probing enzymes such as Complex I, and a variety of hydrophobic and hydrophilic reagents, including cross-linkers, have already been used [1,14–17]. In the latter study, cross-linked dimers were analysed by Western blotting using monospecific antisera to four subunits of the enzyme (75, 49, 30 and 13 kDa) [17]. In the present paper we describe the preparation of antisera to several other subunits and their use in analysing the products of cross-linking. In the following paper [18], we describe the use of impermeable cross-linking agents to study the topology of Complex I in the mitochondrial membrane.

## MATERIALS AND METHODS

### Preparations and chemicals

Complex I (EC 1.6.99.3) was isolated by the method of Hatefi & Rieske [19]. Chaotropic resolution using NaClO<sub>4</sub> and the isolation of the 'flavoprotein', 'iron-protein' and the 'hydrophobic protein' fragments was as described by Ragan *et al.* [6,7]. The cross-linkers, disuccinimidyl tartrate (DST), (ethylene glycol)yl bis(succinimidyl succinate) (EGS) and dimethyl suberimidate (DMS), were obtained from Pierce Chemicals, Chester, Cheshire, U.K.

### Cross-linking

Complex I was dialysed overnight at 4 °C against 200 vol. of 0.25 M-sucrose/50 mM-triethanolamine/HCl, pH 8.0, and stored at –70 °C. The enzyme (4 mg of protein/ml of the same buffer) was incubated for 1 h at 20 °C with the concentrations of cross-linkers indicated in the Figure legends. These were added as freshly prepared solutions in either dimethyl sulphoxide (DST and EGS) or sucrose/triethanolamine buffer (DMS). Reactions were quenched by addition of 50 mM-ammonium acetate (DMS and DST) or 5 mM-glycine (EGS).

### SDS/polyacrylamide-gel electrophoresis

Cross-linked samples were treated with 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol at room temperature and electrophoresed immediately. Separation was achieved on gels (13 cm long × 1.5 mm thick) containing 10% (w/v) acrylamide or a 12–16% (w/v) acrylamide gradient, using the Laemmli [20] buffer system. Gels were stained with Brilliant Blue R (Sigma Chemical Co.,

Abbreviations used: DST, disuccinimidyl tartrate; EGS, (ethylene glycol)yl bis(succinimidyl succinate); DMS, dimethyl suberimidate; DTT, dithiothreitol; TID, 3-(trifluoromethyl)-3-(*m*-[<sup>125</sup>I]iodophenyl) diazirine.

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Poole, Dorset, U.K.). Molecular masses were determined from SDS/polyacrylamide-gel electrophoretograms by comparison with standards obtained from Sigma.

#### Preparation of subunit-specific antisera

The 75, 49, 30 and 13 kDa subunits of the iron-protein fragment of Complex I were purified by the method of Cleeter *et al.* [17]. The same procedure was used to isolate the 51 and 24 kDa subunits of the flavoprotein fragment and the 42 and 39 kDa subunits of the hydrophobic protein fragment. The 33 kDa subunit of Complex I was isolated as follows: the 'hydrophobic protein' fragment (20 mg of protein) was diluted to 6.4 ml with 50 mM-glycine/2 mM-DTT, pH 10.0. Triton X-100 was added to 2% (w/v), and the suspension was adjusted to pH 12.0 with NaOH. After 15 min at 0 °C, the solution was centrifuged at 20000 g for 20 min. The supernatant was discarded, and the small pale-coloured pellet (consisting mainly of the 33 kDa protein) was rinsed with 1% (w/v) Triton X-100 in glycine/DTT buffer and finally dissolved in 1.85 ml of 50 mM-Tris/HCl/2 mM-DTT, pH 7.8, and 0.375 ml of 10% (w/v) SDS by sonication. The 33 kDa protein was then isolated by preparative electrophoresis [17].

Antibodies were raised in rabbits by immunization with purified subunits as described by Cleeter *et al.* [17]. In several attempts, the 33 and 42 kDa proteins failed to elicit any response. However, the former subunit is the product of the mitochondrial ND-1 gene [10,21], and an antiserum directed against a synthetic C-terminal peptide was kindly provided by Professor G. Attardi, California Institute of Technology, Pasadena, CA, U.S.A. An antibody against the 42 kDa protein was obtained by the method of Bisson [22] from an antiserum raised to whole Complex I which had previously been shown to contain antibodies directed against the 42 kDa protein (see Fig. 3 of Cleeter & Ragan [23]). Complex I (2 mg of protein) was dissociated in SDS and electrophoresed on a 10% (w/v) acrylamide gel (13 cm long × 20 cm wide × 1.5 mm thick) by using the Laemmli [20] buffer system. The proteins were electrophoretically transferred on to a nitrocellulose sheet (see below) and stained with 0.1% Amido Black in 45% (v/v) methanol/10% (v/v) acetic acid for 2 min. After destaining, a strip containing the 42 kDa protein was cut out and washed in distilled water. The strip was incubated overnight with phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin and then with the anti-(Complex I) serum (0.1 ml in 2 ml of phosphate-buffered saline) for 1 h. The strip was washed in phosphate-buffered saline/bovine serum albumin and the bound antibody eluted by incubating in 1 ml of 0.2 M-glycine/HCl, pH 2.2, containing 3% (w/v) bovine serum albumin, at 4 °C for 4 min. The strip was discarded and the solution neutralized to pH 7.5 with 1 M-Tris.

#### Western blotting

Western blotting on to nitrocellulose was carried out as described by Erickson *et al.* [24]. When anti-(ND product) antisera were used, 0.5% (w/v) gelatin replaced bovine serum albumin for saturating non-specific sites on the nitrocellulose. Antigen-antibody complexes were revealed with the streptavidin-biotin system (Amersham International, Amersham, Bucks., U.K.) and peroxidase substrate from Bio-Rad Laboratories, Watford, Herts., U.K. [25].

#### Labelling with 3-(trifluoromethyl)-3-(m-[<sup>125</sup>I]iodophenyl)-diazirine (TID)

Complex I or cross-linked Complex I (4 mg/ml of sucrose/triethylamine buffer) was deoxygenated under a stream of N<sub>2</sub> for 30 min. TID (Amersham International) was added to a final concentration of 2 μM and equilibrated at 4 °C for 5 min. Samples (100 μl) were transferred to LP2 glass tubes (Luckhams, Burgess Hill, Sussex, U.K.) and photolysed with a 200 W medium-pressure Hg lamp for four periods of 30 s with intermediate cooling on ice. Labelled proteins were precipitated with 9 vol. of ice-cold ethanol and collected by centrifugation. The pellets were dissolved in 20 μl of 0.5 M-Tris/HCl, pH 6.8, 8 μl of 10% (w/v) SDS, 8 μl of 10% (v/v) 2-mercaptoethanol and water to a final volume of 80 μl before electrophoresis. Autoradiography of TID-labelled proteins, after separation and transfer to nitrocellulose, was carried out at -70 °C with preflashed Kodak X-Omat AR film (Kodak, Hemel Hempstead, Herts., U.K.) and Cronex Lighting Plus intensifying screens (Du Pont U.K., Stevenage, Herts., U.K.)

#### Protein assay

Protein was measured by the method of Lowry *et al.* [26], using bovine serum albumin (fraction V from Sigma) as a standard.

## RESULTS

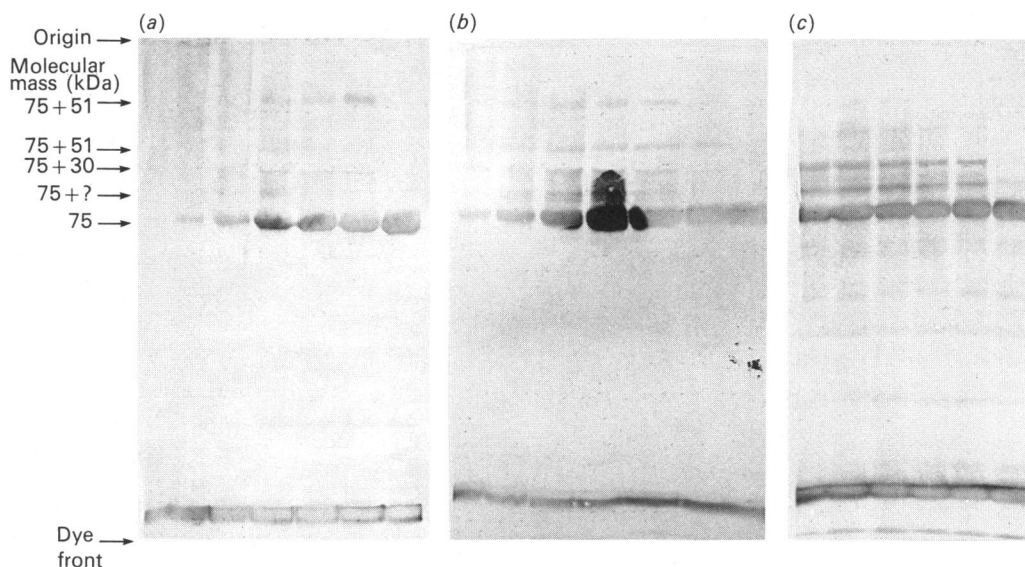
To provide as complete a picture as possible, each cross-linker was tested at six concentrations, typically, and blots were probed with each of the monospecific antisera. Only examples of the results are therefore presented in the present paper. The criteria for identification of the two polypeptides making up a cross-linked dimer were: (a) that the dimer should react with two monospecific antisera on blots; (b) that the dependence of product formation on the nature and concentration of cross-linker should be the same whichever of the two antisera were used for detection, and (c) that the apparent  $M_r$  of the dimer should approximate to the sum of the  $M_r$  values of the two constituents.

Table 1 lists the subunits of Complex I which were examined in the present study, their localization in the

**Table 1. Properties of Complex I subunits to which antisera were raised**

Properties of Complex I subunits are described in [1] and references therein. One Fe-S centre is associated with either, or both, of the 30 kDa and 13 kDa subunits.

$10^{-3} \times$ Subunit $M_r$	Enzyme domain	Prosthetic group or property
51	Flavoprotein	FMN, Fe-S, NADH-binding site
24	Flavoprotein	Fe-S
75	Iron-protein	Fe-S
49	Iron-protein	Fe-S
30	Iron-protein	Fe-S
13	Iron-protein	Fe-S
42	Hydrophobic protein	-
39	Hydrophobic protein	-
33	Hydrophobic protein	ND-1 gene product, rotenone binding site.



**Fig. 1. Cross-linked products containing the 75 kDa subunit**

Complex I was cross-linked with (a) EGS, (b) DST and (c) DMS. Concentrations of cross-linkers were, from left to right: EGS, 1.1, 0.86, 0.49, 0.37, 0.25, 0.12 and 0 mM; DST, 5.0, 3.2, 2.9, 2.1, 1.4, 0.7 and 0 mM; DMS, 15, 12, 8, 4.9, 1.0 and 0 mM. Cross-linked samples were analysed by gel electrophoresis on 10% (w/v) acrylamide gels and Western blotting with antiserum to the 75 kDa subunit. Immunoreactive material of  $M_r$  lower than 75000 is due to proteolytic degradation of the 75 kDa subunit [23].

three domains of the enzyme and their function or prosthetic-group content where known.

#### Cross-linking of 'iron-protein' fragment subunits

In our previous work, detection of cross-linked products was by transfer to diazobenzoyloxymethyl-paper and probing with antiserum and  $^{125}\text{I}$ -labelled protein A [17]. The method used in the present paper afforded much greater resolution and sensitivity, resulting in the detection of a larger number of cross-linked products.

Cross-linking with EGS and DST produced the same pattern of 75 kDa products as reported previously, where two distinct bands were attributed to (75+51) kDa dimers of different degrees of cross-linking [17]. A third band was identified as a (75+30) kDa dimer (Fig. 1). In contrast with EGS and DST, DMS failed to produce either of the (75+51) kDa dimers in other than very small amounts (Fig. 1), a result which we had previously noted with the polar cross-linker di-isethionyl suberimidate [27]. The other subunit of the smallest dimer containing the 75 kDa subunit was not identified. Several dimers containing the 30 kDa subunit were found, particularly with DST [17]. DMS gave rise to the least number of dimers containing the 30 kDa subunit, but all three cross-linkers produced prominent (75+30) kDa and (49+30) kDa dimers [17].

Fig. 2(a) shows the pattern of 49 kDa products after cross-linking with DST. Essentially similar patterns were obtained with EGS and DMS. Two products were identified, the (49+30) kDa dimer and the (49+13) kDa dimer. As shown in Fig. 2(b), the latter is by far the major 13 kDa product. In the previous study [17], this dimer was not seen in cross-linked Complex I probed with antiserum to the 13 kDa subunit, probably because of poorer sensitivity, but was identified in the cross-linked 'iron-protein' fragment. The nature of the other dimers

containing the 30 and 49 kDa subunits is considered in the Discussion section.

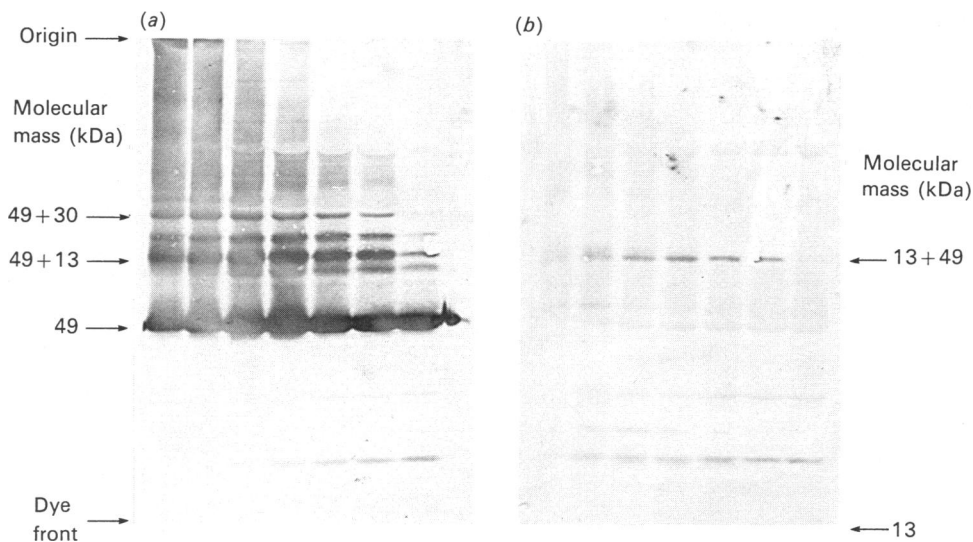
#### Cross-linking of 'flavoprotein' fragment subunits

As shown in Fig. 3(a), DST cross-linking gave rise to two high- $M_r$  dimers containing the 51 kDa subunit which co-migrated with the two large 75 kDa products, thus confirming that both of these are (75+51) kDa dimers [17]. Similar results were found with EGS and, at higher concentrations of either cross-linker, a (51+24) kDa dimer was produced in low concentration. This was the only product containing the 24 kDa subunit (Fig. 3b). With DMS as cross-linker, the (51+24) kDa dimer was also produced, but no (75+51) kDa dimers were detected (Fig. 4), in agreement with the result of Fig. 1. The other subunits in the two smallest dimers containing the 51 kDa subunit were not identified (see the Discussion section).

#### Cross-linking of 'hydrophobic protein' fragment subunits

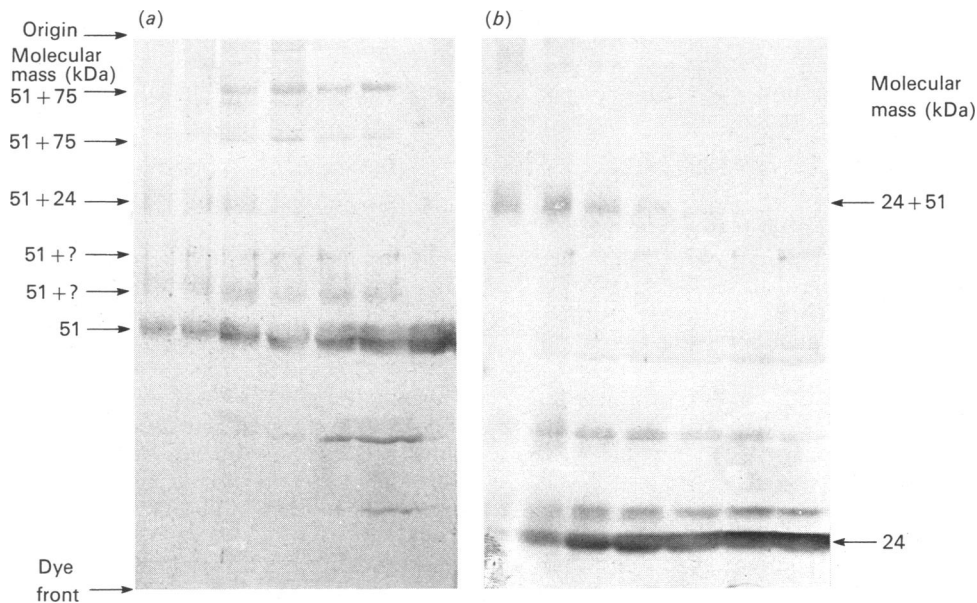
Under a variety of conditions of cross-linker concentration and pH, neither EGS, DST or DMS gave any detectable dimers containing the 39 or 33 kDa subunits. A number of products containing the 42 kDa subunit were detected in low concentration (Fig. 5), but no identification of the other subunits in the dimers could be made.

The failure to detect cross-links between 'hydrophobic protein' subunits and subunits of the other domains can be attributed not only to inaccessibility of some of these subunits (e.g. 39 and 33 kDa), but also to the fact that we had antisera to only three proteins of the many which are present in the 'hydrophobic protein' fragment. An alternative approach was therefore used in which subunits of the hydrophobic domain in contact with the lipid phase were identified by photolabelling with  $^{125}\text{I}$ -labelled TID [28]. As shown in Fig. 6, labelling of Complex I



**Fig. 2. Cross-linked products containing the 49 kDa and 13 kDa subunits**

Cross-linking with DST was performed exactly as in the legend to Fig. 1. Samples were analysed by Western blotting with (a) antiserum to the 49 kDa subunit or (b) antiserum to the 13 kDa subunit. On 10% (w/v) acrylamide gels the 13 kDa subunit migrates close to the dye front [17], which was just off the bottom of the nitrocellulose sheet in this experiment.



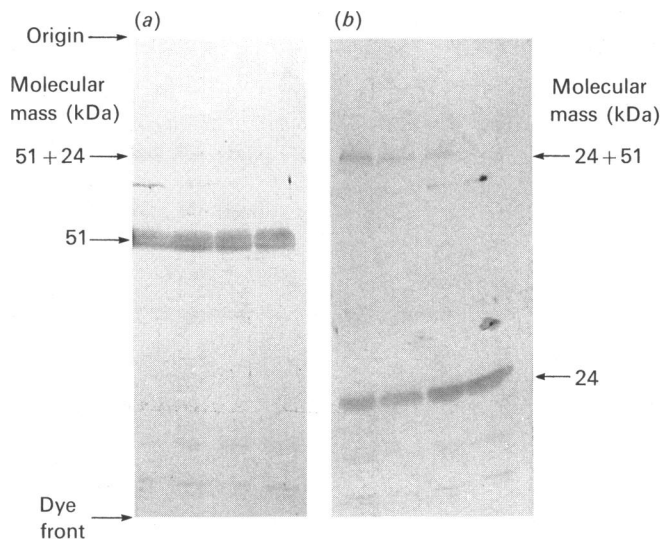
**Fig. 3. Cross-linked products containing the 51 and 24 kDa subunits**

Cross-linking with DST was performed as in the legend to Fig. 1. Samples were analysed by Western blotting with (a) antiserum to the 51 kDa subunit and (b) antiserum to the 24 kDa subunit.

subunits by TID was similar to that reported previously for iodonaphthylazide [15] or arylazido phosphatidylcholines [16]. The most clearly labelled subunits were of low  $M_r$  (less than 20000), with the exception of the 33 kDa subunit (ND-1 product). Chaotropic resolution of the labelled Complex I confirmed that labelling was exclusively confined to subunits of the 'hydrophobic' protein.

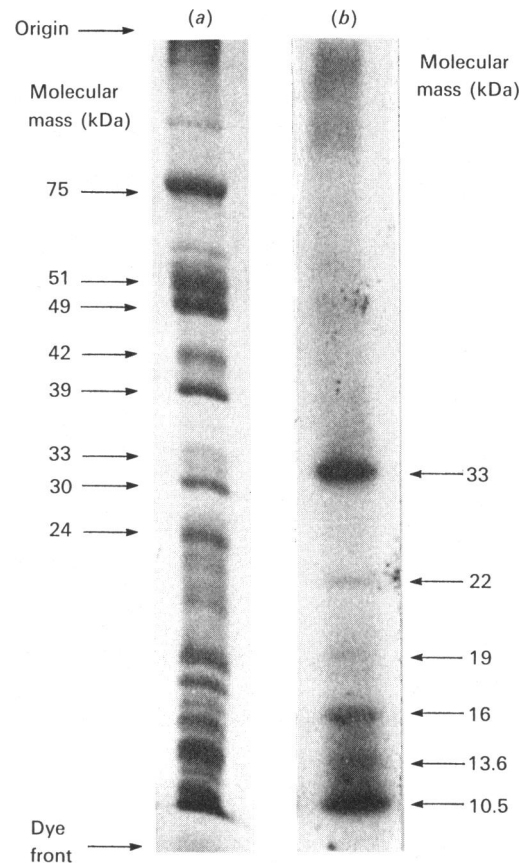
For cross-linking studies, Complex I could be cross-linked either before or after photolabelling with TID, and essentially the same results were obtained by either route. Fig. 7 shows the result of TID-labelling after

cross-linking with 3 mM-DST. Samples were run on a 10% (w/v) acrylamide gel, giving poor resolution of the low- $M_r$  TID-labelled subunits, but allowing better separation of any high- $M_r$  dimers. At higher concentrations of cross-linker only, two TID-labelled dimers were encountered (cf. Fig. 1) and their mobilities correlated with dimers containing the 49 and 30 kDa subunits on the corresponding Western blots (Fig. 7). The resolution and intensity was poor because of the high cross-linker concentration used. The apparent  $M_r$  values of the two products indicated cross-linking to the TID-labelled subunit of approx.  $M_r$  19000, but in view of



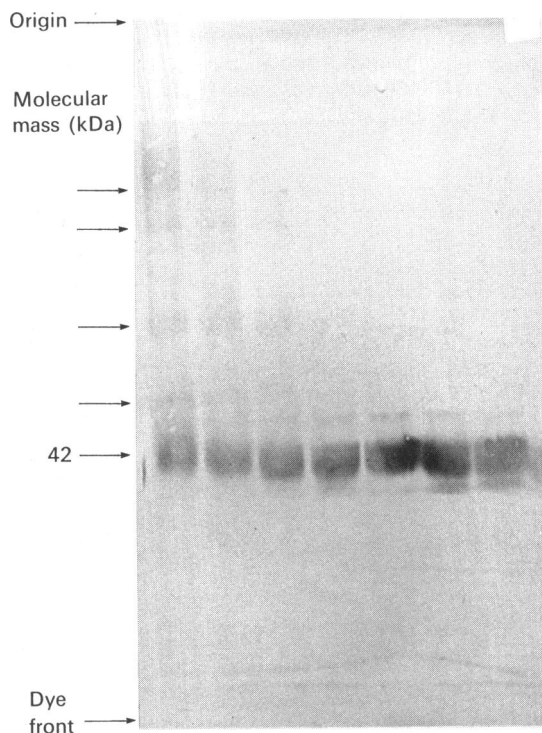
**Fig. 4. Inability of DMS to cross-link the 51 and 75 kDa subunits**

Cross-linking with DMS was performed as in the legend to Fig. 1. Samples were analysed by Western blotting with (a) antiserum to the 51 kDa subunit and (b) antiserum to the 24 kDa subunit. Cross-linker concentrations were, from left to right: 15, 12, 4.9 and 0 mM.



**Fig. 6. Labelling of Complex I subunits with TID**

Complex I was photolabelled with TID as described in the Materials and methods section. Protein samples were electrophoresed on 12–16%-(w/v)-acrylamide gels, which were stained for protein (a) and subjected to autoradiography (b). Molecular masses of some polypeptides are indicated.



**Fig. 5. Cross-linked products containing the 42 kDa subunit**

Cross-linking with EGS was performed as in the legend to Fig. 1 and analysed by using antibody to the 42 kDa subunit.

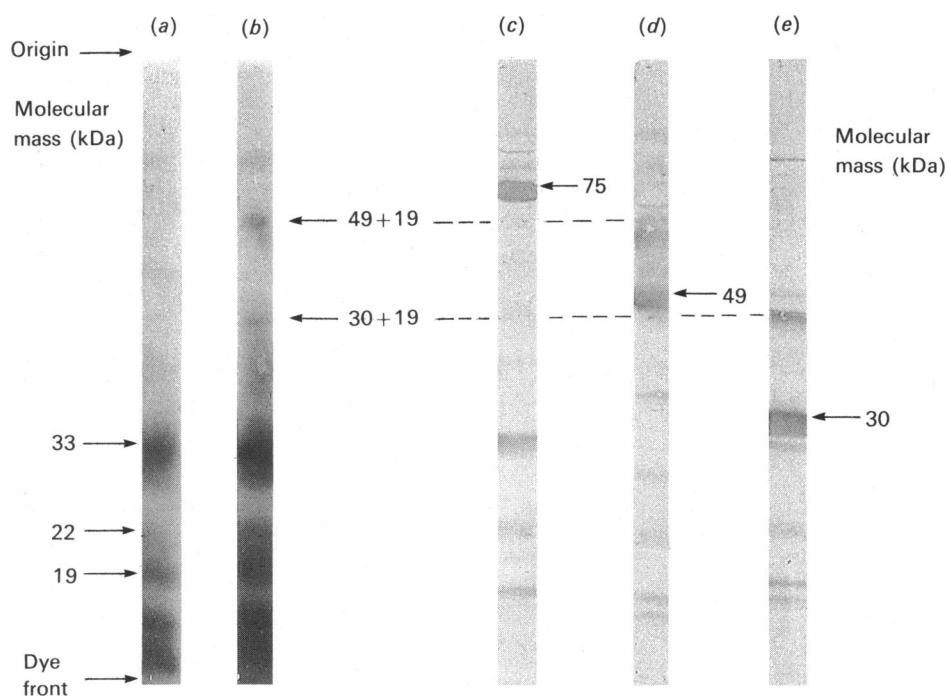
the multiplicity of subunits of similar  $M_r$  in Complex I, it is not possible to conclude definitely that it was the same subunit which was involved in each instance.

Despite the poor resolution of the experiment of Fig. 7, we can be reasonably certain that the larger TID-

labelled dimer contains the 49 kDa subunit. With a dimer  $M_r$  of 68 000, one subunit must have an  $M_r$  of at least 39 000 or thereabouts. Dimers containing the 51 kDa subunit have the 'wrong'  $M_r$ , and the 39 kDa and 33 kDa subunits do not cross-link. The 42 kDa subunit is a possibility, but it cross-links so poorly that it is unlikely to be responsible for the 68 kDa product. This leaves the 49 kDa subunit as the most likely partner, particularly as it forms a product of the correct apparent  $M_r$  and there is a TID-labelled subunit of 19 kDa. Similar arguments apply to the smaller TID-labelled dimer of apparent  $M_r$  50 000. Again, the only alternative to a 30 kDa constituent is the 42 kDa subunit which forms a 49 kDa cross-linked dimer (Fig. 5). However, the yield is very low and we do not know whether the other small subunit can be labelled with TID.

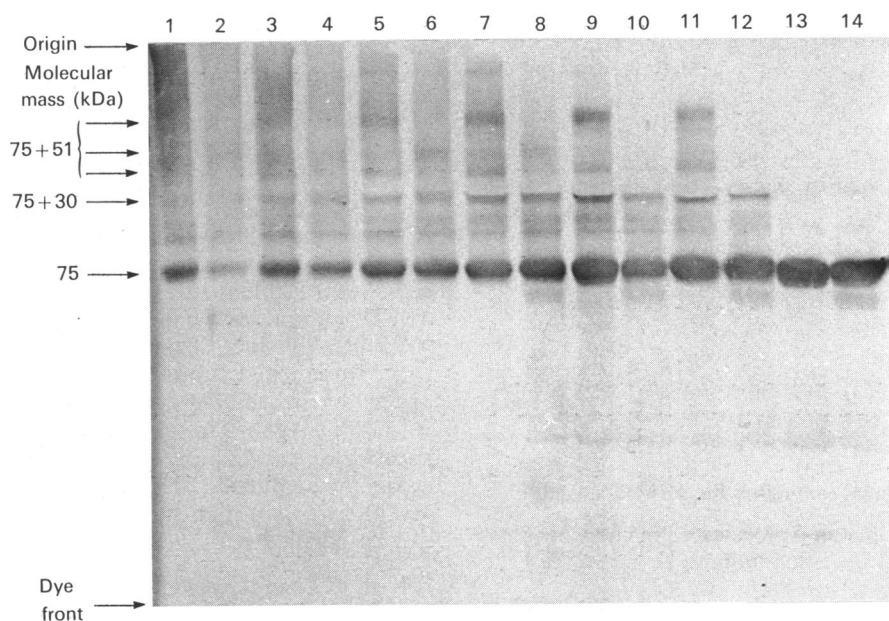
**Effects of Complex I ligands on cross-linking**

The effect of rotenone on the conformation of Complex I was examined by cross-linking the inhibited enzyme. Analysis of the products containing the 49 kDa and 30 kDa subunits revealed no effect of rotenone. On the other hand, there was a marked effect of the substrate, NADH. As shown in Fig. 8, the presence of NADH abolished the pair of (75 + 51) kDa dimers produced by



**Fig. 7. TID-labelling of cross-linked Complex I**

Complex I was cross-linked with 3 mM-DST and labelled with TID as described in the Materials and methods section. Replicate samples containing 140  $\mu$ g of protein were electrophoresed on a 10%-(w/v)-polyacrylamide gel and transferred to nitrocellulose. Samples which had not been cross-linked were treated in parallel. Strips of nitrocellulose were blotted with antisera to the 75, 49 and 30 kDa subunits or subjected to autoradiography. Track 1, autoradiograph of control sample; track 2, autoradiograph of cross-linked sample; track 3, blot of cross-linked sample with antiserum to the 75 kDa subunit; track 4, blot of cross-linked sample with antiserum to the 49 kDa subunit; track 5, blot of cross-linked sample with antiserum to the 30 kDa subunit.



**Fig. 8. Effect of NADH on cross-linking of Complex I**

Cross-linking with DST was performed as in the legend to Fig. 1 in the presence (even-numbered tracks) or absence (odd-numbered tracks) of 0.1 mM-NADH. Samples were analysed by Western blotting with antiserum to the 75 kDa subunit. Concentrations of cross-linker were: tracks 1 and 2, 5 mM; tracks 3 and 4, 3.2 mM; tracks 5 and 6, 2.9 mM; tracks 7 and 8, 2.1 mM; tracks 9 and 10, 1.4 mM; tracks 11 and 12, 0.7 mM, tracks 13 and 14, 0 mM.

**Table 2. Constituent polypeptides of cross-linked products**

Major products only are indicated. The first column shows the molecular mass of the antigen used to produce the antisera used for blotting, and the second column gives the  $M_r$  values of products reacting with the indicated antisera. The composition of the products is shown in the third column, as detailed in the text.

$10^{-3} \times M_r$ of antigen	$10^{-3} \times M_r$ of product	$10^{-3} \times M_r$ values of subunits present
75	165	75 + 51
	125	75 + 51
51	97	75 + small hydrophobic subunit
	165	51 + 75
	125	51 + 75
	74	51 + 24
49	63	51 + small hydrophobic subunit
	59	51 + small hydrophobic subunit
	96	Trimeric product?
	89	Trimeric product?
	84	49 + 30
	75	49 + 30
	68	49 + 19 (TID-labelled)
42	63	49 + 13
	55	49 + small hydrophobic subunit
	105	?
	80	?
	63	42 + small hydrophobic subunit
39	49	42 + small hydrophobic subunit
	None	
33	None	
30	97	30 + 75
	84	30 + 49
	75	30 + 49
	62	30 + ND-2 gene product?
	59	30 + ND-2 gene product?
	53	30 + small hydrophobic subunit
	50	30 + 19 (TID-labelled)
	46	30 + 13
24	74	24 + 51
	13	63

DST, but was without effect on other dimers containing the 75 kDa subunit. However, a product of apparent  $M_r$  intermediate between those two (75 + 51) kDa dimers was produced instead and this was also apparent in the absence of NADH but at higher DST concentration. These results were confirmed using antibody to the 51 kDa subunit. The (51 + 24) kDa dimer was unaltered by the presence of NADH, whereas the two (75 + 51) kDa dimers disappeared to be replaced by a product of intermediate  $M_r$ , thus confirming the latter to be yet another (75 + 51) kDa dimer.

The effect of NADH on cross-linking can be explained either by steric hindrance of the cross-linking site or sites by NADH or by a conformational change induced by substrate binding or reduction of the enzyme. The latter seems more likely, since the production of a third variant of the (75 + 51) kDa dimer is actually promoted by NADH. Thus the three types of dimer can be attributed either to the formation of different numbers of cross-links or, more likely, cross-linking of different pairs of residues. Which pairing is most likely would obviously be affected by conformational changes.

## DISCUSSION

The major cross-linked products identified in the present study are listed in Table 2, together with one or both of their constituent subunits. Since we have looked at cross-linking of all the eight largest subunits of the enzyme, we should be able to make some reasonable guesses about the unidentified constituents of some of these dimers. The multiplicity of subunits of 20 kDa or less in Complex I and the absence of many specific antisera makes it impossible to identify any constituents of small  $M_r$ , e.g. those present in the 63 and 59 kDa dimers containing the 51 kDa subunit. However, possibilities for larger dimers are much more restricted. There are, for example, three clearly visible high- $M_r$  products containing the 49 kDa subunit, whose other constituents have not been identified. As shown in Fig. 2, these appear in a region of the gel that contains many closely migrating minor products. The possibility is therefore that these represent either trimeric and oligomeric products or are dimers of higher degree of cross-linking. The 84 kDa product, for example, does indeed appear at higher concentration of EGS or DST (Fig. 2) and correlates with a band containing the 30 kDa subunit. This dimer is therefore most probably another variant of the (49 + 30) kDa dimer, similar to the situation which occurs with (75 + 51) kDa cross-linking. The 96 and 89 kDa products do not appear to be dimers, since there are no other subunits of appropriate  $M_r$  which form dimers of this mass. They are probably trimeric. The 30 kDa subunit forms two dimers of about 60 kDa, but the 33 kDa subunit is clearly not the other constituent. A possibility, though, is the ND-2 gene product, which has a very similar apparent  $M_r$  to that of the ND-1 product on this gel system, but has not been clearly identified by protein staining of complex I ([29]; G. Gibb & C. I. Ragan, unpublished work). The composition of the products containing the 42 kDa subunit is also not clear. The two largest products would be consistent with dimers of the 42 kDa subunit with 60 or 40 kDa subunits, but there are no likely candidates. These large products may therefore arise from aggregation or other artefacts occurring after SDS treatment.

The results of cross-linking of subunits of the hydrophobic domain were disappointing in that two of the three subunits examined failed to react and the third (42 kDa) produced uninterpretable results. The three hydrophobic subunits (42, 39 and 33 kDa) are all accessible to hydrophilic chemical probes, e.g. [1], and it is therefore unlikely that absence of cross-linking is attributable to their lack of exposure to the aqueous phase on either side of the membrane. Of course, the hydrophobic domain contains many subunits to which we do not have monospecific antisera, and some of these may well be accessible to cross-linkers. This is clearly indicated by the presence of several low- $M_r$  products containing 'flavoprotein' or 'iron-protein' subunits whose other constituent must be a subunit of the hydrophobic domain. Further evidence came from the TID experiments and are consistent with our view [1,18] that the larger 'flavoprotein' and 'iron-protein' subunits are partially surrounded by subunits of the hydrophobic domain.

A different approach to cross-linking of Complex I was taken by Gondal & Anderson [30,31]. In their initial study [30] they used a thiol-cleavable cross-linker and

analysed products by two-dimensional electrophoresis. Dimeric products were not observed, but material remaining at the origin in the first-dimension gel was found to contain seven Complex I subunits, including contributions from the 'iron-protein', 'flavoprotein' and 'hydrophobic' domains. Although the results were generally similar to our own, the problem with their method is that it was not proven that the material running at the origin was indeed cross-linked. For example, the 75 kDa subunit of Complex I is very prone to aggregation in the absence of thiols [17], and the 33 kDa subunit aggregates on heating [10]. Indeed, in their second paper [31], Gondal & Anderson claim that these subunits are disulphide-linked in Complex I on the basis of their lack of migration on gels run in the absence of thiols. However, the 75 kDa subunit migrates perfectly normally when *N*-ethylmaleimide is present instead of a thiol [17], showing that the result in the absence of thiol is due to non-specific oxidation or thiol disulphide exchange.

The claim by Gondal & Anderson [31] that rotenone induces a more open structure of the enzyme, thereby preventing cross-linking, is not supported by our own observations. Whether this is ascribable to methodological differences is unresolved.

In the following paper [18] we describe the use of cross-linking agents to study the sidedness of some of the Complex I subunits.

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