Subunit Arrangement in Beef Heart Complex III†

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ABSTRACT: Beef heart mitochondrial complex III was separated into 12 polypeptide bands representing 11 different subunits by using the electrophoresis conditions described by Schägger et al. [(1986) *Methods Enzymol. 126*, 224–237]. Eight of the 12 polypeptide bands were identified from their NH_2-terminal sequences as obtained by electroblotting directly from the NaDodSO_4–polyacrylamide gel onto a solid support. The topology of the subunits in complex III was explored by three different approaches. (1) Protease digestion experiments of submitochrondrial particles in the presence and absence of detergent showed that subunits II and VI are on the M side of the inner membrane and subunits V and XI on the C side. (2) Labeling experiments with the membrane-intercalated probes [^{125}I]TID and arylazidoPE indicated that cytochrome *b* is the predominant bilayer embedded subunit of complex III, while the non-heme iron protein appears to be peripherally located. (3) Cross-linking studies with carbodiimides and homobifunctional cleavable reagents demonstrated that near-neighbor pairs include subunits I+II, II+VI, III+VI, IV+V, V+X, and VI+VII. The cytochrome *c* binding site was found to include subunits IV, VIII, and X. The combined data are used to provide an updated model of the topology of beef heart complex III.

Ubiquinol-cytochrome *c* oxidoreductase (EC 1.10.2.2), here referred to as complex III but also termed bc_1 complex, is an oligomeric protein that is involved in electron transport and coupled proton movements across the mitochondrial inner membrane. The complex contains *b* heme, c_1 heme, and a non-heme iron center in the molar ratio of 2:1:1 [for reviews see Rieske (1976, 1986)].

The polypeptide composition of complex III has been studied extensively (Das Gupta & Rieske, 1973; Yu et al., 1974; Gellerfors & Nelson, 1975; Bell & Capaldi, 1976; Schägger et al., 1986). The apoproteins of cytochrome *b*, cytochrome c_1, and the non-heme iron protein have each been identified, and anywhere from five to eight additional polypeptides have been resolved in complex III preparations, depending on the electrophoretic conditions used. Recently, Schägger et al. (1986) have separated beef heart complex III into 11 different subunits by using highly resolving conditions of NaDodSO_4[1]–polyacrylamide gel electrophoresis.

The arrangement of component polypeptides in complex III has been explored by a variety of chemical approaches, including protease digestion (Mendel-Hartvig & Nelson, 1983; Sidhu et al., 1983), chemical modification (Gellerfors & Nelson, 1977; Bell et al., 1979; Beattie et al., 1981; D'Souza & Wilson, 1982; Ho & Rieske, 1985), and electron microscopy and image reconstruction analysis (Wingfield et al., 1979; Leonard et al., 1981). These studies have been conducted over a period of years in different laboratories and have not consistently taken into account the number of components now thought to be present. In addition, studies in different laboratories have used different separating procedures and have sometimes identified the subunits by different nomenclatures, so that models of the structure of complex III built on the accumulated data are confusing. To clarify this situation, we have begun by analyzing the subunit composition of beef heart complex III, exploring a variety of conditions of NaDodSO_4–polyacrylamide gel electrophoresis to obtain optimal resolution of components. Subunits have been sequenced directly from the resolving gels after electroblotting, thereby allowing the identification of components unambiguously. In addition, chemical labeling and cross-linking experiments were carried out to generate a more complete model of the subunit arrangement in complex III than has previously been possible.

Experimental Procedures

Enzyme Preparations. Active beef heart ubiquinol-cytochrome *c* oxidoreductase (complex III) was prepared according to the method of Rieske (1967) and stored at −70 °C until used. Beef heart submitochondrial particles were prepared according to the method of Loyter et al. (1969). Reconstituted vesicles of the complex were made as described by Leung and Hinkle (1975).

Protease Digestion Experiments. Detergent-solubilized complex III, obtained as described by Rieske (1967), was diluted to a protein concentration of 5 mg/mL in 20 mM Tris-HCl (pH 8.2) and was incubated with protease at a ratio of 1:100 or 1:1000 (w/w) for 30 min at room temperature. Proteases, purchased from Sigma Chemical Co., included trypsin (type XIII, TPCK treated), thermolysin (protease type X, from *Bacillus thermoproteolyticus Rokko*), subtilisin (type VIII, from *Bacillus subtilis*), Pronase E (type XXV, from *Streptomyces griseus*), and chymotrypsin (type VII, TLCK treated). Proteolytic reactions were stopped by the addition of 1 mM PMSF, or in the case of trypsin, with trypsin inhibitor (type I-P, from bovine pancreas) in a 1:3 ratio (w/w) of inhibitor to protease. The thermolysin reaction was stopped by addition of 10 mM EDTA and 10 mM 1,10-phenanthroline. Where indicated, sucrose gradient centrifugation was carried

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[1] Abbreviations: arylazidoPE, 1,2-[^{3}H]dipalmitoyl-*sn*-glycerol 3-[2-[(4-azido-2-nitrophenyl)amino]ethyl phosphate]; DABS, diazobenzenesulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; DSP, dithiobis(succinimidyl propionate); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, ethylenediaminetetraacetic acid; NaDodSO_4, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PVDF, poly(vinylidene difluoride); [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine.

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out as described by Zhang et al. (1984).

Cleavage of submitochondrial particles (2 mg of protein/mL) was conducted in 25 mM Tris-HCl (pH 7.5), both in the presence and in the absence of 0.5% potassium deoxycholate, by using a ratio of 1:5 (w/w) of protease to total protein. After the inhibition of the protease reaction, samples were taken to a final concentration of 0.5% $NaDodSO_4$ and centrifuged for 5 min in a tabletop centrifuge. The small tight pellet containing insoluble material was discarded. The supernatant was subjected to polyacrylamide gel electrophoresis.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Schägger et al. (1986) with 1.2 mm thick slab gels. For comparative purposes, gels were run according to the methods of Swank and Munkres (1971) and Weber and Osborn (1969), using a 16% acrylamide concentration. A stacking gel of 5% acrylamide was added for the Weber and Osborn system. Densitometric traces of gels were obtained with a scanning densitometer Zeidel Model SL-50h-XL soft laser.

Isolation of Subunits and Antibody Formation. Antibodies against subunits I+II, III, IV, VI, X, and XI were obtained after purification of the individual subunits. Complex III was separated into five main fractions following the method reported by Shimomura et al. (1986) using hydrophobic chromatography with phenyl-Sepharose 4B. Fractions enriched in subunits I+II, IV, and VI were obtained after complex III was bound to the column and fractionated with guanidine treatment as described (Shimomura et al., 1986). A fraction containing cytochrome c_1 (subunit IV) and several of the small subunits was subsequently eluted by substituting the detergent dodecyl octaethylene glycol monoether ($C_{12}E_8$) with TX-100 (1%). Finally, a cytochrome *b* (subunit III)-enriched fraction was eluted by using a buffer containing 2% $NaDodSO_4$. Guanidine-containing fractions were dialyzed, and all fractions were concentrated and denatured completely in the presence of 5% $NaDodSO_4$ containing 2% (v/v) β-mercaptoethanol. All subunits were further purified in denatured form by gel filtration, as described for the subunits of beef heart cytochrome *c* oxidase by Takamiya et al. (1987). Samples were loaded on a Bio-Gel P-60 (<400 mesh) column (1.5 × 116 cm), previously equilibrated with 2% $NaDodSO_4$ and eluted at 1 mL/h. The eluent was monitored at 280 nm. The small subunits X and XI were resolved by high-performance liquid chromatography (HPLC), using a Beckman gradient liquid chromatograph equipped with a Brownlee Laboratories column (type BU-300), Aquapore Butyl, 300-Å pore size, 7 μm spherical, 4.6 × 30 mm) at a flow rate of 0.7 mL/min. The proteins were eluted in a linear gradient from 30 to 80% solvent A in B. [A: 0.05% (v/v) triethylamine and 0.05% (v/v) trifluoroacetic acid in acetonitrile. B: 0.05% (v/v) triethylamine and 0.05% (v/v) trifluoroacetic acid in 5% (v/v) acetonitrile.]

Pure core protein II was obtained by preparative electrophoresis. Partial digestion of core II by trypsin treatment of the intact complex was used to increase the distance in migration between the two core proteins. Gels were stained and the bands excised and electroeluted in an ISCO Model 1750 electrophoretic concentrator in the presence of 25 mM Tris, 200 mM glycine, and 0.1% $NaDodSO_4$.

Pure subunits, obtained as above, were dialyzed against 50% ethanol and then against water to remove the $NaDodSO_4$. Samples were lyophilized, resuspended in 0.9% NaCl, mixed with Freund's complete adjuvant, and injected into rabbits (100 μg of protein per injection). A total of three booster injections were performed every three weeks, and antibodies were recovered from the serum of the rabbits by ammonium sulfate precipitation (Goding, 1983). The 0–40% ammonium sulfate cuts were resuspended in 0.5 M NaCl and 20 mM Tris-HCl (pH 7.5) and kept at −20 °C until used. Antibody titers were assayed by immunoblotting as described below by using 30 μg of complex III and different antibody dilutions ranging from 1:50 to 1:5000.

Immunodetection of Subunits after Gel Blotting. Immunoblotting was conducted according to the procedure of Towbin et al. (1979) using a Hoefer TE22 transblot apparatus and transferring at 250 mA for 4 h or at 180 mA overnight and using a buffer containing 0.19 M glycine, 0.02 M Tris, 0.1% $NaDodSO_4$, and 20% methanol. Additional protein binding sites on the blot were saturated with either 3% gelatin or 5% nonfat dry milk (Carnation). Either alkaline phosphatase or horseradish peroxidase conjugated goat anti-rabbit antibodies were used to develop color (Hawkes et al., 1982) in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium or 4-chloro-1-naphthol, respectively. All saline buffers used during antibody incubations contained 0.05% Tween 20.

Electroblotting onto Activated Glass Fiber Paper and PVDF Membrane. Protein samples resolved by polyacrylamide gel electrophoresis were transferred electrophoretically onto derivatized glass filter paper sheet by following the procedure of Aebersold et al. (1986) as modified by Yuen et al. (1986). Glass fiber paper (Whatman GF/F) was pretreated with trifluoroacetic acid and derivatized with *N*-[(trimethoxysilyl)propyl]-*N*,*N*,*N*-trimethylammonium chloride (Petrarch Systems) in the presence of acetonitrile. Electroblotting was performed at room temperature for 3 h at 240 mA in a 20 mM *N*-ethylmorpholine buffer (Pierce Chemical Co.) adjusted to pH 8.2 with formic acid. The blot was stained with 3,3′-dipentyloxacarbocyanine iodide (Molecular Probes, Inc.), and the polypeptide bands were visualized under UV light. Bands were excised with a razor blade and kept at −20 °C until sequence analysis was done.

Alternatively, gels were transferred onto poly(vinylidene difluoride) (PVDF) membrane (Immobilon, Millipore) following the procedure described by Matsudaira (1987). Since 1.2 mm thick gels were used, the electroblotting time was increased to 2.5 h at 400 mA. After staining, the blots were air-dried and stored at −20 °C.

Sequence analysis was performed by a gas-phase protein sequencer Applied Biosystems Model 470A) equipped with an on-line PTH analyzer (Applied Biosystems Model 120A).

Photolabeling of Complex III with the ArylazidoPE. 1,2-[^{3}H]Dipalmitoyl-*sn*-glycerol 3-[2-[(4-azido-2-nitrophenyl)amino]ethyl phosphate] (arylazidoPE) was synthesized as described by Aggeler et al. (1987) and was kindly provided by Dr. R. Aggeler. The reagent had a specific activity of 200 mCi/mmol.

ArylazidoPE (50 nmol) in toluene was mixed with L-α-phosphatidylcholine (2 mg, from egg yolk, type III-E, hexane solution, Sigma) in a glass vial and dried under a nitrogen stream for 30 min. The phospholipids were sonicated in a water bath sonicator (Branson 2200) for 30 min in the presence of 500 μL of a buffer containing 2% potassium cholate and 10 mM potassium phosphate (pH 7.8). Complex III (1 mg), obtained as described by Rieske (1967), was added to this mixture, making a final volume of 530 μL. The mixture was dialyzed according to the reconstitution procedure described by Leung and Hinkle (1975). The protein concentration was adjusted to 1 mg/mL, part of the sample was saved as dark control, and the rest was irradiated at room temperature for

Table I: Sequence Homology between Yeast and Beef Heart Core Proteins

Core 1																													
						20								25							30								
YEAST	L	A	T	-	A	V	A	T	P	K	A	E	V	T	Q	L	S	N	.	.	.	.	.						
BEEF HEART	T	A	T	Y	A	Q	A	L	Q	S	V	P	E	T	Q	V	S	Q	.	.	.	.	.						
						5					10					15													
Core 2																													
					50					55					60					65					70				
YEAST	T	R	S	A	L	K	L	V	R	E	S	E	L	L	G	G	T	F	K	S	T	L	D	R	E	Y	I	T	L
BEEF HEART	S	L	K	V	A	P	K	V	K	A	T	E	A	P	A	G	V	P	P	H	P	Q	D	L	E	F	R	R	L
					5				10					15					20					25					

40 min in a Pyrex cuvette with an 18-W 366-nm UV lamp (UVP, Inc., Model UVL-56, BLAK-RAY lamp). After irradiation, $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described above, using 19 cm long slab gels. Gels were first stained and then sliced, dissolved, and counted as described by Aggeler et al. (1987).

Photolabeling of Complex III with [^{125}I]TID. Complex III was incorporated into vesicles following the procedure of Leung and Hinkle (1975), except that a lower ratio of lipid to protein was used (2:1, lipid:protein w/w). Reconstituted vesicles (2 mg of protein/mL) were incubated with 5 μM [^{125}I]TID (ethanolic solution, Amersham, 10 Ci/nmol) for 15 min in the dark (the final concentration of ethanol was 1%). Samples were photolyzed for 15 min by using the same conditions that have been described above for the arylazidoPE. After photolysis, vesicles were diluted 20-fold with 0.1 M potassium phosphate (pH 7.8) containing 1% bovine serum albumin and centrifuged at 45 000 rpm in a Beckman 60 Ti rotor for 90 min. Pellets were resuspended in 20 mM potassium phosphate (pH 7.8) containing 5% potassium cholate and loaded on a discontinuous sucrose gradient as described by Zhang et al. (1984). This centrifugation procedure was carried out twice to remove excess phospholipids and label. Samples were subjected to polyacrylamide gel electrophoresis as described above. After fixing, staining, and destaining, gels were sliced on a Mickel gel slicer (Brinkmann Instruments). Radioactivity in gel slices was determined in an Automatic Gamma Counter 4/200 Micromedic Systems).

Other Methods. Protein concentrations were determined according to the method of Lowry et al. (1951) as modified by Markwell et al. (1978). Cross-linking experiments with DSP were carried out essentially as described by Smith and Capaldi (1977), except that first- and second-dimensional electrophoresis was performed by using the system described by Schägger et al. (1986). Cross-linking experiments with DCCD were done as described by Nalecz et al. (1983) and those with EDC as reported by Gutweniger et al. (1983), using the conditions described by Kadenbach and Stroh (1984) for cytochrome *c* oxidase except that the final concentration of complex III was 11 μM and the acetone precipitation step was omitted.

Ubiquinol-cytochrome *c* oxidoreductase activity was measured as described in Rieske (1967), using ubiquinol 1 as substrate. Uniquinone 1 was a kind gift from Drs. S. Weber and J. P. Vuilleumier, Hoffmann-La Roche, Basel, Switzerland.

Results

Subunit Separation and Identification. Four different systems of $NaDodSO_4$–polyacrylamide gel electrophoresis have been used in studies of mammalian complex III (Weber & Osborn, 1969; Laemmli, 1970; Swank & Munkres, 1971; Schägger et al., 1986). Each was examined by using high concentrations of acrylamide in order to establish optimal resolving conditions. The best procedure for resolving all components proved to be the buffer conditions of Schägger et al. (1986) with a 16% acrylamide gel. Typical polypeptide profiles of beef heart complex III using these conditions are shown in Figure 1. The sample in gel A was dissociated in $NaDodSO_4$ alone; the one in B in the presence of β-mercaptoethanol as a reducing agent. Twelve Coomassie blue staining bands contributed by complex III were resolved in both gels but with differences in migration of some components.

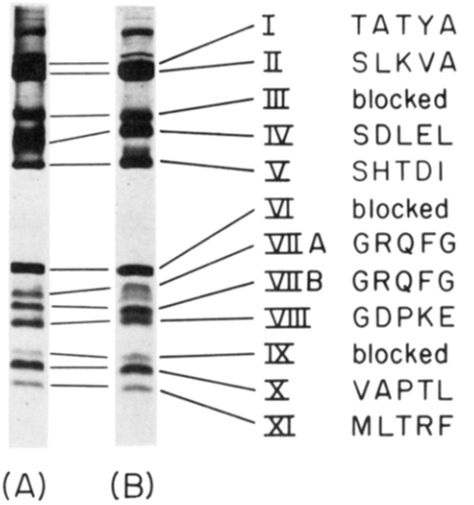

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of complex III run in the presence (B) and absence (A) of β-mercaptoethanol. To the right are listed the subunit nomenclature used in the text and the amino-terminal sequence of the polypeptides as obtained after electroblotting onto derivatized glass fiber paper on PVDF membrane.

Electrophoresed samples, analogous to those in Figure 1, were transferred by electroblotting to a solid support (sometimes glass fiber paper but usually PVDF membrane; Matsudaira, 1987). The NH_2-terminal sequences obtained in this way are listed next to the corresponding bands in Figure 1. Nine of these polypeptides could be identified from published sequence data for complex III components.

Subunits I and II in Figure 1 are the so-called core proteins. The mammalian forms of these polypeptides have not been sequenced. However, the genes for core proteins of yeast have been cloned and their protein sequences deduced from the DNA sequences (Tzagoloff & Crivellone, 1986; Oudshoorn et al., 1987). The NH_2-terminal sequences of the beef heart core proteins as determined in our study show weak sequence

homology to their yeast counterparts (Table I).

Polypeptides IV and V were identified by NH_2-terminal sequencing as cytochrome c_1 and the non-heme iron protein, confirming identifications of these bands made previously by staining for covalently bound heme in the case of cytochrome c_1 (Bell & Capaldi, 1976), and by subunit depletion studies in the case of the non-heme iron protein (Trumpower & Edwards, 1979; Shimomura et al., 1984).

The other bands that could be related to published amino acid sequences were bands VI, VII, VIII, X, and XI (Borchart et al., 1986; Wakabayashi et al., 1982; Schägger et al., 1983, 1985). Polypeptide VI is the so-called "quinone binding protein" sequenced by Wakabayashi et al. (1985). This polypeptide had a blocked NH_2-terminus when sequenced directly after electroblotting from the gel shown in Figure 1, and identification required treatment of complex III with thermolysin. Detergent-solubilized complex III was incubated with thermolysin for 30 min at 100:1 (w/w) ratio to protease. This treatment converted subunit VI to a component with a slightly faster migration, as demonstrated by a protease time course and immunoblotting with a specific anti-subunit VI antibody (data not shown). The NH_2-terminal sequence of the thermolysin-modified subunit VI was AVS, establishing its identity as the putative quinone binding protein and showing that thermolysin removes the NH_2-terminal 5 residues from this subunit.

The polypeptide represented by band III has been identified by subunit purification studies as cytochrome *b* (Shimomura et al., 1986). This is the only mitochondrially synthesized subunit of complex III and begins with F-Met (Anderson et al., 1982), explaining the blocked NH_2-terminus when sequenced from transferred gels. Another polypeptide with blocked NH_2-terminus is subunit IX. This subunit has been related to the "DCCD-binding protein" of complex III (Lorusso et al., 1983), which has an acetylated methionine at the NH_2-terminus (Borchart et al., 1985).

Two bands on the gel, VII A and VII B, had the identical NH_2-terminal sequence GRQ [see Borchart et al. (1986)] and are electrophoretic variants of the same subunit. Such anomalous migration of the same polypeptide as two bands is also seen with subunit C_{VI} of cytochrome *c* oxidase (Y. Z. Zhang and R. A. Capaldi, unpublished results) and might be related to incomplete reduction of disulfide bonds. Polypeptide VIII was identified from sequencing as the hinge protein (Kim & King, 1981; Wakabayashi et al., 1982).

Three components migrated differently depending on the presence or absence of β-mercaptoethanol. These are cytochrome c_1 (subunit IV), which runs with a slightly lower apparent molecular weight and as a broader band in the absence of the reducing agent, and subunits VII and VIII.

The other two most frequently used gel systems in studies of complex III are the ones described by Weber and Osborn (1969) and Swank and Munkres (1971). These were compared with the gel system used in Figure 1B by using two-dimensional gel electrophoresis as shown in Figure 2. The subunits all ran at the same relative positions in the Schägger et al. (1986) system and in the Swank and Munkres system. However, the order of migration of subunits VI and VII was reversed in the Weber and Osborn system. As discussed later, this difference in migration affects identification of the quinone binding protein by different laboratories.

Protease Digestion of Complex III. All of the structural studies described below use the gel conditions and the nomenclature of subunits presented in Figure 1. The orientation of beef heart complex III has been explored by chemical labeling, and it has been found that this complex spans the mitochondrial inner membrane (Gellerfors & Nelson, 1977; Bell et al., 1979; D'Souza & Wilson, 1982). Protease digestion experiments offer an alternative approach to studying the orientation of subunits in the complex. As a preliminary assay to these studies, the accessibility of the polypeptides to protease was screened by using detergent-solubilized complex III. The result of this experiment is presented in Figure 3. Trypsin, thermolysin, subtilisin, Pronase E, and chymotrypsin all cleaved both subunits V and XI. Other subunits cleaved by one or more proteases are II, VI, and VII. The cleavage sites in subunits II and V could be identified by protein sequencing as described for subunit VI above. Similar patterns of cleavage of complex III have been obtained with papain by Lorusso et al. (1985).

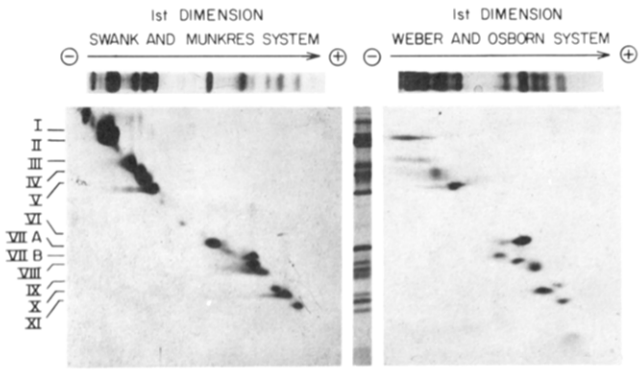

FIGURE 2: Two-dimensional gel electrophoresis of complex III using 16% Swank and Munkres (1971) and 16% Weber and Osborn (1969) gels in the first dimension and a Schägger et al. (1986) gel system in the vertical dimension.

Fragments of core protein II generated by thermolysin treatment included a fragment of 42 kDa with a NH_2-terminus VKA, indicating that this cleavage removes the first 7 residues from the subunit. The major trypsin fragment had a similar apparent molecular weight and began ATE, showing that 10 residues were removed from the NH_2-terminal portion of the protein. Additional fragments were generated by prolonged digestion with trypsin, starting with the NH_2-terminus ATE. The smaller apparent molecular weights (25 kDa) of these fragments indicate cleavage from the COOH-terminus of subunit II.

The cleavage products of the non-heme iron protein were identified initially by antibody binding. These fragments (V′ and V″) were collected by electroblotting and sequenced as above. The cleavage sites are shown in Figure 4.

In one set of experiments, protease-treated complex III was centrifuged through sucrose gradients to separate fragments released by the treatment from those retained by the major part of the complex. Fragments in the supernatant and pellet were identified immunologically. The fragments of subunit II remained in the core complex. However, the large fragment (V″) containing the non-heme iron center of subunit V was released into solution by protease treatment (Figure 5). Small amounts of intact non-heme iron protein were also found in these supernatants, probably dissociated from the complex by the presence of Triton X-100 in the gradients [see Weiss et al. (1978)].

Trypsin and thermolysin were used in the orientation studies of complex III in submitochondrial particles. Previous studies have shown our preparation of submitochondrial particles to be 95% oriented with the matrix face of the inner membrane outermost (Zhang et al., 1988). The time course of cleavage of individual subunits of complex III was followed immunologically after incubation of submitochondrial particles with

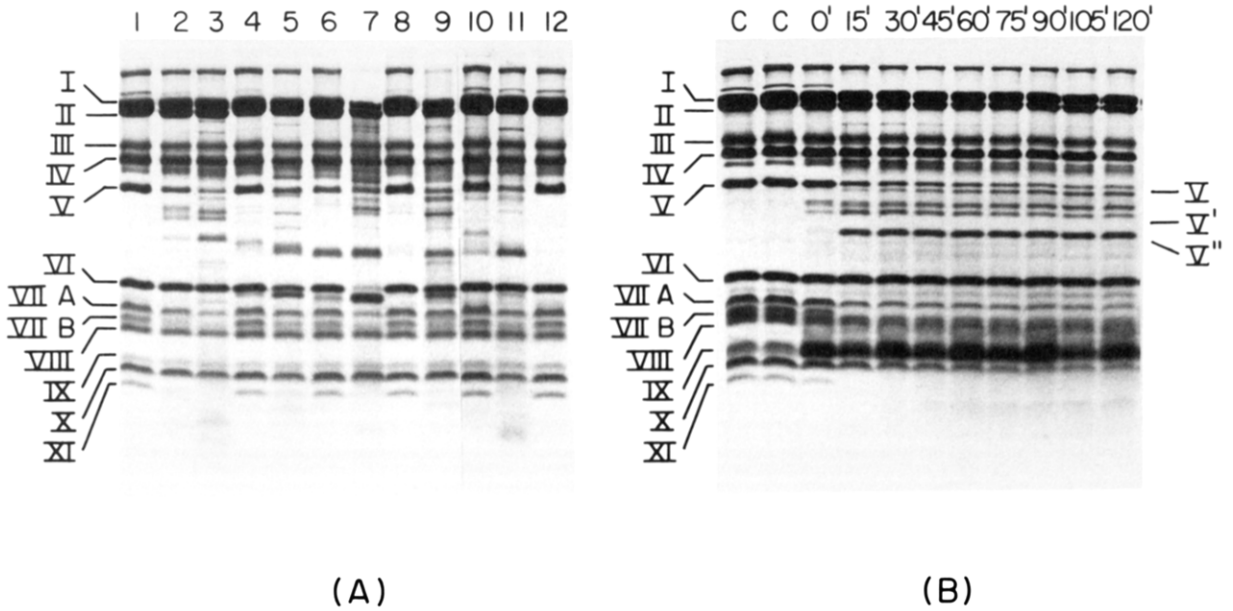

FIGURE 3: (A) Effect of different proteases on the polypeptide profile of beef heart complex III. Detergent-dispersed complex III (5 mg/mL in 20 mM Tris-HCl, pH 8.2) was incubated with trypsin (1:1000 w/w, lane 2; 1:100 w/w, lane 3), thermolysin (1:1000 w/w, lane 4; 1:100 w/w, lane 5), subtilisin (1:1000 w/w, lane 6; 1:100 w/w, lane 7), Pronase E (1:1000 w/w, lane 8; 1:100 w/w, lane 9), and chymotrypsin (1:1000 w/w, lane 10; 1:100 w/w, lane 11). Protease digestion was stopped after 30 min by the addition of 1 mM PMSF. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out as described under Experimental Procedures by using 30 μg of protein per lane. Lanes 1 and 12 show control samples without protease treatment. (B) Time course of proteolytic digestion of beef heart complex III by trypsin. Detergent-dispersed complex III (5 mg/mL in 20 mM Tris-HCl, pH 8.2) was incubated with trypsin (1:100 w/w) at room temperature. Protease digestion was stopped at the indicated times by the addition of PMSF and trypsin inhibitor, as described under Experimental Procedures. Gels were loaded with 30 μg of protein per lane. Lanes marked C show control samples without protease treatment. The position of the non-heme iron protein fragments are indicated as V′ and V″.

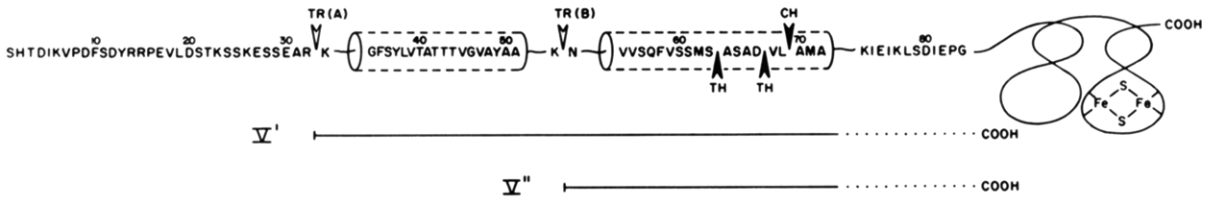

FIGURE 4: Sequence of beef heart mitochondrial non-heme iron protein according to Schägger et al. (1987), showing the cleavage sites of trypsin (TR), thermolysin (TH), and chymotrypsin (CH). These cleavage sites were characterized by amino-terminal sequencing of the fragments generated by these proteases in the intact, detergent-dispersed complex. The diagram shows the two putative hydrophobic stretches suggested by Schägger et al. (1987). The trypsin sites of cleavage A and B generate the products V′ and V″, respectively. A gel showing these products is shown in Figure 3B.

either of these proteases (Figure 6). Both subunits II and VI were cleaved to fragments identical in size with those produced by using detergent-solubilized enzyme (II′ and VI′, respectively). The non-heme iron subunit and subunit XI were both inaccessible from the M side (the data for subunit XI is not shown). Addition of detergent to disrupt the submitochondrial particles increased the rate of cleavage of subunits II and VI marginally but facilitated the extensive cleavage of subunits V and XI. These protease results indicate that subunits II and VI are exposed on the M side of the inner membrane and the non-heme iron protein and subunit XI on the C side. This localization of the non-heme iron protein to the cytoplasm face of the inner membrane is consistent with functional studies and previous chemical labeling experiments (Ohnishi et al., 1982; Bell et al., 1979; D'Souza & Wilson, 1982).

Protease digestion experiments were also conducted on reconstituted vesicles and digitonin particles from bovine heart mitochondria (Loyter et al., 1969) as approaches to cleaving components from the cytoplasmic (C) side selectively. However, both preparations proved to be scrambled, and there was slow but significant cleavage of both subunits II and VI (up to 40% that in submitochondrial particles) along with the cleavage of the non-heme iron protein and subunit XI.

Labeling of Complex III with Bilayer Intercalated Probes. Identification of those subunits of complex III with parts inside the bilayer has been attempted by using arylazidophospholipids (Gutweniger et al., 1981). The carbene-generating reagent [^{125}I]TID is another reagent for labeling within the bilayer, but with a broader reactivity than arylnitrenes (Brunner & Semenza, 1981). The labeling of complex III in lipid vesicles with [^{125}I]TID is shown in Figure 7. By far the major site of labeling is subunit III (cytochrome *b*). There are smaller amounts of radioactivity associated with subunit II along with subunits VI, VII, IX, X, and XI (Figure 7, inset) but essentially no labeling of core protein I, cytochrome c_1, and the iron–sulfur protein (V). Oxidoreductase activity of the vesicles remained unchanged after photolabeling. It was observed that centrifugation through the detergent containing sucrose gradients (Zhang et al., 1984) partially removed subunits V and XI. Therefore, parallel experiments were conducted omitting the lipid removal. No significant labeling of the non-heme iron protein was observed in these experiments (not shown).

As a companion experiment to the labeling with [^{125}I]TID, complex III was reacted with [^{3}H]arylazidophosphatidylethanolamine, a membrane-intercalated probe in which the

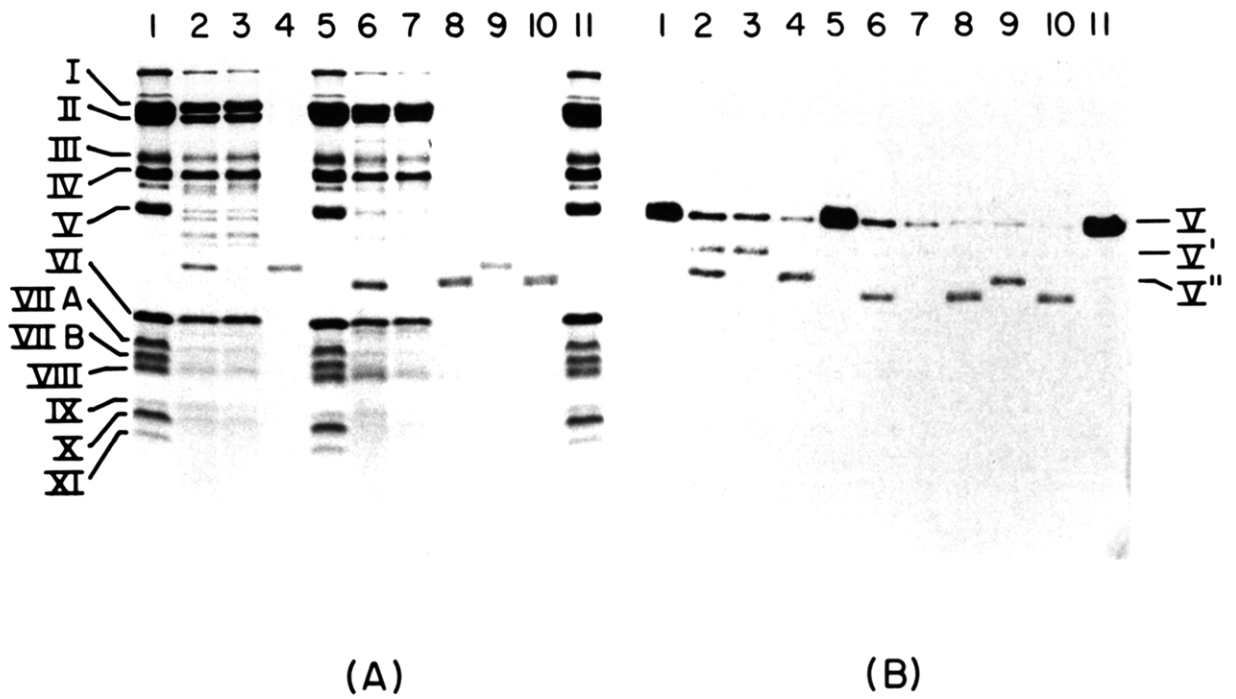

FIGURE 5: Recovery of the trypsin and chymotrypsin fragments from the non-heme iron protein after sucrose gradient centrifugation: (A) $NaDodSO_4$–polyacrylamide gel electrophoresis; (B) immunoblot of an identical gel, incubated in the presence of anti-non-heme iron protein antibody. Complex III (5 mg/mL) was reacted with trypsin (1:100 w/w, lane 2) and chymotrypsin (1:100 w/w, lane 6) for 30 min at room temperature, and the reaction was stopped with the addition of 1 mM PMSF. A fraction of this sample was subjected to sucrose gradient centrifugation as described under Experimental Procedures. The pellet obtained for the trypsin-treated complex is shown in lane 3, while the corresponding (10-fold concentrated) supernatant is shown in lanes 4 and 9. The pellet obtained for the chymotrypsin-treated complex is shown in lane 7, and its supernatant is shown in lanes 8 and 10. Lanes 1, 5, and 11 show control samples in which both protease treatment and sucrose gradient centrifugation were omitted.

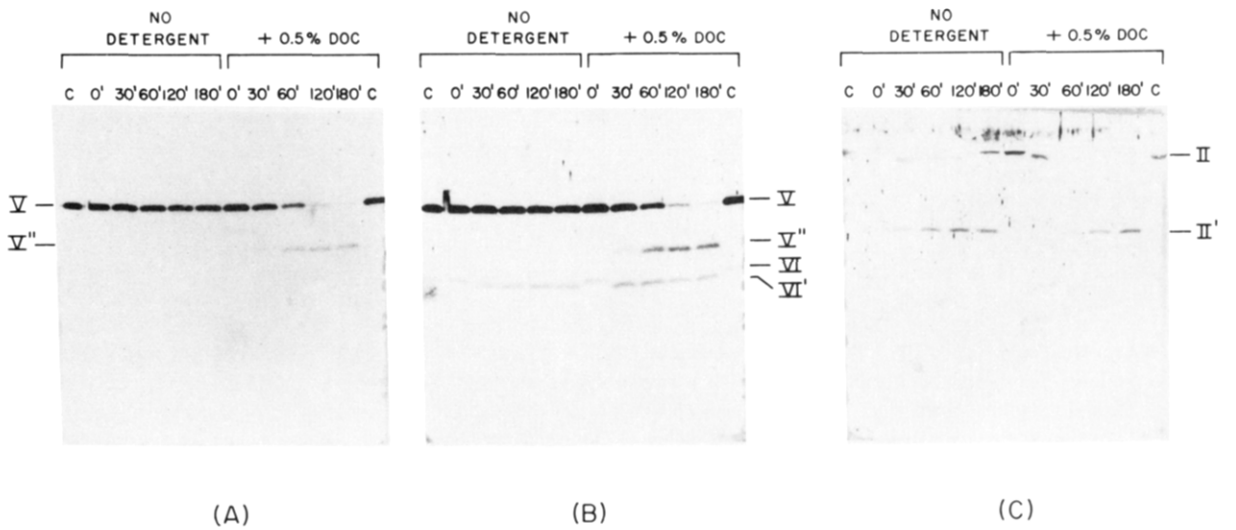

FIGURE 6: Thermolysin cleavage of complex III in submitochondrial particles. Digestion was carried out as described under Experimental Procedures and stopped at the indicated times with the appropriate inhibitors. Digestion was carried out both in intact submitochondrial particles (no detergent) and in membranes disrupted by the addition of 0.5% potassium deoxycholate (+0.5% DOC). Control lanes (C) show untreated samples. Gels and immunoblots were done as described: (A) Immunoblot using antibodies against the non-heme iron protein (V); (B) immunoblot with anti-subunit V antibodies as in (A), which was then reacted with antibodies against subunit VI; (C) immunoblot using anti-core II.

reactive group is in the head-group region rather than being buried among the fatty acid chains (as in the case of [^{125}I]-TID). Figure 8 shows the labeling profile with arylazidoPE. The predominant site of labeling with this probe is cytochrome c_1 rather than cytochrome *b*. In addition, there is some labeling of cytochrome *b*, along with subunits VI and VII. Again, there is little or no labeling of core proteins or the non-heme iron protein.

Cross-Linking Experiments To Identify Near-Neighbors in Complex III. Previous cross-linking experiments on beef heart complex III (Smith & Capaldi, 1977; Smith et al., 1978) were conducted without the benefit of optimally resolving gel conditions and without all of the subunits of the complex having been identified. Figure 9 shows a two-dimensional gel of complex III that has been cross-linked by DSP and then electrophoresed in the buffer system of Schägger et al. (1986) in both dimensions. This is essentially the same experiment reported by Smith and Capaldi (1977) (their Figure 4), but in the gel system used now, cytochromes *b* and c_1 run as discrete spots rather than smears across the gel. The cross-linked products resolved are between subunits I+II, II+VI, V+X, and VI+VIII, all near-neighbor pairs identified by

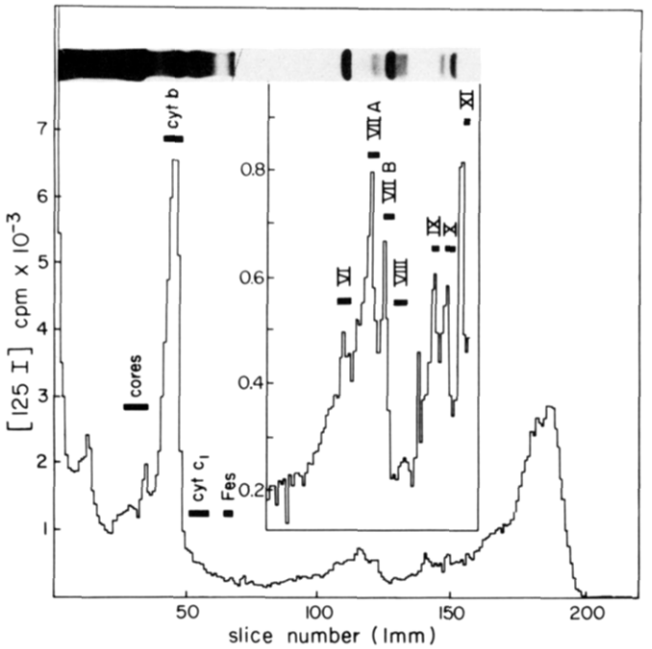

FIGURE 7: Photolabeling of complex III with [^{125}I]TID. Labeling conditions were the ones described under Experimental Procedures. Gels (22 cm long) were loaded with 60 μg of protein per lane. The inset shows an amplified scale for the lower molecular weight subunits.

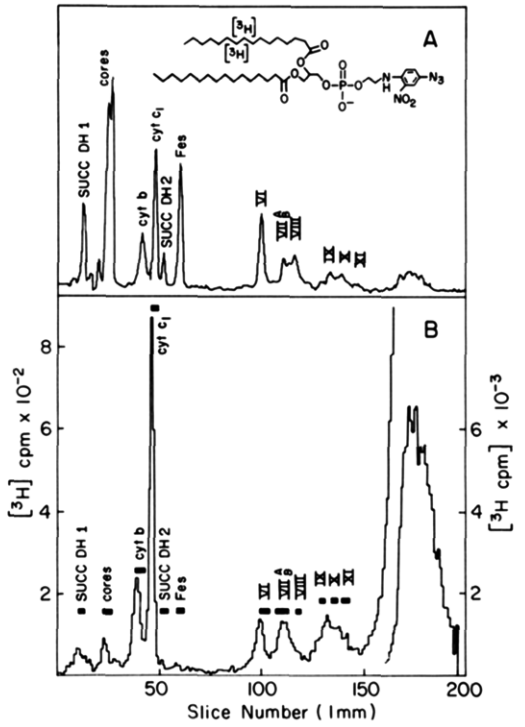

FIGURE 8: Photolabeling of complex III with arylazidoPE. Labeling conditions were the ones described under Experimental Procedures. Gels (22 cm long) were loaded with 40 μg of protein per lane: (A) Densitometer tracing of the Coomassie blue stained gel; (B) radioactivity distribution pattern in the gel. No significant incorporation of radioactivity over background was observed in the dark control (not shown).

Smith and Capaldi (1977). Also resolved are cross-linked products of IV+V and III+VI, which were hidden by smearing in the previous study. The cross-linked product between I+V previously reported was not confirmed since additional spots for subunits III and IV were also present in the same vertical position.

Cross-linking of subunits within complex III has also been seen by reaction with carbodiimides. Nalecz et al. (1983) and

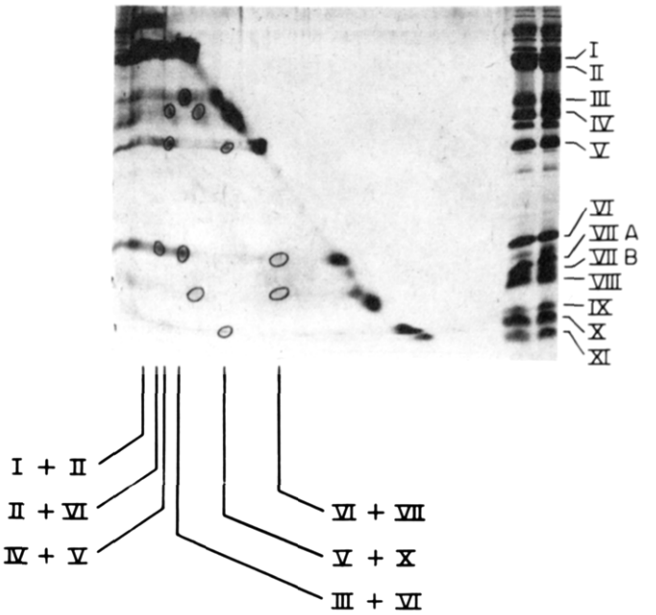

FIGURE 9: Two-dimensional analysis of the cross-linked aggregates obtained with DSP (0.05 mg/mg for 5 min). The first-dimensional gel was run in the Schägger et al. (1987) system, loading 60 μg of protein per lane. After incubation in a buffer containing 0.1 M Tris, 0.1 M Tricine, 0.1% $NaDodSO_4$, and 4% mercaptoethanol for 1 h at room temperature, the gels were run in a second dimension, using the same electrophoresis system. Control lanes were loaded with 30 μg of protein.

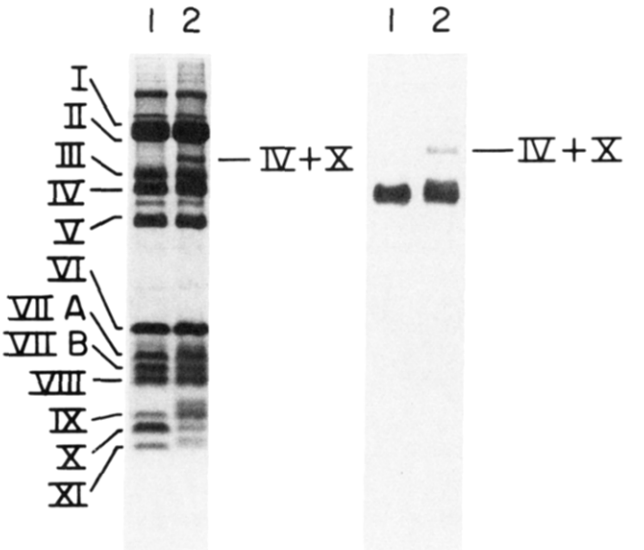

FIGURE 10: Cross-linking of subunits IV and X after treatment of complex III with DCCD. Cross-linking conditions were the ones described under Experimental Procedures: (A) $NaDodSO_4$–polyacrylamide gel, loaded with 30 μg of protein per lane; (B) immunoblot of an identical gel decorated with antibody against subunit IV (cytochrome c_1); (lane 1) untreated complex III; (lane 2) DCCD-treated complex III.

Lorusso et al. (1983) both reported that the hydrophobic carbodiimide DCCD generated a cross-link between the iron–sulfur protein (subunit V) and subunit VII in high yield. The authors identified the components in this covalently cross-linked product by the disappearance of these subunits from the gel in proportion to the amount of cross-linked

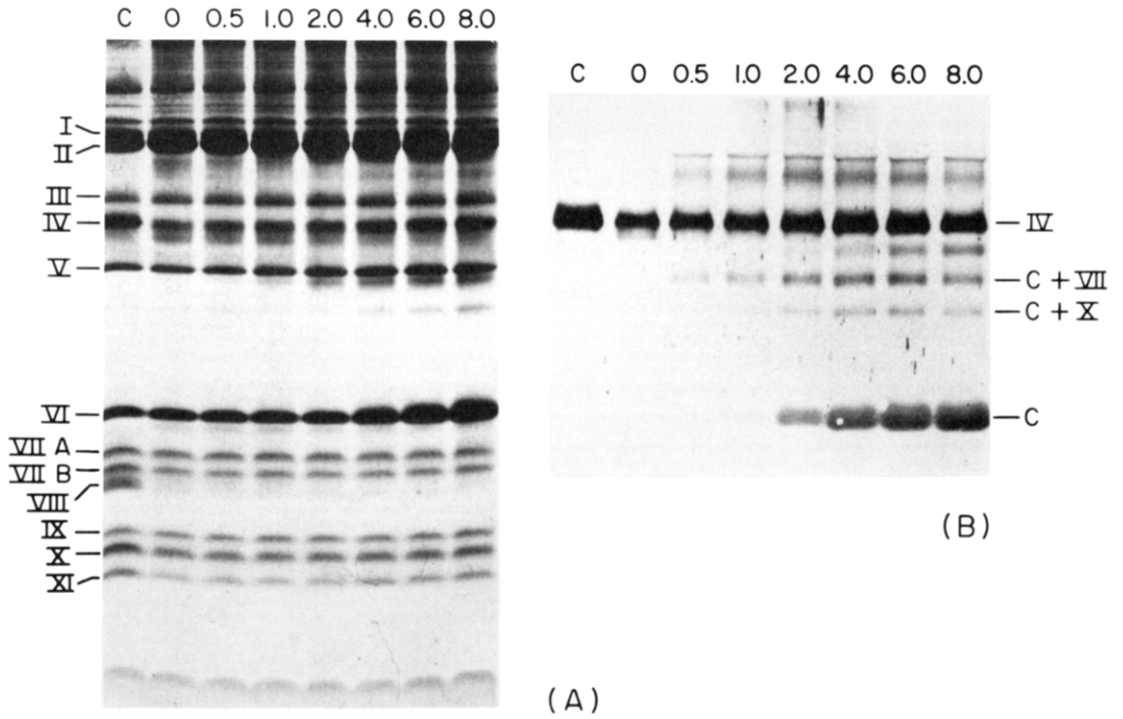

FIGURE 11: Cross-linking of complex III by EDC in the presence and absence of horse heart cytochrome *c*: (A) $NaDodSO_4$–polyacrylamide gel, loaded with 40 μg (complex III) of protein per gel; (B) immunoblot of an identical gel decorated with antibodies against subunit IV (cytochrome c_1) and anti-yeast cytochrome *c*. The experiment was carried out in the presence of increasing amounts of horse heart cytochrome *c*. Numbers indicate mole/mole ratios of cytochrome *c* to complex III. Control lane (C) contains untreated complex III.

Table II: Identification of Carbodiimide Cross-Link Products by Amino-Terminal Sequence Analysis

DCCD X-link product:	
	5 10
cyt c_1 (band IV)	S D L E L H P P S Y P . . . . .
+	
subunit X	V A P T L T A R . . . . .
	5
EDC X-link products:	
cyt c	blocked (identified by immunoblotting)
+	
subunit VIII	G D P K . . . . .
cyt c	blocked (identified by immunoblotting)
+	
subunit X	V A P T . . . . .

product present. Figure 10 confirms that reaction of complex III with DCCD in the conditions described by Nalecz et al. (1983) generates a cross-linked product of approximately 42 kDa that gives positive reaction when blotted and reacted with an anti-cytochrome c_1 antibody. This product was electroblotted onto derivatized glass fiber paper and sequenced in order to obtain and unambiguous identification of the subunits involved. Two sequences were obtained, consistent with the presence of the NH_2-termini of cytochrome c_1 and subunit X (Table II).

Reaction of complex III with the water-soluble carbodiimide EDC has also been found to generate a cross-linked product in high yield, identified as between cytochrome c_1 and the hinge protein (subunit VIII) on the basis of molecular weight considerations (Gutweniger et al., 1983). The effect of EDC on the subunit profile of complex III in the presence and absence of cytochrome *c* is shown in Figure 11. The cross-linked product first identified by Gutweniger et al. (1983) migrates between subunits II and III. The presence of cytochrome *c* bound to complex III reduced the extent of cross-linking between cytochrome c_1 and the hinge protein and induced the cross-linking of cytochrome c_1, hinge protein, and subunit X

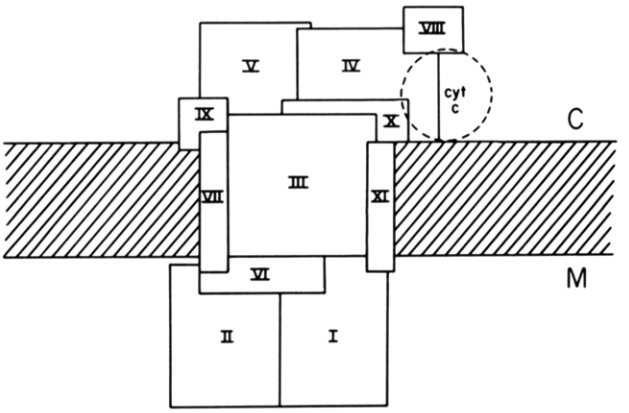

FIGURE 12: Schematic of the arrangement of polypeptides in the beef heart mitochondrial complex III.

to cytochrome *c* (Figure 11 and Table II).

Discussion

The topology of complex III from yeast and from beef heart has been studied extensively by chemical labeling, protease digestion, and cross-linking studies (Schneider & Racker, 1971; Case & Leigh, 1976; Mendel-Hartvig & Nelson, 1978; Bell et al., 1979; Beattie et al., 1981; Ohnishi & Salerno, 1982; D'Souza & Wilson, 1982). In most cases, these experiments were conducted before gel systems were available to resolve all of the component polypeptides of the complex and before the various subunits could be identified unambiguously. This last point is important because, until now, subunits have been identified by their relative migration on gels, which varies with different gel systems, as shown here.

In the present study a number of different approaches have been used to examine the topology of beef heart complex III. In some cases, previously established methods have been repeated when there was an opportunity to obtain more definitive data. Several new and important conclusions can be drawn

from the results presented here, leading to an updated model of the arrangement of subunits in complex III (Figure 12).

Complex III Is Made Up of 11 Different Subunits. There has been no consensus on the number of different subunits in mammalian complex III, and it has variously been reported that the enzyme is made up of 9, 10, and 11 different polypeptides [see Trumpower and Katki (1979)]. Our results indicate that beef heart complex III is composed of 11 different components as claimed by Schägger et al. (1986). These can be resolved completely in the gel buffers of Swank and Munkres (1971) and Schägger et al. (1986), provided the appropriate concentration of acrylamide is used (16%).

It has recently become possible to sequence the small amounts of protein used in $NaDodSO_4$–polyacrylamide gel electrophoresis by transferring the protein electrophoretically to a support [glass fiber paper or a hydrophobic poly(vinylidene difluoride) membrane] and by locating the protein with fluorescent staining or Coomassie blue (Aebersold et al., 1986; Matsudaira, 1987). We have used this procedure to obtain sequence data on all 12 bands resolved when complex III is electrophoresed under optimally resolving gel conditions. The use of PVDF membranes, as described by Matsudaira (1987), allows the sequencing of picomole quantities of electroblotted proteins, avoiding pretreatment of the membrane support. In our hands, this method gave low recovery of PTH derivatives of the positively charged amino acids: arginine, histidine, and lysine. Ten of the 12 bands were identified with previously published sequences for complex III subunits. Thus, band III is cytochrome *b* (Anderson et al., 1982), IV is cytochrome c_1 (Wakabayashi et al., 1980), V is the non-heme iron protein (Schägger et al., 1987), VI is the polypeptide called quinone binding protein (Wakabayashi et al., 1985), VIII is the hinge protein (Wakabayashi et al., 1982), and IX is the DCCD binding protein (Borchart et al., 1985).

Subunit VII ran as two bands on all the gels tested, as evidenced by sequencing data. This result is at variance with the report of Schägger et al. (1986), who also found that one component runs as a doublet but identified this as subunit VIII (hinge protein). Our conclusion that subunit VII has two electrophoretic forms comes not only from sequencing but is evident in labeling and protease digestion studies. Bands VIIA and VIIB are both labeled by [^{125}I]TID, while subunit VIII is not. Both bands VIIA and VIIB are cleaved by proteases, while subunit VIII is not. Upon cross-linking in the presence of EDC, subunit VIII disappears from the gel electrophoresis pattern, while bands VIIA and VIIB remain. Whether the anomalous behavior of band VII is due to the hydrophobic character of this subunit, its cysteine content, or a combination of properties is not clear.

Two subunits of the mammalian complex III remain to be sequenced, namely, the two core proteins. NH_2-terminal sequences obtained in this study show significant sequence homology with the core proteins from yeast as deduced from the DNA sequences of their genes (Tzagoloff & Crivellone, 1986; Oudshoorn et al., 1987).

Cytochrome b Is the Predominant Bilayer Intercalated Part of Complex III. A majority of the subunits of complex III are proposed to span the membrane on the basis of labeling with bilayer intercalated probes (Gutweniger et al., 1981) or by inference from experiments that show the same subunit labeled from both sides of the mitochondrial inner membrane (Gellerfors & Nelson, 1977; Bell et al., 1979f D'Souza & Wilson, 1982). The labeling studies with [^{125}I]TID reported here show that cytochrome *b* is the major transmembrane component of the complex. Photoactivation of [^{125}I]TID generates a short-lived but highly reactive carbene (Brunner & Semenza, 1981; Hoppe et al., 1984), more reactive than previously used arylnitrene reagents (Gutweniger et al., 1981). Cytochrome *b* is labeled by [^{125}I]TID at least 20-fold more heavily than any other subunit of the complex. Hydropathy plots of cytochrome *b* predict that this polypeptide spans the membrane a total of nine times (Widger et al., 1984; Saraste, 1984). The other subunits labeled to any significant extent by [^{125}I]TID are subunits II, VII, IX, X, and XI. Hydropathy plots of these subunits (except core protein II) show a single transmembrane region in each (calculations not shown). Two other subunits of complex III have putative transmembrane stretches, as can be seen from hydropathy profiles of their sequences: These are cytochrome c_1 and the non-heme iron protein. Neither of these polypeptides was significantly labeled by [^{125}I]TID.

The Non-Heme Iron Protein Appears To Be Peripherally Located. Functional studies place the non-heme iron protein on the cytoplasmic (C) side of the mitochondrial membrane and close to cytochrome c_1 [for a review, see Rich (1984)]. This localization is confirmed in our studies by protease digestion and cross-linking experiments. It is also suggested by the dissociation studies conducted by Li et al. (1981). From sequence data, Schägger et al. (1987) have proposed a structure for the non-heme iron protein in which the polypeptide is anchored into the membrane by two hydrophobic helices arranged as a hairpin spanning the bilayer twice. This model is not supported by our data. The non-heme iron protein is not labeled by [^{125}I]TID and is not labeled significantly by arylazidoPE, as would be expected if it made contact with the lipid head groups. Also it was not labeled in the experiments of Gutweniger et al. (1981) using photoreactive lipid analogues. Moreover, the non-heme iron protein can be cleaved by proteases at several sites within the predicted bilayer spanning sequences. We conclude that the non-heme iron protein is peripherally located, possibly bound to cytochrome *b* (at least partly) through hydrophobic interactions involving the NH_2-terminal region. It is important to note that the non-heme iron protein can be released from membranous complex III in *Neurospora crassa* by conditions as mild as bicarbonate washing (Hartl et al., 1986).

The Cytochrome c Binding Site Includes Cytochrome c_1, Hinge Protein, and Possibly Subunit X. The binding of cytochrome *c* to cytochrome c_1 has been established by kinetic studies (Ahmed et al., 1978; König et al., 1980), direct binding assays (Bosshard et al., 1979), cross-linking experiments (Erecinska et al., 1980; Broger et al., 1980), and labeling studies, in which it was shown that cytochrome *c* protects cytochrome c_1 from reaction with protein-modifying reagents (Gutweniger et al., 1983). This places cytochrome c_1 on the C side of the mitochondrial inner membrane.

The cross-linking data presented here show that the hinge protein and polypeptide X are both near neighbors of cytochrome c_1, each being linked to cytochrome c_1 by DSP reaction. A cross-linked product between cytochrome c_1 and subunit X is formed in high yield by reacting complex III with DCCD. Cytochrome c_1 (subunit IV), the hinge protein (subunit VIII), and subunit X are cross-linked to cytochrome *c* when a complex III–cytochrome *c* complex is reacted with the water-soluble carbodiimide EDC. The hinge protein and subunit X are highly negatively charged, suggesting that they may attract cytochrome *c* by electrostatic forces.

The Core Proteins and Subunit VI Are Located on the M Side of the Complex. Electron microscopy and image reconstruction studies of two-dimensional crystals of *Neurospora*

complex III reveal a protein complex approximately 150 Å long, spanning the mitochondrial inner membrane. The structure is asymmetrically arranged with most of the protein outside the bilayer on the matrix (M) side of the membrane (Wingfield et al., 1979; Leonard et al., 1981).

Our results indicate that the core proteins and subunit VI contribute the major part of this M domain. Core proteins I and II are cross-linked in high yield, with core protein II also cross-linked to subunit VI, indicating the close proximity of these components in the complex. Neither core protein was significantly labeled by [^{125}I]TID (consistent with the hydrophilic character of the known yeast sequences) or arylazidoPE. These labeling results indicate a mostly peripheral location of the beef heart mitochondrial core proteins rather than the transmembrane, mostly buried location, as claimed in some recent structural studies in the yeast mitochondrial system (Beattie et al., 1981; Sidhu & Beattie, 1982).

Protease digestion experiments place both core protein II and subunit IV (and hence by inference core protein I as well) on the M side of the complex. This localization of subunit VI to the M side agrees with the recent antibody binding studies of Japa et al. (1987) but is not consistent with [^{35}S]-DABS labeling results that had localized this polypeptide to the C side (Bell et al., 1979).

Link et al. (1987) have recently proposed a model, based on hydropathy plots, in which the amino-terminal portion of subunit VI is buried in the membrane. Our protease digestion experiments indicate to the contrary that the NH_2-terminus is exposed, being rapidly cleaved by thermolysin, even in the membrane-bound complex. Subunit VI could span the membrane, but this is unlikely given the very hydrophilic amino acid sequence of this polypeptide and its insignificant labeling by [^{125}I]TID. It may be that the labeling by [^{35}S]DABS attributed to subunit VI from the C side of the mitochondrial inner membrane (Bell et al., 1979) was instead labeling of subunit Cii-3 of complex II. This polypeptide of complex II, which copurifies with complex III in immunoprecipitation experiments, migrates very close to subunit VI and could comigrate with the complex II subunit when modified by [^{35}S]DABS.

The location of subunit VI on the M side has important functional implications if this is the quinone binding protein, as discussed by Japa et al. (1987). However, the assignment of beef heart subunit VI as a quinone binding protein is far from certain. Identification of the quinone binding protein involved the reaction of complex III with radioactive photoaffinity analogues of ubiquinone and then a determination of which components were labeled by these probes using $NaDodSO_4$–polyacrylamide gels (Yu & Yu, 1982; Yu et al., 1985). The gel system used in these experiments was the Weber and Osborn (1969) system, and as described above, band VI on these gels is not subunit VI in the nomenclature used here [or by Wakabayashi et al. (1985)] but is subunit VII, a component that is in the bilayer and closely associated with cytochrome *b*. Preliminary experiments using the quinone analogues synthesized by Yu et al. (1985) show that subunit VII and cytochromes *b* and c_1) are the most highly labeled sites for quinone binding (D. Gonzalez-Halphen and C. A. Yu, unpublished results).

In summary, the combined results from different biochemical approaches used in this study lead to a model of the beef heart complex III that fits well the low-resolution structure of *Neurospora* complex III determined by Wingfield et al. (1979). In particular, the locations of core proteins, cytochromes *b* and c_1, and the non-heme iron protein are those predicted by electron microscopy and image reconstruction of partial complexes (Weiss et al., 1986). The arrangement of subunits in our model, shown in Figure 12, is consistent with the fractionation studies of Schägger et al. (1986) in which the non-heme iron protein is released along with subunit XI; cytochrome c_1 purifies as a water-soluble complex with subunits VIII and X; the core proteins copurify along with subunit VII; and cytochrome *b*, subunit VI, and the subunit IX are fractionated as a subcomplex associated with lipid. The implication is that the associations retained during the fractionation scheme are not adventitious but are reflections of topological relationships in the intact complex.

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