Chemical cross-linking of mitochondrial NADH dehydrogenase from bovine heart

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The structure of bovine heart mitochondrial NADH dehydrogenase was investigated by using two cleavable cross-linking agents, disuccinimidyl tartrate and (ethylene glycol)yl bis-(succinimidyl succinate). Cross-linking was analysed primarily by immunoblotting to detect products containing subunits of the iron-protein fraction from chaotropic resolution of the enzyme, namely those of 75, 49, 30 and 13 kDa. By using both the isolated iron-protein fraction and the intact dehydrogenase, cross-links were identified between these four subunits, from these subunits to the largest subunit of the flavoprotein fraction, which contains the active site for NADH, and from these subunits to polypeptides in the hydrophobic shell, which surrounds the hydrophilic iron-protein and flavoprotein fractions.

Mitochondrial NADH dehydrogenase may be isolated from bovine heart as a multisubunit lipoprotein complex (Complex I) that catalyses the reduction of ubiquinone by NADH (Hatefi *et al.*, 1962). It is by far the most complicated of the respiratory-chain enzymes, consisting of more than 20 different polypeptide subunits (Heron *et al.*, 1979) and containing, in addition to FMN, eight or nine distinct iron-sulphur centres (Ragan *et al.*, 1982a,b; Ohnishi *et al.*, 1985). Structural analysis has been greatly aided by fragmentation of the enzyme with chaotropic agents (Hatefi & Stempel, 1969), which leads to separation of the enzyme into three well-defined fractions. These are, firstly, the soluble flavoprotein fraction, which retains NADH dehydrogenase activity and contains the FMN and two iron-sulphur centres (Hatefi & Stempel, 1969; Galante & Hatefi, 1979; Ragan *et al.*, 1982a; Ohnishi *et al.*, 1985), secondly, the soluble iron-protein fraction, which lacks flavin but contains four iron-sulphur centres (Ragan *et al.*, 1982b; Ohnishi *et al.*, 1985), and thirdly, the insoluble residue, which contains a further two or three iron-sulphur centres (Ohnishi *et al.*, 1985). The polypeptide compositions of the first two fractions are relatively simple compared with Complex I, the flavoprotein fraction consist-

ing of three polypeptides (51, 24 and 9 kDa; Galante & Hatefi, 1979; Heron *et al.*, 1979), and the iron-protein fraction consisting of approximately six to eight polypeptides plus smaller amounts of others (Heron *et al.*, 1979; Earley & Ragan, 1981).

In the present paper we describe the results of chemical cross-linking of the isolated iron-protein fragment and of intact Complex I. To aid interpretation we have identified cross-linked products by their reaction with monospecific antibodies to four subunits of the iron-protein fraction by using the technique of immunoblotting. The subunits chosen (75, 49, 30 and 13 kDa) have been identified by fragmentation studies (Ragan *et al.*, 1982b) as iron-sulphur protein subunits. The 75 kDa subunit carries two binuclear centres, and the other three carry between them a binuclear and a tetranuclear centre, one on the 49 kDa subunit and the other on the 30 kDa or 13 kDa subunits. The results of the cross-linking experiments provide support for the model of Complex I structure proposed in previous publications (Earley & Ragan, 1980; Ragan, 1980, 1985; Ohnishi *et al.*, 1985).

Materials and methods

Preparations and chemicals

Complex I (EC 1.6.99.3) was isolated by the method of Hatefi & Rieske (1967), and the iron-protein fraction was prepared as described by Ragan *et al.* (1982b). For cross-linking, the pre-

Abbreviations used: DST, disuccinimidyl tartrate; EGS, (ethylene glycol)yl bis-(succinimidyl succinate); SDS, sodium dodecyl sulphate.

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parations were dialysed overnight at 4°C against 200 vol. of 0.25M-sucrose/50mM-triethanolamine/HCl buffer, pH8.0, and stored at -70°C. The cross-linkers, disuccinimidyl tartrate (DST) and (ethylene glycol)yl bis-(succinimidyl succinate) (EGS), were obtained from Pierce Chemicals, Chester, Cheshire, U.K.

Cross-linking

Complex I (4mg/ml of sucrose/triethanolamine buffer) and the iron-protein fraction (2mg/ml of buffer) were incubated for 1 h at 20°C with the cross-linkers. These were added as freshly prepared solutions in dimethyl sulphoxide such that the final solvent concentration was not more than 4% (v/v), a concentration that had no effect on the electrophoretic mobility of Complex I proteins. Quenching of the reaction was achieved with 50mM-ammonium acetate (DST) or 5mM-glycine (EGS). These general procedures were adapted from Smith *et al.* (1978) for DST and from Abdella *et al.* (1979) for 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 either electrophoresed immediately or stored at -20°C for no more than 1 week. Separation was achieved on gels (13cm long × 1.5mm thick) containing 12% (w/v), 10% (w/v) or 7% (w/v) acrylamide, with the Laemmli (1970) buffer system. Gels were stained with Brilliant Blue R (Sigma Chemical Co., Poole, Dorset, U.K.). For analysis of cross-linked products, bands were excised from parallel unstained tracks on the gel, and incubated for 90 min at 37°C with either 30mM-NaIO₄/0.1% SDS/2% (v/v) 2-mercaptoethanol/0.125M-Tris/HCl buffer, pH6.8 (for DST products; Smith *et al.*, 1978), or with 1M-NH₄OH/0.1% SDS/2% (v/v) 2-mercaptoethanol/50mM-Tris/HCl buffer, pH8.5 (for EGS products; Abdella *et al.*, 1979). The gel slices were then incubated for 30 min at 37°C with 0.125M-Tris/HCl buffer, pH6.8, containing 0.1% SDS, 2% (v/v) 2-mercaptoethanol and Bromophenol Blue tracking dye, and inserted into sample slots in a stacking gel (1cm long) on top of a 12-16% (w/v) acrylamide concave-gradient gel (11cm long × 1.5mm thick). After electrophoresis with the Laemmli (1970) buffer system, gels were stained with Brilliant Blue R and then silver-stained (Irie *et al.*, 1982). Molecular masses were determined from SDS/polyacrylamide-gel electrophoretograms, by comparison with standards obtained from Sigma Chemical Co.

Preparation of subunit-specific antisera

The iron-protein fraction (5mg of protein) was

dissociated in 25mM-Tris/HCl buffer, pH6.8, containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 5% (w/v) sucrose and 0.05% Bromophenol Blue (1ml final volume) at 100°C for 2 min. The sample was electrophoresed on a 15%-(w/v)-polyacrylamide gel (10cm long × 20cm wide × 0.3cm thick) overlaid with a 5%-(w/v)-polyacrylamide stacking gel (2cm long) with the Laemmli (1970) buffer system. The gel was stained for 1 h with aq. 1% Brilliant Blue R, and destained for 1 h in water. Strips of gel containing the 75kDa, 49kDa, 30kDa and 13kDa subunits were excised, and the protein was electroeluted as described by Walker *et al.* (1982). Despite the presence of several proteins running in the 13kDa region (Earley & Ragan, 1981), the subunit that is associated with the 30kDa protein in fragmentation studies runs at the dye front on 15% preparative gels as a very tight band and is well separated from others of similar apparent molecular mass.

The yield of purified subunits was approx. 75%. If necessary, the preparative gel electrophoresis was repeated, to ensure homogeneity of the subunits.

The subunits (200-500µg of protein in 0.5ml) were mixed with 0.5ml of Freund's complete adjuvant. New Zealand White rabbits were immunized by multiple subcutaneous injections into the back. A booster, with the same amount of protein in Freund's incomplete adjuvant, was given 4 weeks later intramuscularly into the flank. Rabbits were bled from the marginal ear vein after a further 2 weeks. Blood was left at room temperature for 4h, and the serum collected by centrifugation and stored at -20°C.

Immunoblotting

Aminobenzyloxymethyl-paper was made from Whatman 540 filter paper (Whatman, Maidstone, Kent, U.K.) and diazotized by the procedure of Christophe *et al.* (1982). Proteins were electrophoretically transferred from gels on to diazotized paper, and treated with antiserum and ¹²⁵I-labelled protein A much as described by Symington *et al.* (1981), except that radioiodination of protein A was achieved with Bolton-Hunter reagent (Langone, 1980). Autoradiography used pre-flashed Kodak XOMAT-S film (Kodak, Hemel Hempstead, Herts., U.K.) and a Cronex Lightning Plus intensifying screen (Dupont U.K., Stevenage, Herts., U.K.) for 48 h at -70°C.

Immunoblots were erased by treatment for 1 h at room temperature with 0.2M-glycine/HCl buffer, pH2.8, followed by 30 min at 100°C with 0.2M-2-mercaptoethanol/2% (w/v) SDS. The procedure of Symington *et al.* (1981) was found to cause insufficiently complete erasure.

Protein assay

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V from Sigma Chemical Co.) as a standard.

Results

Cross-linking of the iron-protein fraction

Fig. 1 shows that both DST and EGS cause cross-linking of the subunits of the iron-protein fraction in a concentration-dependent manner, leading eventually to high-molecular-mass aggregates largely excluded from the gel. The two cross-linkers used produce qualitatively similar effects, despite the greatly differing lengths of the molecules [0.6 nm for DST (Smith *et al.*, 1978); 1.8 nm for EGS].

The results of immunoblotting experiments are shown in Fig. 2. Polypeptides were transferred to diazotized paper to immobilize them covalently and then successively probed with antisera to the 75 kDa, 49 kDa, 30 kDa and 13 kDa subunits, with erasure between each probing. In the absence of cross-linker, each antiserum identified only a

single polypeptide of the appropriate molecular mass, with the exception of the anti-(75 kDa subunit) serum. The smaller polypeptides showing antigenicity are proteolytic breakdown products of the 75 kDa protein, produced on storage of the iron-protein fraction. The sensitivity of this protein to proteolysis is much less marked in intact Complex I (see Fig. 4), and immunoblots of mitochondria identify only the undegraded protein.

In the cross-linked samples, all antisera identify immunoreactive proteins of higher molecular mass than the original protein. No clear-cut products containing the 75 kDa subunit could be identified because of smearing and poor resolution in the upper part of the gel. The cross-linked products containing the other three subunits are listed in Table 1, with their apparent molecular masses. Qualitatively similar results were obtained with either DST or EGS. Complete identification of cross-linked products was possible in some instances where a band on the gel reacted with two different antisera and was of the appropriate apparent molecular mass. Thus products of 75 kDa, 62 kDa and 45 kDa were identified as cross-links between the 49 kDa and 30 kDa sub-

Fig. 1. Cross-linking of the iron-protein fraction

The iron-protein fraction was cross-linked with EGS or DST and electrophoresed on a 12%-(w/v)-acrylamide gel as described in the Materials and methods section. Each track contained 40 µg of protein. Track a, no cross-linker; track b, 0.04 mM-EGS; track c, 0.2 mM-EGS; track d, 0.4 mM-EGS; track e, 1 mM-DST; track f, 2 mM-DST; track g, 4 mM-DST. Molecular masses of major subunits are shown to the left of track a.

Fig. 2. Immunoblotting of cross-linked iron-protein fraction

The iron-protein fraction was cross-linked with a range of either EGS or DST concentrations and analysed by gel electrophoresis on a 10%-(w/v)-acrylamide gel and immunoblotting as described in the Materials and methods section. The results obtained with no cross-linker (tracks a, b, c, and d), 0.08 mM-EGS (tracks e, f, g, and h) and 0.5 mM-DST (tracks i, j, k and l) are shown. Immunoblotting was done with antiserum to the 75 kDa subunit (tracks a, e and i), the 49 kDa subunit (tracks b, f and j), the 30 kDa subunit (tracks c, g and k) and the 13 kDa subunit (tracks d, h and l). Cross-linked products are shown to the right of track l. Only the upper part of the gel was analysed for 75 kDa products.

units, the 49 kDa and 13 kDa subunits and the 30 kDa and 13 kDa subunits respectively. Other products were not completely analysed, but their molecular masses could readily be made up from the known remaining subunits or even associated impurities.

It is obvious from Fig. 1 that the yield of simple cross-linked products is extremely low, and, in view of the multiplicity of products, we did not attempt further analysis by the 'diagonal' method commonly used with cleavable cross-linkers (e.g. Capaldi *et al.*, 1979).

Cross-linking of intact Complex I

As expected, similar cross-linking of intact Complex I led to a multitude of products, generally present in very small amounts (Fig. 3). Again, DST and EGS gave qualitatively similar patterns. Two high-molecular-mass bands were produced in relatively high yield, the larger by both DST and EGS and the smaller predominantly by EGS alone.

Immunoblotting experiments are shown in Fig. 4. Clear-cut products containing the 75 kDa subunit were now identified, the two larger ones being the two high-yield products of Fig. 3. However, these bands were not immunoreactive with the

other subunit-specific antisera, and direct identification of the other constituent polypeptides was carried out by cleaving the cross-link in excised gel slices as described in the Materials and methods section. As shown in Fig. 5, both the products contain, in addition to the 75 kDa protein, a 51 kDa polypeptide. The aggregate molecular mass of 126 kDa agrees very well with that measured (125 kDa) for the smaller of these two products, but fails to account for the larger product of molecular mass 165 kDa (measured on 7%-acrylamide gels). The possible nature of this product is discussed below.

Many different cross-linked products containing the 49 kDa or 30 kDa subunits were obtained, and these are listed in Table 2. Again, some of these could be unambiguously characterized by immunoreaction with two different antisera. Thus the 106 kDa and 75 kDa products were identified as cross-links between the 75 kDa and 30 kDa subunits and the 49 kDa and 30 kDa subunits respectively. In addition, the 108 kDa and 83 kDa products reacting with antisera to the 49 kDa and 30 kDa subunits respectively are likely to contain the 51 kDa subunit as the other component, since this is the only subunit of the appropriate mole-

Table 1. *Cross-linking of the iron-protein fraction*

The products of cross-linking with both EGS and DST were analysed as indicated in Fig. 2 legend, by using antisera to the 75 kDa, 49 kDa, 30 kDa and 13 kDa subunits. Concentrations of cross-linkers were varied from 0.04 mM- to 0.8 mM-EGS and from 0.5 mM- to 10 mM-DST. The first column shows the molecular mass of the immunogen that produced the antisera used for immune blotting, and the second column shows the molecular masses of products reacting with the indicated antisera. The composition of the products is shown in the third column.

Molecular mass of antigen (kDa)	Molecular mass of product (kDa)	Molecular masses of subunits present (kDa)
75	None	
49	85	
	75	49 + 30
	66	
	62	49 + 13
30	75	30 + 49
	60	
	54	
	51	
	45	30 + 13
13	62	13 + 49
	54	
	45	13 + 30
	33	
	27	

cular mass. Attempts to verify this by cleavage of the cross-link were frustrated by the large number of closely migrating bands on the gels. Thus the region of the gel containing the 108 kDa product is composed of several cross-linked species containing subunits of 51 kDa, 49 kDa, 39 kDa, 33 kDa, 30 kDa and 24 kDa, and resolution was insufficient to decide which subunit belonged with which. This problem was encountered in attempts to analyse other cross-linked species in this manner.

The cross-linked patterns obtained with the 49 kDa and 30 kDa subunits are quite similar to those obtained with the isolated iron-protein fraction (compare Figs. 2 and 4), except that more cross-linked species were obtained with Complex I. This suggests that the regions of these proteins involved in cross-linking in the iron-protein fraction are still exposed when part of the intact enzyme. However, this is not true of the 13 kDa subunit. Several products, including cross-links to the 30 kDa and 49 kDa subunits, were obtained with the iron-protein fraction (Fig. 2), but only one in Complex I (Fig. 4). The molecular mass (32 kDa) implies a cross-link to a protein of approx. 19 kDa.

These experiments were carried out on dialysed and therefore membranous Complex I. Identical

Table 2. *Cross-linking of Complex I*

The products of cross-linking with EGS (0.16 mM to 0.8 mM) and DST (1 mM to 10 mM) were analysed as indicated in Fig. 4 legend. Data are presented as in Table 1.

Molecular mass of antigen (kDa)	Molecular mass of product (kDa)	Molecular masses of subunits present (kDa)
75	165	75 + 51
	125	75 + 51
	106	75 + 30
	85	
49	108	49 + 51
	82	
	78	
	75	49 + 30
	71	
	66	
	63	
	61	
	59	
	52	
30	106	30 + 75
	83	30 + 51
	78	
	75	30 + 49
	58	
	53	
	51	
	46	
	40	
13	32	

results were obtained in the presence of 0.5% Triton X-100, in which Complex I is dimeric (Dooijewaard *et al.*, 1978). Thus the cross-linked products did not arise from random protein-protein contacts in the aggregated state, but could have arisen from subunits in different monomers of the regular dimeric structure (Boekema *et al.*, 1982).

Discussion

Fragmentation of the iron-protein fraction with detergents and chaotropic agents (Ragan *et al.*, 1982b) had indicated that the 75 kDa, 18 kDa and 15 kDa subunits (Fig. 1) could be relatively easily dissociated from each other and from the other subunits present, whereas the 49 kDa, 30 kDa and 13 kDa subunits were strongly associated. These observations are supported by the cross-linking experiments reported in the present paper. The identification of cross-links between the 49 kDa, 30 kDa and 13 kDa subunits indicates the close association of these three proteins.

Chemical-modification studies of isolated Complex I and intact membranes with hydrophilic and

Fig. 3. *Cross-linking of Complex I*

Complex I was cross-linked with EGS or DST and electrophoresed on a 10%-(w/v)-acrylamide gel. Each track contained 140 μ g of protein. Track a, no cross-linker; track b, 0.16 mM-EGS; track c, 0.4 mM-EGS; track d, 0.8 mM-EGS; track e, 1 mM-DST; track f, 2 mM-DST; track g, 5 mM-DST. Arrows adjacent to track b identify the two high-molecular-mass products described in the text.

Fig. 4. *Immunoblotting of cross-linked Complex I*

Complex I was cross-linked with a range of either EGS or DST concentrations. The results obtained with no cross-linker (tracks a, b, c and d), 0.16 mM-EGS (tracks e, f, g and h) and 1 mM-DST (tracks i, j, k and l) are shown. Data presentation is otherwise as in Fig. 2.

Fig. 5. Analysis of the high-molecular-mass cross-linked products

Complex I was cross-linked with 0.16 mM-EGS and electrophoresed on a 10%-(w/v)-acrylamide gel. Slices containing the high-molecular-mass products were removed, and analysed as described in the Materials and methods section. Track a, 6 μ g of un-cross-linked Complex I protein; track b, the 125 kDa product after cleavage; track c, the 165 kDa product after cleavage. Molecular masses of the residual uncleaved material and the constituent subunits are shown to the right of track c. The presence of the 51 kDa subunit was also confirmed on 10%-(w/v)- and 7%-(w/v)-acrylamide gels, where the separation from the 49 kDa subunit is greater (see Fig. 3).

hydrophobic probes (Smith & Ragan, 1980; Earley & Ragan, 1980, 1981) led to a model of Complex I structure in which the hydrophilic flavoprotein fraction was largely buried within the enzyme, with specific and restricted access for NADH only. The iron-protein fragment was transmembranous and exposed on both sides of the membrane, but prevented from contact with the membrane lipid by a shell of hydrophobic proteins (the insoluble residue from NaClO_4 resolution). More specifically, only the 75 kDa, 49 kDa, 30 kDa and 13 kDa subunits of the iron-protein fraction are exposed to the aqueous phase (Smith & Ragan, 1980; M. W. J. Cleeter & C. I. Ragan, unpublished work), and, of these four, the three largest are transmembranous. In addition, labelling of Complex I with a photoactivatable NAD^+ analogue (Chen & Guillory, 1981) had led to the suggestion that the NADH-binding polypeptide of molecular mass 51 kDa was in close proximity to the 75 kDa subunit of the iron-protein fraction.

The results of cross-linking of Complex I provide good evidence for the basic correctness of these proposals. The accessibility of the 75 kDa, 49 kDa, 30 kDa and 13 kDa proteins to polar cross-linkers confirms the exposure of these proteins to the aqueous phase. For the three largest subunits, greater numbers of products were observed in cross-linked Complex I than in the iron-protein fraction. This strongly suggests that these subunits have contacts with several subunits other than those of the iron-protein fraction itself. One of these is the NADH-binding site of the flavoprotein fraction, the 51 kDa protein, which forms a high-yield product with the 75 kDa polypeptide and may also cross-link to the 49 kDa and 30 kDa proteins. Other cross-links of these three iron-protein fraction subunits are presumably to subunits of the hydrophobic shell, equated with the insoluble residue from NaClO_4 resolution, but this awaits direct confirmation.

The largest product (165 kDa) containing the 75 kDa protein is something of a mystery, since the only other detectable constituent is the 51 kDa protein. We consider that an undetected third constituent is unlikely, since trimeric cross-linked products are invariably much less abundant than related dimeric products (e.g. Coggins *et al.*, 1977; Briggs & Capaldi, 1977; Smith & Capaldi, 1977). If this species were trimeric, we would expect to see even higher yields of either the (75+51) kDa dimer or dimers of the third protein with either of the other two. This is clearly not the case, and the (75+51) kDa dimer is present in high yield only with EGS as cross-linker. The most likely explanation is that the 165 kDa species arises from cross-linking of the 75 kDa and 51 kDa subunits in such a way that polypeptide unfolding in SDS is extensively interfered with and the dimer migrates with a much higher apparent molecular mass.

The 13 kDa polypeptide, unlike the others, behaves quite differently in the iron-protein fraction and Complex I. Clearly, this subunit is buried in the holoenzyme in a way that largely hinders access for the cross-linkers. Its association with the 49 kDa and 30 kDa proteins is therefore only visible in the iron-protein fraction.

A schematic summary of our findings is shown in Fig. 6. The 75 kDa subunit appears to be linked to the other three iron-protein-fraction proteins through the 30 kDa subunit only. Also shown is the close association of the iron-protein subunits with the largest subunit of the flavoprotein fraction (51 kDa). The scheme bears a strong resemblance to structures derived from fragmentation and topological studies (Ragan, 1980, 1985; Ohnishi *et al.*, 1985), although it is not intended to indicate the positions of subunits relative to the two sides of the membrane.

Fig. 6. Scheme of results from cross-linking

A possible arrangement of Complex I subunits based on the cross-linking studies. The lines linking molecular masses (in kDa) indicate cross-links identified in this paper.

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