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Topology of Subunits of the Mammalian Cytochrome *c* Oxidase: Relationship to the Assembly of the Enzyme Complex†

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ABSTRACT: The arrangement of three subunits of beef heart cytochrome *c* oxidase, subunits Va, VIa, and VIII, has been explored by chemical labeling and protease digestion studies. Subunit Va is an extrinsic protein located on the C side of the mitochondrial inner membrane. This subunit was found to label with *N*-(4-azido-2-nitrophenyl)-2-aminoethane[^{35}S]sulfonate and sodium methyl 4-[^{3}H]formylphenyl phosphate in reconstituted vesicles in which 90% of cytochrome *c* oxidase complexes were oriented with the C domain outermost. Subunit VIa was cleaved by trypsin both in these reconstituted vesicles and in submitochondrial particles, indicating a transmembrane orientation. The epitope for a monoclonal antibody (mAb) to subunit VIa was lost or destroyed when cleavage occurred in reconstituted vesicles. This epitope was localized to the C-terminal part of the subunit by antibody binding to a fusion protein consisting of glutathione *S*-transferase (G-ST) and the C-terminal amino acids 55–85 of subunit VIa. No antibody binding was obtained with a fusion protein containing G-ST and the N-terminal amino acids 1–55. The mAb reaction orients subunit VIa with its C-terminus in the C domain. Subunit VIII was cleaved by trypsin in submitochondrial particles but not in reconstituted vesicles. N-Terminal sequencing of the subunit VIII cleavage product from submitochondrial particles gave the same sequence as the untreated subunit, i.e., ITA, indicating that it is the C-terminus which is cleaved from the M side. Subunits Va and VIII each contain N-terminal extensions or leader sequences in the precursor polypeptides; subunit VIa is made without an N-terminal extension.

The mammalian cytochrome *c* oxidase is a multisubunit enzyme made up of 13 different subunits, each of which has been fully sequenced [e.g., see Anderson et al. (1981), Meinecke et al. (1984), and Meinecke and Buse (1985)] and for many of which the genes have been cloned and characterized [reviewed in Cao et al. (1988), Lomax and Grossman (1989),

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and Capaldi (1990a)]. Approximately half of the mass of the enzyme complex forms a large (C) domain extending from the outer (cytoplasmic) face of the mitochondrial inner membrane (Henderson et al., 1977; Deatherage et al., 1982; Valpuesta et al., 1990). Around 40% of the protein is buried inside the lipid bilayer with a small part of the complex (10%) extending into the matrix space (M_1 and M_2 domains) (Henderson et al., 1977; Fuller et al., 1979).

Ongoing studies in this laboratory are directed at determining the arrangement of subunits in the enzyme. Subunit II has been examined in detail and found to span the membrane twice with both N- and C-termini in the C domain [reviewed in Capaldi (1990b)]. There is good evidence that the C-terminal half of subunit II contributes the cytochrome *c* binding site (Millett et al., 1982, 1983; Bisson et al., 1978, 1983) and this subunit probably contains Cu_A (Steffens & Buse, 1979). Subunit I appears to contain the other prosthetic groups, heme *a*, heme a_3, and Cu_{A3} (Holm et al., 1987). This large polypeptide has been proposed to span the membrane 12 times (Holm et al., 1987). No direct experimental data on the topology of subunit I are available. Subunit III, another relatively hydrophobic subunit, has been proposed to span the membrane 7 times (Capaldi et al., 1983; Holm et al., 1987). A segment of this polypeptide around Cys-112 has been localized to the C domain (Malatesta & Capaldi, 1982).

We have recently determined the arrangements of subunits IV, VIb, and VIc by protease digestion studies of submitochondrial particles in which the M side is available for proteolytic attack, and in reconstituted vesicles in which as many as 95% of the enzyme molecules are oriented C domain out (Zhang et al., 1988). Subunits IV and VIc were concluded to be transmembranous, with their N-termini in the matrix. Subunit VIb was concluded to be a peripheral subunit, located in the C domain. We have now studied the topology of three additional subunits of cytochrome *c* oxidase, Va, VIa, and VIII. The arrangement of all of these subunits is discussed in relation to the targeting and sorting of subunits to and within the mitochondrion.

Experimental Procedures

Enzyme Purification. Bovine heart cytochrome *c* oxidase was purified according to the method of Capaldi and Hayashi (1972) and stored in small aliquots at −70 °C until use. Bovine heart submitochondrial particles were isolated by the procedure of Lee and Ernster (1967). Reconstituted vesicles of cytochrome *c* oxidase were made as described by Zhang et al. (1984). Bovine brain cytochrome *c* oxidase was isolated by the published procedure of Yanamura et al. (1988).

Protease Digestion and $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Cytochrome *c* oxidase (1 mg/mL), dissolved in 0.1% Triton X-100 and 50 mM potassium phosphate (pH 8.2), was incubated with trypsin (TPCK-treated, type XIII, Sigma) or proteinase K at a ratio of 1:40 (w/w) at room temperature. The proteolytic reaction was stopped by addition of 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma) at the desired time (see figure legends). Cleavage of membranous cytochrome *c* oxidase (submitochondrial particles or reconstituted vesicles) was conducted as described by Zhang et al. (1988).

Polyacrylamide gel electrophoresis in $NaDodSO_4$ was carried out according to Zhang et al. (1988). The isoelectrofocusing gel and two-dimensional gel electrophoresis were performed as described by Merli et al. (1979).

Immunodetection of Proteolytic Products. Protein samples, resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis, were electroeluted onto nitrocellulose membranes following the precedure of Towbin et al. (1979). Protein binding sites on the membrane were saturated with 5% nonfat dry milk (Carnation). The nitrocellulose membrane was then incubated with subunit-specific polyclonal antibodies raised in rabbits or monoclonal antibodies raised in mice, and the immunoreactive polypeptides were detected by the use of alkaline phosphatase conjugated goat anti-rabbit or goat anti-mouse antibodies (Bio-Rad) according to Hawkes et al. (1982).

Electroblotting of Electrophoretically Resolved Protein Samples onto a PVDF Membrane. Electroblotting was conducted according to Matsudaira (1987) as modified by Gonzalez-Halphen et al. (1988). N-Terminal sequence analysis was performed on a gas-phase protein sequencer (Applied Biosystems Model 470A) equipped with an on-line PTH analyzer (Applied Biosystems Model 120A).

Covalent Labeling of Detergent-Solubilized and Membranous Cytochrome c Oxidase with Water-Soluble Reagents. [^{35}S]NAP-taurine (12 mCi/mol) was synthesized from [^{35}S]taurine (Amersham) according to the method of Tinberg et al. (1974). Isolated cytochrome *c* oxidase (1 mg/mL) in 0.1% Tween 20, 100 mM NaCl, and 50 mM sodium phosphate (pH 7.5) was incubated with 1.3 mM [^{35}S]NAP-taurine for 10 min in the dark on ice. The sample was then illuminated with high-intensity white light (filtered through a 3-cm-thick 2% $CuSO_4$ solution) for 20 min at 4 °C. Reconstituted vesicles of cytochrome *c* oxidase prepared according to Zhang et al. (1984) were suspended in 100 mM NaCl/50 mM sodium phosphate (pH 7.5) and reacted with NAP-taurine as above. The labeled cytochrome *c* oxidase was then separated from free [^{35}S]NAP-taurine (and from phospholipids, in the case of labeling of reconstituted vesicles) by sucrose gradient centrifugation prior to $NaDodSO_4$–polyacrylamide gel electrophoresis and isoelectrofocusing, as described by Zhang et al. (1984).

Sodium methyl 4-[^{3}H]formylphenyl phosphate (MFPP) (5.5 mCi/mmol) was synthesized according to the procedure of McMillen et al. (1986). MFPP was placed in a glass vial and solvent removed under nitrogen for 15 min. Purified cytochrome *c* oxidase and reconstituted oxidase vesicles, respectively, were added at 1 mg/mL. The mixture was equilibrated for 30 min at 4 °C with occasional vortexing. Sodium cyanoborohydride was added in three portions over 1.5 h to a final concentration of 100 mM. The reactions were quenched by the addition of 0.5 M glycine. Sucrose gradient centrifugation was used to collect labeled enzyme as described above.

Chemical Cross-Linking of Detergent-Dispersed Cytochrome c Oxidase. Cross-linking of cytochrome *c* oxidase dissolved in 0.1% potassium cholate/50 mM sodium phosphate, pH 7.4, by dithiobis(succinimidyl propionate) (DSP) was conducted as described by Briggs and Capaldi (1977). Unmodified subunits and cross-linked products were resolved by two-dimensional polyacrylamide gel electrophoresis using the conditions of Fuller et al. (1981) in both dimensions but without β-mercaptoethanol in the sample buffer of the first-dimensional electrophoresis.

Overexpression of the N- and C-Terminal Peptides of Subunit VIa in Escherichia coli. The glutathione *S*-transferase gene fusion system (Pharmacia LKB Biotech.) was employed for overexpression in *E. coli* of N- and *C*-terminal portions of bovine cytochrome *c* oxidase subunit VIa. With this system, the VIa-derived polypeptides were expressed as C-terminal extensions of the 26-kDa protein glutathione *S*-transferase (G-ST) from *Schistosoma japonicum* (Smith et al., 1986). The appropriate gene fusions were constructed in the pBR322-based plasmid pGEX-3X. Transcription (and hence

translation) of the fusion gene in *E. coli* is under control of the isopropyl β-D thiogalactoside (IPTG)-inducible *tac* promoter.

cDNA fragments corresponding to the bovine cytochrome *c* oxidase subunit VIa heart isoform were amplified by reverse transcription (AMV-reverse transcriptase) and polymerase chain reaction (PCR) from 5 μg of total RNA isolated from beef heart. The oligonucleotides used for this purpose were derived from sequence data kindly provided before publication by M. Lomax (University of Michigan). A 282 bp fragment of the subunit VIa gene was produced encompassing the entire coding region for the mature subunit. An internal *Bam*HI site in this cDNA was used to create two subclones encoding the N-terminal (amino acids 1–55) and C-terminal (amino acids 55–85) portions. The products of the PCR reaction were phosphorylated with T4 polynucleotide kinase (PNK), digested with *Bam*HI, and then ligated to *Bam*HI- and *Sma*I-treated vector pGEX-3X. After transformation of *E. coli* MC1061 and selection on ampicillin, clones were isolated which contained plasmids with the desired 5′- or 3′-terminal fragments of subunit VIa fused to the 3′ end of the glutathione *S*-transferase gene. Dideoxy sequencing was performed to confirm the orientation and reading frame of the inserts.

For induction of the G-ST COX VIa fusion proteins, 2 mL of LB/ampicillin (50 μg/mL) containing medium was inoculated with 20 μL of an overnight culture of *E. coli* containing the appropriate plasmid and grown for 1 h at 37 °C before IPTG (1 mM final concentration) was added and growth continued for a further 3–4 h. Noninduced cultures were grown simultaneously as controls. After induction, 50-μL samples of the culture were taken, dissociated in 5% SDS, and electrophoresed on a 12% acrylamide gel. Gels were either stained with Coomassie Blue or transferred to a PVDF membrane for immunodetection.

Results

Resolution of Subunits Va and Vb in Isoelectric Focusing Gels. The highly resolving polyacrylamide gel electrophoresis conditions of Kadenbach and colleagues (Kadenbach & Merle, 1981; Kadenbach et al., 1986) have provided new insight into the subunit structure of cytochrome *c* oxidase. Also they have facilitated topology studies by improving the separation of polypeptides for detection of labeling by various membrane-impermeant and bilayer-intercalated reagents. However, the gel system does not efficiently resolve subunits Va and Vb. Thus, in our previous studies, we were unable to determine whether Va, Vb, or both subunits were reactive to diazobenzenesulfonate or *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate (NAP-taurine) when these reagents were used to label cytochrome *c* oxidase in reconstituted vesicles (Zhang et al., 1984). Figure 1 shows the resolution of Va from Vb produced by a gel electrophoresis method in which cytochrome *c* oxidase is denatured in $NaDodSO_4$/urea, diluted with a large excess of Triton X-100, and then subjected to isoelectric focusing using a procedure described in Merli et al. (1979). Subunits separated by isoelectric focusing are then resolved and identified by second-dimension electrophoresis in $NaDodSO_4$. Three subunits, II, Va, and Vb, were well focused in this two-dimensional gel system. The identity of Va and Vb on the gel was determined by electrotransferring polypeptides to Immobilon and then N-terminal sequencing. Subunit Va runs to a more acid pH than Vb, as expected from the amino acid compositions of the two proteins. Subunit Vb often ran as two spots, as in Figure 1. Both spots had the same N-terminal sequence, and both reacted with a monoclonal antibody to Vb (see later). The minor spot may represent a small amount of subunit Vb in which Cys residues had not been fully reduced.

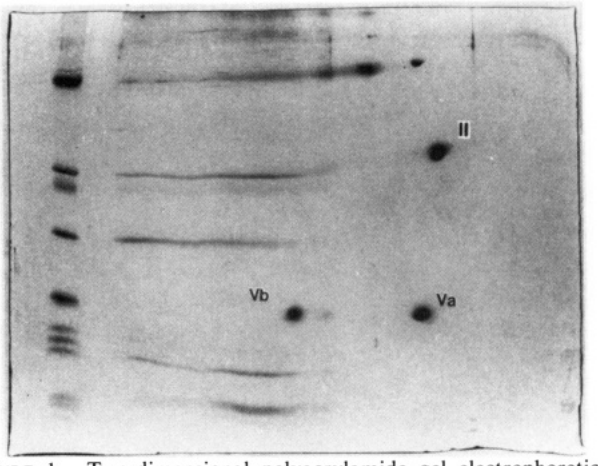

FIGURE 1: Two-dimensional polyacrylamide gel electrophoretic separation of subunits of beef heart cytochrome *c* oxidase using the procedure of Merli et al. (1979). The subunit separation on the left side shows the migration of subunits of the enzyme in the second dimension only. The positions of II, Va, and Vb are labeled.

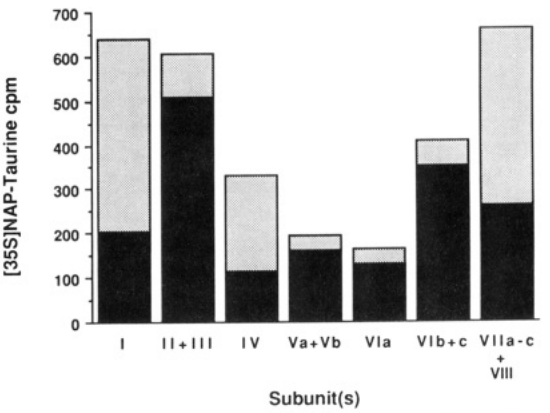

FIGURE 2: Labeling of subunits of membrane-bound cytochrome *c* oxidase by [^{35}S]NAP-taurine. Labeled subunits were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis and the counts incorporated into each determined by slicing the gel in 1-mm slices and counting as described in Zhang et al. (1984). Counts in subunits II + III, Va + Vb, VIb + c, and VIIa,b,c + VIII were combined, respectively, as these subunits were not resolved completely. The black part of each bar gives the number of counts in subunit(s) in intact reconstituted vesicles; the gray part is the number of counts incorporated when the vesicles were disrupted with Triton X-100 prior to being labeled (see Experimental Procedures).

Disposition of Subunit Va in the Cytochrome c Oxidase Complex. In order to determine the orientation of subunit Va, cytochrome *c* oxidase in reconstituted vesicles was labeled with the membrane-impermeant reagent [^{35}S]NAP-taurine, and subunits were separated in one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis, as well as by the two-dimensional gel system described above. In these reconstituted vesicles, 90% of the cytochrome *c* oxidase molecules were oriented with their C domain outside, based on protease digestion of subunit IV and on heme reduction experiments [see also Zhang et al. (1988)]. Figure 2 shows that the amount of radioactivity incorporated into individual subunits in a typical experiment where intact vesicles (solid) and vesicles made leaky by addition of 1% Triton X-100 (shaded) were reacted with the reagent. Subunits Va + Vb (not resolved) were labeled almost as heavily in intact vesicles as after disruption of the vesicles with detergent. Figure 3B shows a histogram of the radioactivity in slices cut through a horizontal

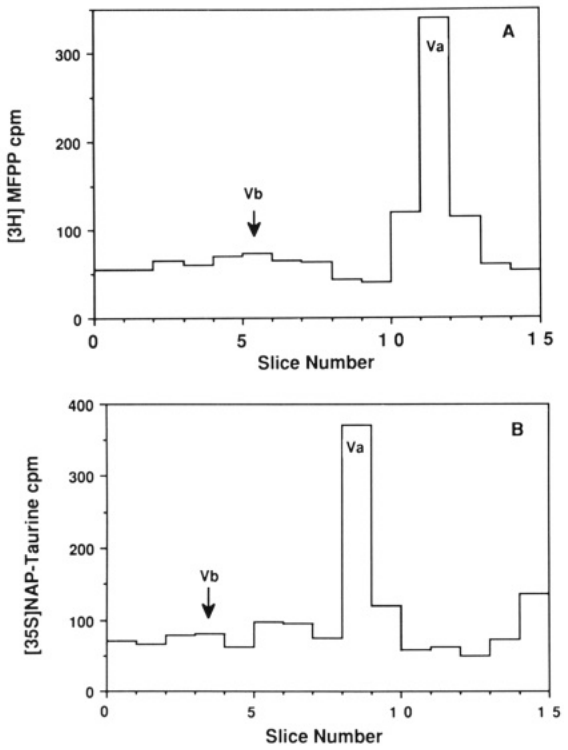

FIGURE 3: Labeling of subunits Va and Vb by membrane-impermeant reagents. (A) Labeling of subunits Va and Vb by [^{3}H]MFPP and (B) labeling of these subunits with [^{35}S]NAP-taurine. In each experiment, reconstituted vesicles of cytochrome *c* oxidase were reacted with reagent, subunits were resolved by two-dimensional electrophoresis, and a horizontal section of the gel containing subunits Va and Vb was excised and then sliced into 15 segments which were then dissolved and counted for radioactivity.

section of a two-dimensional gel of [^{35}S]NAP-taurine-labeled vesicles in a companion experiment to that in Figure 2. The reagent was incorporated into subunits II (not shown) and Va, but there was no labeling of Vb. In detergent-disrupted vesicles, subunits II and Va were labeled, but again there was no radioactivity incorporated into subunit Vb. When detergent-dissolved cytochrome *c* oxidase was reacted with [^{35}S]-NAP-taurine, there was heavy labeling of Va but no labeling of Vb (results not shown). A set of experiments were also conducted with a second membrane-impermeant reagent methyl 4-[^{3}H]formylphenyl phosphate (MFPP), which reacts to form a Schiff base with lysine residues in the presence of borohydride (McMillen et al., 1986). Analysis of enzyme labeled with MFPP in reconstituted vesicles and in detergent-dispersed form showed similar results to those with NAP-taurine: subunit Va was labeled in intact vesicles (Figure 3A), detergent disruption of these vesicles gave only a small amount of additional labeling of this subunit, while Vb was not labeled even in detergent-solubilized cytochrome *c* oxidase (result not shown). These labeling data indicate that subunit Va is located in the C domain of the oxidase complex. The locus of subunit Vb cannot be obtained from labeling experiments because of lack of reactivity with the reagents used.

Analysis of the cross-linking of detergent-solubilized cytochrome *c* oxidase by DSP is shown in Figure 4. Two major cross-linked products were found to contain Va or Vb, as reported previously (Briggs & Capaldi, 1977, 1978), one involving subunit I, the second subunit II. We have recently obtained monoclonal antibodies specific to subunits Va and Vb, respectively, and these were used to identify the partners in cross-links with the mitochondrially coded subunits. The mAb to subunit Va reacts with the off-diagonal spots (Figure 4C) but not that to Vb (Figure 4B), indicating that the

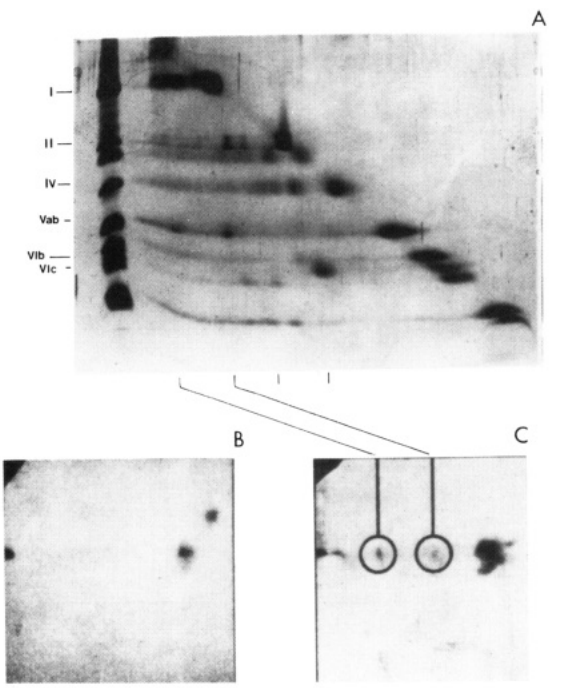

FIGURE 4: Cross-linking of beef heart cytochrome *c* oxidase by DSP. Detergent-dissolved enzyme was reacted with DSP, and then cross-linked products were identified by two-dimensional gel electrophoresis (panel A). Companion gels to the one stained with Coomassie brilliant blue were electrotransferred onto Immobilon, which reacted with mAbs to subunits Vb (panel B) and Va (panel C).

cross-linked products are I + Va and II + Va. Subunit II is predominantly located in the C domain of cytochrome *c* oxidase, supporting a placement of subunit Va to the C side of the inner membrane.

Orientation of Subunit VIa. In recent studies, we found that subunit VIa was cleaved by trypsin both when cytochrome *c* oxidase was reconstituted into vesicles (C side out) and in submitochondrial particles (M side out) (Zhang et al., 1988). These results indicate that subunit VIa spans the membrane, as predicted from features of its sequence and from labeling of this subunit by 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID) (Zhang & Capaldi, 1988), but they do not define the orientation of subunit VIa.

We have obtained a mAb specific for VIa [heart isoform: see Yanamura et al. (1988) for a discussion of isoforms of subunits of beef cytochrome *c* oxidase]. This mAb was used to follow subunit VIa cleavage by trypsin in intact vesicles, in submitochondrial particles, and in detergent-dispersed enzyme (Figure 5). The mAb identified two steps in the cleavage of VIa in reconstituted vesicles (Figure 5C, left lanes); first, there is a product migrating at the position of subunit VIc, and this is followed by a second cleavage in which the epitope for the mAb is lost. In submitochondrial particles, VIa is cleaved to a slightly smaller polypeptide, and then there is no further cleavage (Figure 5C, right lanes). As expected in detergent-dispersed enzyme (Figure 5A), where cleavage from both ends can take place, mAb binding to the subunit is rapidly lost (Figure 5B).

Molecular biological approaches were used to identify the epitope for the mAb and hence the part of subunit VIa exposed at the C side. Fusion genes of glutathione *S*-transferase and cDNA sequences corresponding to the N-terminal part (residues 1–55) or C-terminal part (residues 55–85) of subunit VIa were constructed in plasmid pGEX-3X and expressed in *E. coli* under control of the *tac* promoter. Figure 6 shows a Western analysis of total protein from cell lysates of *E. coli*

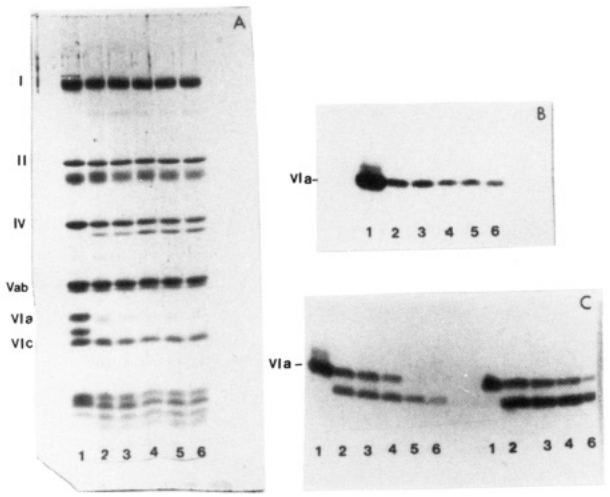

FIGURE 5: Cleavage of subunit VIa with trypsin in detergent-dispersed (panel A and B) and membrane-bound (panel C) cytochrome *c* oxidase. Panel A shows $NaDodSO_4$–polyacrylamide gel analysis of the time course of cleavage of cytochrome *c* oxidase with trypsin (1:50 w/w) stained with Coomassie brilliant blue. Panel B is an immunoblot of a companion gel to that in panel A that was incubated with a mAb specific to subunit VIa. Lanes 1–6 are control untreated enzyme and 5-, 15-, 30-, 60-, and 120-min treated samples, respectively. Panel C shows an immunoblot of the cleavage of subunit VIa by trypsin in reconstituted vesicles (left part) and in submitochondrial particles (right part). Samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis; gels were transferred to Immobilon which was probed with the mAb to VIa. Lanes 1–6 are control and 15-, 30-, 45-, 60-, and 120-min samples respectively.

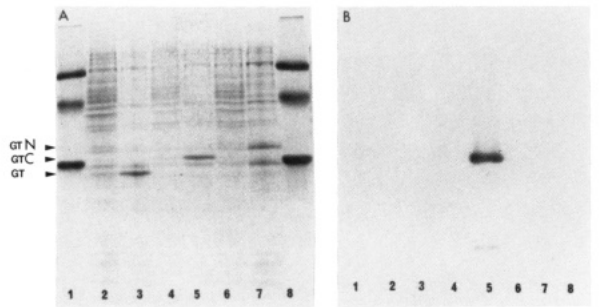

FIGURE 6: Expression of glutathione *S*-transferase–subunit VIa fusion proteins in *E. coli* and identification of the mAb binding domain. (Panel A) $NaDodSO_4$–polyacrylamide gel electrophoretic separation of polypeptides stained with Coomassie brilliant blue. (Panel B) Western blotting of samples in which polypeptides were separated in a companion gel to that in Panel A, electrotransferred in nitrocellulose, and then reacted with the subunit VIa mAb. Lanes 1 and 8, molecular weight markers. Lanes 2 and 3, whole cell lysate of *E. coli* containing pGEX-3X; lane 2, noninduced, lane 3, induced. Lanes 4 and 5, whole cell lysate with plasmid pGEX-3X containing subunit VIa C-terminal fragment; lane, 4 noninduced, lane 5, induced. Lanes 6 and 7, whole cell lysate with plasmid pGEX-3X containing subunit VIa N-terminal fragment; lane 6, noninduced, lane 7, induced.

containing these fusion genes with their expression either induced with IPTG or not induced. The mAb was found to react exclusively with the fusion protein containing the C-terminal part of VIa.

Orientation of Subunit VIII. The proteinase K cleavage of subunit VIII was followed in detergent-dissolved enzyme, in reconstituted vesicles, and in submitochondrial particles (Figure 7). This polypeptide was cleaved rapidly in detergent-solubilized cytochrome *c* oxidase to a product approximately 500 daltons smaller (result not shown). The same sized cleavage product was obtained by proteinase K treatment of submitochondrial particles. This fragment of subunit VIII was identified by Western blotting with a subunit-specific antibody

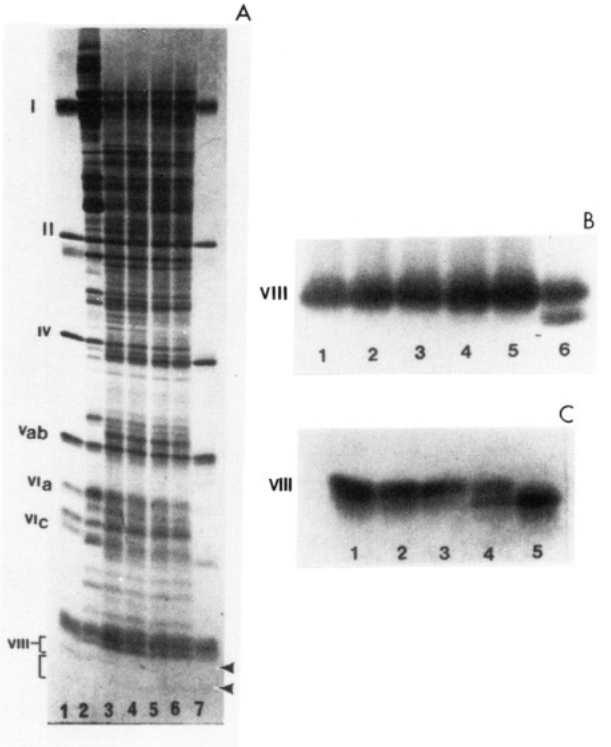

FIGURE 7: Proteinase K cleavage of subunit VIII of cytochrome *c* oxidase. (Panel A) $NaDodSO_4$–polyacrylamide gel of samples of submitochondrial particles which were reacted with proteinase K for different lengths of time. Lane 1, control untreated bovine heart cytochrome *c* oxidase. Lane 7, detergent-solubilized enzyme treated with proteinase K for 2 h. Lanes 2–6, membranes incubated with trypsin for 15, 30, 60, 90, and 120 min, respectively. (Panel B) Western blot of reconstituted vesicles of cytochrome *c* oxidase treated with trypsin for 0, 15, 30, 60, and 120 min in lanes 1–5, respectively. Lane 6 shows a control of detergent-solubilized enzyme after treatment with proteinase K for 30 min. Samples were run on a $NaDodSO_4$–polyacrylamide gel, transferred to Immobilon, and reacted with a polyclonal antibody to VIII. (Panel C) Western blot following the cleavage of subunit VIII in submitochondrial particles. Lanes 1–4 are for 0-, 30-, 60-, and 120-min proteolysis, respectively. Lane 5 shows detergent-solubilized enzyme incubated with proteinase K for 120 min.

(Figure 7C) and could be seen in Coomassie blue stained gels (Figure 7A) because, apparently, subunit VIII is the smallest polypeptide of the mitochondrial inner membrane. There was no cleavage of subunit VIII in reconstituted vesicles (Figure 7B).

The major cleavage product of subunit VIII was obtained by electrotransfer onto Immobilon from detergent-solubilized enzyme and from submitochondrial particles treated for 2 h with proteinase K to ensure complete cleavage of this polypeptide. Care was taken to excise a region of the gel containing cleavage product and excluding any uncleaved subunit VIII. Amino acid sequencing gave the same N-terminal sequence, ITAK..., as the mature, uncleaved polypeptide. Thus, subunit VIII is cleaved in submitochondrial particles from the C-terminus.

Topology of Cytochrome c Oxidase Subunits in Relation to the Precursor Sequences. The orientation of subunits Va, VIa, and VIII in the cytochrome *c* oxidase complex based on the data reported here is shown in Figure 8. The sequences of these three subunits, as well as of all of the other nuclear-coded subunits, have been obtained by cloning and sequencing of cDNAs for these polypeptides (e.g., see references in Table I). Most but not all are made as precursor proteins with an N-terminal extension or leader peptide. The N-terminal sequences of the precursors for subunits where the orientation is known are given in Table I with cleavable leader

Table I: N-Terminal Sequences of Precursors of Cytochrome *c* Oxidase Subunits

	C-Side Subunits
Va, human (Rizzuto et al., 1988)	MLGAALRRCAVATTRADRRGLLHSARTPGPAVAIQSVRCY-
Va, rat (Droste et al., 1989)	MLAAALRRC---TAAAAARGLLHPVSAPSPAAAVCSIRCY
VIb, human (Taanman et al., 1989)	M-aedmetkiknykta
VIb, beef (Lightowlers & Capaldi, 1989)	M-aediqakiknyqta
	Transmembrane Subunits
IV, human (Zeviani et al., 1987)	MLATRVFSLVGKRAISTSVCVR
IV, rat (Gopdan et al., 1989)	MLATRALSLIGKRAISTSVCLR
IV, beef (Lomax et al., 1984)	MLATRVFSLIGRRAISTSVCVR
VIa, human L (Fabrizi et al., 1989)	M-ssgahgeegsar
VIa, rat H (Schlerf et al., 1988)	M-asaskedhggag
VIc, human (Otsuka et al., 1988)	M-apevlpkprmrgllarrlr
VIc, rat (Suske et al., 1987)	M ssgallpkpqmrgllakrlr
VIIb, beef (Lightowlers et al., 1989)	(MFNLR)MFPLAKNALSRLRVQSIQQAVQAVARQ
VIII, human (Rizzuto et al., 1989)	MSVLTPLLLRGLTGSARRLPVPRAK-
VIII, beef L (Lightowlers et al., 1990)	MSVLTPLLLRGLTGPARRLPVPRAQ-
VIII, beef H (Lightowlers et al., 1990)	MLRLAPT-VRLLQAPLRGWVVPKAH-

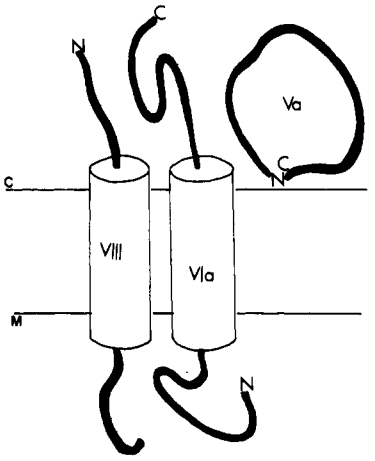

FIGURE 8: Schematic showing the arrangement of subunits Va, VIa, and VIII in the mitochondrial inner membrane.

sequences shown in capital letters and mature sequences given in lower case letters.

Discussion

The results presented here extend our picture of the arrangement of subunits in cytochrome *c* oxidase. We find that subunit Va is located on the C side of the mitochondrial inner membrane. This contrasts with previous reports that have placed subunit Va on the M side (Freedman & Chan, 1983; Freedman et al., 1988; Jarausch & Kadenbach, 1985). Freedman and colleagues used polyclonal antibody prepared against subunit Va that had been isolated by isoelectric focusing to localize this subunit in immunological experiments (Freedman & Chan, 1983; Freedman et al., 1988). They found that their antibody binds to submitochondrial particles when oxidase was in a reduced form (but not if the enzyme was oxidized). Their antibody did not react with mitoplasts (right side out inner membrane preparations where the C side is outermost). The difficulty in using polyclonal antibodies is the lack of clear definition of reactivity. Thus, the results of Freedman et al. (1986) could be explained if their antibody contains immunoglobulins reacting to Va in Western analysis which do not react with membrane-bound oxidase, and *also* immunoglobulins to contaminating subunits of oxidase (present in the antigen) which fail to react with denatured oxidase in Western analysis but bind to the native enzyme in membranes.

Jarausch and Kadenbach (1985b) reported cleavage of subunit Va by protease treatment of submitochondrial particles. Cleavage was only partial, and the experiments appear to have been conducted with membrane preparations of scrambled orientation.

We find that subunit Va is labeled by membrane-impermeant chemical-modifying reagents in the C domain. This polypeptide is very hydrophilic, without the long stretches of hydrophobic residues that characterize the majority of transmembrane polypeptides [see Capaldi (1990a)]. Moreover, this subunit is not labeled by any of the bilayer-intercalated reagents that have been used to date (Bisson et al., 1979; Georgevich & Capaldi, 1982; Zhang & Capaldi, 1988), making a transmembrane arrangement unlikely. Subunit Va is cross-linked to subunit II in relatively high yield by DSP, a reagent that reacts with lysine residues (Briggs & Capaldi, 1977, 1978; Jarausch & Kadenbach, 1985a). Most of the mass of subunit II including seven of the eight lysines in the polypeptide are on the C domain of cytochrome *c* oxidase (Bisson et al., 1982a,b).

A transmembrane arrangement of subunit VIa is consistent with protease digestion studies and labeling with membrane-intercalated reagents (Zhang et al., 1988; this study; Zhang & Capaldi, 1988). Subunit VIa has an amino acid sequence typical of many transmembrane polypeptides, containing one stretch of predominantly hydrophobic amino acids long enough to span the bilayer in an α-helical structure (Meinecke & Buse, 1985), a feature conserved in VIa from different species as well as in different isoforms of this subunit (Schlerf et al., 1988). Our results place the C-terminus of subunit VIa on the C side, which would put the N-terminus on the M side.

Subunit VIII also has all the features of a transmembrane polypeptide. It has one segment of predominantly hydrophobic amino acids which is conserved in different species of both the liver and heart isoforms (Meinecke et al., 1984; Lightowlers et al., 1990). The C-terminus of subunit VIII is on the M side, which would put the N-terminus on the C side. Alternative, but less likely, arrangements of this subunit are ones with both the N- and C-termini on the M side and with the hydrophobic segment either parallel to the bilayer or spanning the membrane twice in a β structure.

There is as yet no clear role for any of the nuclear-coded subunits in cytochrome *c* oxidase activity, and therefore it is premature to discuss the functional significance of the topology data. However, one point of functional interest is the location of regions of structural difference between isoforms of cytochrome *c* oxidase. It is now established that there are at least two isoenzyme forms of mammalian cytochrome *c* oxidase, a heart form found in heart and skeletal muscle and a liver form found in liver, kidney and brain (Kadenbach, 1986; Yanamura et al., 1988; Schlerf et al., 1988; Lightowlers et

al., 1990; Lomax & Grossman, 1989). These forms differ in one or more of subunits VIa, VIIa, and VIII depending upon the species. The orientation of subunits VIa and VIII based on our labeling data is such that there are isoform differences in both C and M domains, with the C domain having a net charge difference of −5 and −2 contributed by VIa and VIII, respectively, in liver compared to heart. In the M domain, there is no net charge difference between the two forms of the enzyme. The significance of these structural differences remains to be worked out.

The topology data are most useful at present in considering the mechanism of assembly of cytochrome *c* oxidase. This multisubunit enzyme is made up of 3 mitochondrially coded subunits and 10 nuclear-coded subunits in mammals [reviewed in Capaldi (1990b) and Hartl et al. (1989)]. The nuclear-coded subunits are synthesized in the cytoplasm as precursors which are targeted to mitochondria and are then incorported into the oxidase complex. It is thought that the information targeting mitochondrial proteins to the organelle is encoded in the sequence of the precursors of these proteins, often within a N-terminal extension or leader sequence [see Hartl et al. (1989)]. The precursors of the cytochrome *c* oxidase subunits must sort to the correct compartment, i.e., intracristal space, inner membrane, or matrix space, in order to attain the proper topology and form an active complex. The information for this intermitochondrial sorting is also thought to be encoded in primary sequences of the precursor polypeptides [reviewed in Hartl et al. (1989)]. The N-terminal sequences of the precursors of subunits IV, Va, VIa, VIb, VIc, VIIb, and VIII, subunits whose arrangement in the oxidase complex are known, are listed in Table I but divided in two classes: subunits localized exclusively on the C side (intracristal space) and transmembrane subunits (inner membrane). The precursor of subunit Va, one of the two subunits directed to the intracristal space, has been reported to include an N-terminal leader of 27 amino acids (Rizzuto et al., 1988; Droste et al., 1989). This leader sequence has features similar to the leader peptides of proteins destined for the matrix space in that it has several positively charged residues and a number of serines and threonines (Hartl et al., 1989; Schatz & Butow, 1983); it is different from most proteins directed to the matrix in that the human form has a glutamate in the N-terminal extension. Note that the Glu residue is not conserved in rat (Table I).

We have recently cloned and sequenced a cDNA for beef cytochrome *c* oxidase subunit VIb (Lightowlers & Capaldi, 1989): a cDNA for the human protein has also been cloned (Taanman et al., 1989). These contain a Met immediately N-terminal to the sequence of the mature polypeptide but do not extend far enough 5′ to the Met to decide unequivocally whether the precursor of subunit VIb has a N-terminal extension. A clue that subunit VIb does not have a leader peptide is the fact that the mature polypeptide is N-acetylated (Steffens et al., 1979). Subunit VIb may be taken up into mitochondria and localized to the C side of the inner membrane by a similar mechanism to cytochrome *c* (Hartl et al., 1989).

The transmembrane subunits of cytochrome *c* oxidase, IV, VIa, VIc, VIIb, and VIII, show a variety of precursor structures that emphasizes the variability in the targeting information at the primary sequence level. Subunits IV and VIIb have classical precursor structures in that they each contain an N-terminal extension with positively charged residues, hydroxyl-containing amino acids, and no negative charges (Schatz & Butow, 1983; Hartl et al., 1989). With both of these subunits, the N-termini became localized to the matrix side. The leader sequence of subunit IV in mammals has features typical of a precursor that is processed in two steps, i.e., there is an Arg at position −10, a hydrophobic residue at −8, and a hydroxyl-containing residue (Ser) at −5 (Hendrick et al., 1989). The analogous subunit in yeast (subunit V) does not have these features (Poyton et al., 1988). Subunit VIc is without an N-terminal extension. The N-terminus of the mature subunit in rat has only positive charges (Suske et al., 1987) and could both target this polypeptide to the mitochondrion and sort the N-terminus to the matrix. The N-terminus of the mature subunit VIc in humans is also highly positively charged, but the published sequence contains a Glu at residue 3. The N-terminus of subunit VIa is on the M side in the same way as the subunits described above, the precursor of this subunit is without an N-terminal extension, and both the heart and liver forms of VIa have negatively charged residues close to the N-terminus in the mature polypeptide (Schlerf et al., 1988; Fabrizi et al., 1989). Subunit VIII is another anomaly if it spans the membranes as we predict from our structural studies. The precursor of this subunit has an N-terminal extension with all of the same features as those polypeptides where the N-terminus resides in the matrix (Lightowlers et al., 1990), and yet the protein appears to be oriented with its N-terminus on the C side. Subunit VIII could be targeted to the matrix space and then the N-terminus returned to the intracristal space by the conservative pathway proposed for localizing cytochrome c_1 and the non-heme iron protein of complex III. Both of these proteins are processed twice to completely remove the N-terminal extension, once in the matrix and a second time in the intracristal space (Hartl et al., 1989). The leader sequence of subunit VIII, however, does not show features typical of precursors processed to the mature subunit in two steps.

Registry No. Cytochrome *c* oxidase, 9001-16-5.

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