Orientation of the Cytoplasmically Made Subunits of Beef Heart Cytochrome *c* Oxidase Determined by Protease Digestion and Antibody Binding Experiments†

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ABSTRACT: The topology of several of the cytoplasmically made subunits of beef heart cytochrome *c* oxidase has been determined by protease digestion of oriented membrane preparations, using subunit-specific antibodies to identify cleavage products. Reconstituted vesicles of cytochrome *c* oxidase and asolectin were used as a vesicle preparation with the C domain of the enzyme available for protease digestion. Submitochondrial particles were used as vesicles with the M domain outermost. Trypsin and/or proteinase K cleaved polypeptides C_{IV}, ASA, AED, STA, and IHQ. Cleavage of C_{IV}, STA, and IHQ was from the M domains only and involved the removal of a fragment from the N-terminus in each case. Polypeptide AED was cleaved from the C side in the N-terminal part, while ASA was cleaved from both the C and M domains. Polypeptide fragments were electroblotted from polyacrylamide gels onto derivatized glass paper and sites of proteolytic cleavage determined by N-terminal sequence analysis.

Cytochrome *c* oxidase, the terminal member of the respiratory chain, is a complex of two hemes and two copper atoms in association with a variable number of polypeptides, depending on the source of the enzyme [reviewed in Azzi (1980), Capaldi et al. (1983), and Wilkstrom et al. (1984)]. In prokaryotes, the enzyme complex contains three different subunits (Poole, 1983; Saraste et al., 1986). In eukaryotes, from 9 to 13 different polypeptides are present in the cytochrome *c* oxidase complex (Capaldi et al., 1983; Kadenbach & Merle, 1981). Three of these are coded for by mitochondria DNA (mtDNA) and are the homologues of the subunits of the prokaryote enzyme (Saraste et al., 1986; Steffens et al., 1983). In lower eukaryotes, such as yeast, the cytochrome *c* oxidase complex includes a total of nine different subunits, six made in the cytoplasm (Power et al., 1984; Wright et al., 1986). In higher eukaryotes, including beef and human, there is an additional level of complexity, with the cytochrome *c* oxidase complex containing 4 more polypeptides, for a total of 10 cytoplasmically made subunits (Kadenbach & Merle, 1981; Capaldi et al., 1986).

The cytoplasmically made subunits are made on free ribosomes as precursor molecules that are targeted to the mitochondrion by N-terminal leader sequences (Schatz & Butow, 1983). The available evidence is that these enter the mitochondrion individually and assemble into the enzyme complex at the inner membrane, either during or shortly after their processing by a matrix-localized protease (Hurt et al., 1986; van Steeg et al., 1986).

Three of the cytoplasmically made subunits of beef heart cytochrome *c* oxidase are hydrophilic polypeptides, probably extrinsic to the lipid bilayer (Capaldi et al., 1986). The others all have a similar structural motif, with a hydrophilic N-terminal domain, a central hydrophobic domain, and a hydrophilic C-terminus (Capaldi et al., 1986). This study focuses on the arrangement of several of these likely transmembrane polypeptides, using protease digestion to establish their orientation in the mitochondrial inner membrane.

Experimental Procedures

Enzyme Preparations. Beef heart cytochrome *c* oxidase was prepared according to the method of Capaldi and Hayashi (1972). Beef heart mitoplasts were prepared by digitonin treatment as described by Loyter et al., 1969). Submitochondrial particles were isolated according to Lee and Ernster (1967). Reconstitited vesicles of cytochrome *c* oxidase were made as described by Zhang et al. (1984).

Protease Digestion Experiments. Detergent-solubilized cytochrome *c* oxidase, dissolved in 0.1% Triton X-100 and 25 mM potassium phosphate (pH 8.2), was incubated with protease at a ratio of 1:20 or 1:50 (w/w) for 1 h at room temperature, and then the reaction was stopped by adding 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma). The proteases used included trypsin [type XIII, N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone (TPCK) treated], chymotrypsin [type VII, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone (TLCK) treated], proteinase K (protease type XI fungal), thermolysin (protease type X, thermophilic bacterial protease), subtilisin (protease type VIII), and Pronase E (protease type XXV from *Streptomyces grisens*). All were purchased from Sigma Chemical Co. Cleavage of membranous cytochrome *c* oxidase (mitoplasts, submitochondrial particles, or reconstituted vesicles) was conducted as described by Malatesta et al. (1983). Enzyme in reconstituted vesicles was separated from the protease and from phospholipids by sucrose gradient centrifugation prior to sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis, as described by Zhang et al. (1984).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in $NaDodSO_4$ was carried out according to Fuller et al. (1981) using 15 cm long gels, and according to Kadenbach et al. (1982) using 25 cm long gels, 0.12-cm thickness, but with 19.2% acrylamide and 0.5% *N*,*N*′-methylenebis(acrylamide).

Immunodetection of Subunits on Gels. Protein samples, resolved in $NaDodSO_4$–polyacrylamide gel electrophoresis, were transferred electrophoretically to nitrocellulose paper by a modification of the method of Towbin et al. (1979), using a Hoefer TE22 transblot apparatus at 250 mA for 3–5 h and a buffer of 0.19 M glycine, 0.02 M tris(hydroxymethyl)-aminomethane (Tris), 0.1% $NaDodSO_4$, and 20% methanol. The nitrocellulose paper was incubated with subunit-specific antibodies raised in rabbits, and the immunoreactive polypeptides were detected by reaction of horseradish peroxidase conjugated goat antibodies against rabbit immunoglobulins

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(Bio-Rad) (Hawkes et al., 1982).

Electroblotting onto Activated Glass. Protein samples resolved in $NaDodSO_4$–polyacrylamide gel electrophoresis were transferred electrophoretically onto derivatized glass filter paper sheets, by using the procedure of Aebersold et al. (1986) with modifications according to Yuen et al. (1986). Trifluoroacetic acid etched glass filter paper (Whatman GF/F) was derivatized by immersing into a silane solution containing 97 mL of acetonitrile, 2 mL of *N*-[(trimethoxysilyl)-propyl]-*N*,*N*,*N*-trimethylammonium chloride (Petrach Systems), and 1 mL of water. The electrophoresis buffer was freshly made 20 mM *N*-ethylmorpholine (Pierce Chemical Co.), titrated to pH 8.3 with 20% formic acid. Electroblotting was performed for 1–3 h at 250–350 mA at 15 °C, depending upon the efficiency of transfer of the polypeptide(s) of interest. Sequence analysis was performed by using an Applied Biosystems Model 470A protein sequenator and an Applied Biosystems Model 120A phenylthiohydantoin (PTH) analyzer.

Assays. Protein concentrations were determined according to Lowry et al. (1951). Cytochrome *c* oxidase activity was measured as described by Vik and Capaldi (1977). Heme reduction experiments were conducted as described by Zhang et al. (1984).

Results

The approach taken to determine the orientation of the cytoplasmically made subunits of cytochrome *c* oxidase involved protease digestion of oriented membranes in which the C or M faces of the enzyme were available for cleavage, in conjunction with sequencing to determine the site(s) of cleavage, i.e., from the N-terminus, C-terminus, or both ends of the individual polypeptides.

As a preliminary experiment, the effect of different proteases on the polypeptide profile of beef heart cytochrome *c* oxidase in detergent solution was surveyed. The cytochrome *c* oxidase preparations used had a heme *a* content of 8.9–9.7 nmol/mg of protein and showed high specific activities in the range 380–450 s^{-1}, indicating that there was very little denatured protein whose cleavage would complicate the interpretation of the protein digestion patterns. Figure 1 shows the cleavage patterns obtained with a variety of proteases when reacted with cytochrome *c* oxidase dissolved in 0.1% Triton X-100 in pH 8.2 buffer. The gel system employed resolved 13 different components, labeled in Figure 1 by our recently adopted nomenclature (Capaldi et al., 1986); these include the 3 mitochonrially coded polypeptides (Mt_{I-III}), 6 cytoplasmically made components that have counterparts in the yeast enzyme (C_{IV}–C_{IX}), and 4 polypeptides that appear to be present only in higher eukaryotes, and which are labeled by their N-terminal 3 amino acids (i.e., ASA, AED, STA, and IHQ). This last polypeptide, IHQ, is subunit VIIb in the nomenclature of Kadenbach and colleagues [e.g., see Merle and Kadenbach (1981) and Jarausch and Kadenbach (1985)], a component claimed to be absent from preparations made in the laboratory of Buse [e.g., see Buse et al. (1985)]. We have recently sequenced this polypeptide and find that it is similar to subunit C_{VII}–C_{IX} in having three domains, i.e., hydrophilic N- and C-termini separated by a central hydrophobic domain (S. Takamiya and R. A. Capaldi, unpublished results). Three polypeptides, C_{IV}, ASA, and AED, were cleaved by all of the proteases tested: Mt_{III}, STA, IHQ, and C_{IX} were cleaved by at least one protease; C_V, C_{VI}, C_{VII}, and C_{VIII} were resistant to cleavage by any of the proteases tried.

Orientation studies were conducted on three membrane preparations. Mitoplasts, prepared by digitonin treatment of beef heart mitochondria (Lee et al., 1967), as well as reconstituted vesicles of purified cytochrome c oxidase and asolectin (Zhang et al., 1984), were used as vesicles with the C domain available for cleavage. Submitochondrial particles were used as vesicles in which the M side is outermost for cleavage by proteases. The orientation of these preparations was evaluated by reduction of heme *a* by ascorbate plus cytochrome *c* in the presence of cyanide. The amount of cytochrome *c* oxidase with the C domain exposed (for reaction with cytochrome *c*) ranged from 50% to 75% in mitoplasts, 75% to 90% in reconstituted vesicles, and from 10% to 20% in submitochondrial particles.

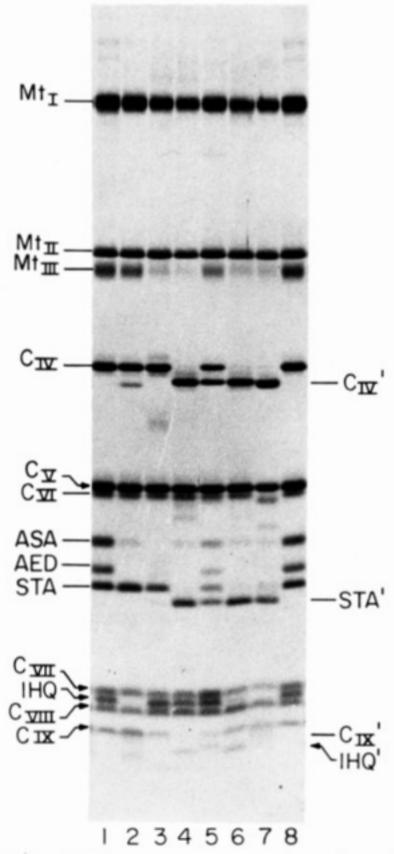

FIGURE 1: Effect of different proteases on the polypeptide profile of beef heart cytochrome *c* oxidase. Detergent-dispersed cytochrome *c* oxidase samples (2 mg/mL, in 0.1% Triton X-100 and 25 mM potassium phosphate, pH 8.2) were incubated with trypsin (1:20 w/w, lane 2), chymotrypsin (1:20 w/w, lane 3), proteinase K (1:50 w/w, lane 4), thermolysin (1:50 w/w, lane 5), subtilisin (1:35 w/w, lane 6), and pronase (1:35 w/w, lane 7), respectively. Protease digestion was stopped by addition of PMSF (1 mM). The samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (19% acrylamide), and the gel was stained with Coomassie Brilliant blue as described under Experimental Procedures. Lanes 1 and 8 show the control without protease treatment.

We have previously established that subunit Mt_{III} is cleaved by chymotrypsin exclusively from the C side of the mitochondrial inner membrane, while subunit C_{IV} is cleaved by trypsin only from the M side (Zhang et al., 1984). Figure 2 shows the cleavage of Mt_{III} by chymotrypsin in reconstituted vesicles and in submitochondrial particles, as monitored by immunoblotting with an antibody specific to this subunit. Antibody binding reveals the same peptide fragments after cleavage of subunit Mt_{III} in reconstituted vesicles as previously identified by [^{14}C]dicyclohexylcarbodiimide ([^{14}C]DCCD) labeling, the major produot being a fragment comigrating with subunit C_{IV}. As evident in Figure 2, there is little or no cleavage of subunit Mt_{III} in submitochondrial particles, confirming that this vesicle preparation is mostly oriented with the M domains of the enzyme outside and the C domain in the lumen of the vesicles.

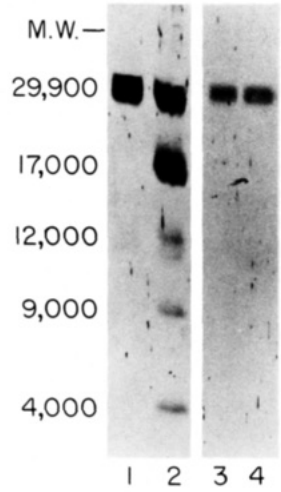

FIGURE 2: Chymotrypsin cleavage of subunit Mt_{III} in cytochrome *c* oxidase vesicles (lanes 1 and 2) and in submitochondrial particles (lanes 3 and 4). Twenty micrograms of cytochrome *c* oxidase was loaded onto the $NaDodSO_4$–polyacrylamide gel (lanes 1 and 2) after being isolated from phospholipids by step sucrose gradient centrifugation, and 40 μg of mitochondrial protein was loaded in lanes 3 and 4 after $NaDodSO_4$ solubilization and centrifugation as described under Experimental Procedures. Subunit Mt_{III} and its cleavage products were identified by immunoblotting on nitrocellulose paper with subunit Mt_{III} specific antibody. The controls of untreated enzyme are shown in lanes 1 and 3, while the effect of chymotrypsin treatment on subunit Mt_{III} is shown in lane 2 (in reconstituted vesicles) and in lane 4 (in submitochondrial particles), respectively. The anti-Mt_{III} antibody was visualized by using immunochemical techniques as discussed under Experimental Procedures.

Cleavage of subunit C_{IV} by trypsin and proteinase K in reconstituted vesicles and in submitochondrial particles was followed in a time course experiment (Figure 3), using antibody blotting with a subunit-specific antibody. A small amount of subunit C_{IV} was cleaved in reconstituted vesicles, ranging from 5% to 20% in different experiments, and this cleavage occurred relatively rapidly, with the remainder of the subunit being resistant to proteolysis. In mitoplasts, the amount of cleavage of C_{IV} was much greater than in reconstituted vesicles, ranging from 25% to 40%. This confirms the heme reduction experiments in showing that reconstituted vesicles are better oriented for the C side outermost than are mitoplasts (Figure 4). In submitochondrial particles, almost all of the subunit IV was cleaved rapidly.

We have previously used trypsin cleavage, as well as chemical labeling with ^3H- and ^{14}C-labeled arylazidophospholipids, [^{3}H]-*N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate and [^{35}S]diazobenzenesulfonate, to examine the topology of C_{IV} in beef heart cytochrome *c* oxidase (Malatesta et al., 1983). We concluded that this polypeptide was oriented with its N-terminus on the M side and its C-terminus on the C side of the inner membrane, but with the C-terminus mostly buried by protein–protein interactions. More recently, Jarausch and Kadenbach (1985) have claimed that C_{IV} is cleaved by trypsin from both the M and C sides in rat liver mitochondria. Also, Buge and Kadenbach (1986) have claimed that 50% of C_{IV} can be cleaved in reconstituted vesicles of beef heart cytochrome *c* oxidase. They argue that this represents cleavage of the polypeptide from the C domain, rather than scrambling of their membrane preparations.

If C_{IV} is accessible from both the C and M sides of the mitochondrial membrane, then the size of the major fragment generated by trypsin cleavage from either side alone should be larger than that obtained in leaky membranes or in the detergent-dispersed enzyme. This was not the case as shown in Figure 5. The cleavage of subunit IV was from the N-terminus, as indicated by protein sequencing of the subunit IV fragments generated by trypsin cleavage of detergent-solubilized cytochrome *c* oxidase (Table I).

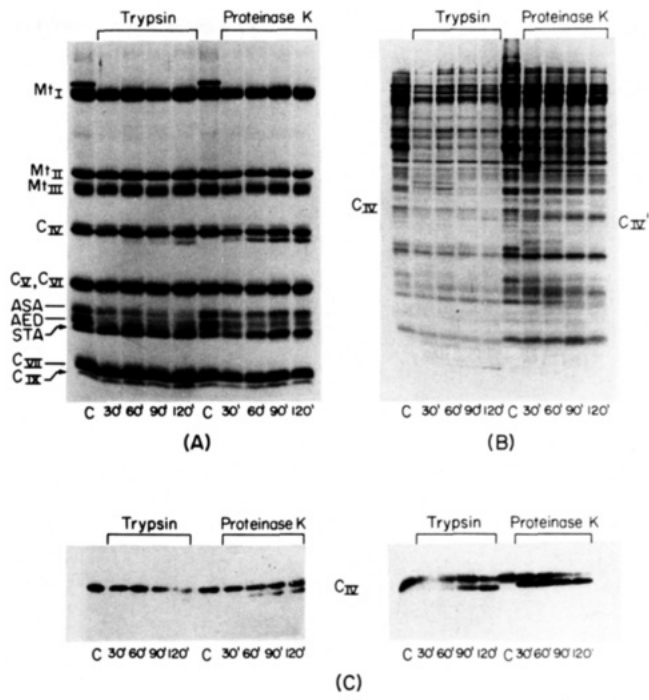

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of the time course of trypsin and proteinase K cleavage of reconstituted cytochrome *c* oxidase vesicles (A) and submitochondrial particles (B). The ratios of protease to membranous protein were 1:20 (w/w) for trypsin and 1:50 (w/w) for proteinase K. Proteolytic digestions were terminated at the indicated times by adding PMSF (1 mM). Before being subjected to gel electrophoresis, the proteins were isolated from phospholipids either by step sucrose gradient centrifugation (A) or by $NaDodSO_4$ solubilization and centrifugation (B). C = control of untreated cytochrome *c* oxidase vesicles and/or submitochondrial particles. The left part of (C) is a blot of the same lanes of gel A using antibodies that react only with subunit C_{IV}, while the right part of (C) is a blot of gel B with the same anti-C_{IV} antibodies. The experimental conditions are the same as in Figure 2.

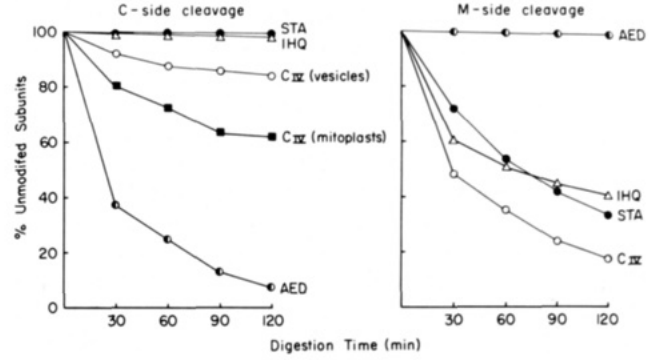

FIGURE 4: Time course of cleavage of individual subunits of cytochrome *c* oxidase in reconstituted vesicles (left) and in submitochondrial particles (right) during protease digestion experiments. The amount of subunit remaining was calculated from the densitometric scans of the $NaDodSO_4$–polyacrylamide gels and/or from scans of the negative film of the immunoblots with the different subunit-specific antibodies. The data for C_{IV}, AED, and IHQ are with a 1:20 (w/w) ratio of trypsin to membrane protein while the cleavage of STA is with a 1:50 (w/w) ratio of proteinase K.

The small amount of cleavage of subunit C_{IV} in reconstituted vesicles was also from the N-terminus. This was determined by electroblotting the subunits of trypsin-cleaved or proteinase K cleaved cytochrome *c* oxidase onto glass paper, cutting out the subunit IV cleavage product, and sequencing in an Applied Biosystems 470A sequencer. The N-terminus began SEDY

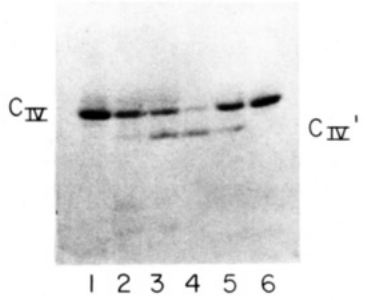

FIGURE 5: Subunit C_{IV} and its major fragment after treatment with trypsin. Trypsin cleavage was performed in 25 mM potassium phosphate, pH 8.2, at the ratio 1:20 (w/w with respect to protein), for 1 h at room temperature. Lanes 1 and 6 show the control of purified cytochrome *c* oxidase without trypsin treatment. Lanes 2–5 show the effect of trypsin on reconstituted cytochrome *c* oxidase vesicles without (lane 2) and with (lane 3) prior treatment of the membranes with 2% Triton X-100, on submitochondrial particles (lane 4), and on the detergent-dispersed enzyme (in 0.1% Triton X-100) (lane 5). Subunit C_{IV} and its major trypsin cleavage fragment were immunodetected after the proteins were blotted from the $NaDodSO_4$–polyacrylamide gel onto nitrocellulose paper as described in Figure 3.

Table I[a]

SUBUNITS	AMINO ACID SEQUENCES	
	1 ▼ 10 20	
C_{IV}	NH_2 - A H G S V V K S E D Y A L P S Y V D R R	.....
	Δ	
	1 10 20	
AED	NH_2 - A E D I Q A K I K N Y Q T A P F D S P F	.....
	Δ	
	1 ▼ 10 20	
STA	NH_2 - S T A L A K P Q M R G L L A R R L R F H	.....
	1 10 20	
IHQ	NH_2 - I H Q K R A P D F H D K Y G N A V L A S	.....
	Δ Δ	

[a] Cleavage sites of trypsin and protease K in the cytoplasmically made subunits of beef heart cytochrome *c* oxidase complex. (Δ) Trypsin; (▼) proteinase K.

and thus began at residue 8 of the intact polypeptide. These sequencing data confirm that protease cleavage of subunit C_{IV} in reconstituted vesicles represents cleavage of the small amount of wrongly oriented enzyme in the vesicle preparations. Cytochrome *c* oxidase with M domains exposed has been as much as 20% of the total enzyme present in some of our preparations of reconstituted vesicles, but never the 50% reported by Buge and Kadenbach (1986).

The orientation of other polypeptides of cytochrome *c* oxidase was determined by time course experiments using the cleavage of C_{IV} as a control. Time courses were followed for 2 h by which time the limited proteolysis of both C_{IV} and AED was essentially complete. The cleavage of IHQ and STA ranged from 50% to 70% in this time period in different experiments. More extensive digestion of these last two subunits (in submitochondrial particles) could be obtained by using larger amounts of protease (e.g., 1:10) or by longer times of incubation (result not shown). The cleavage of individual polypeptides in reconstituted vesicles could be followed from the Coomassie blue stained gels (Figure 3) as well as by antibody blotting. The cleavage of C_{IV} in submitochondrial particles could also be followed from the Coomassie blue stained gels (Figure 3), but the degradation of other polypeptides was only resolved by using subunit-specific antibodies.

Immunoblotting data for IHQ and STA are presented in Figure 6. Both are cleaved in submitochondrial particles, although more slowly than C_{IV} (time course in Figure 4):

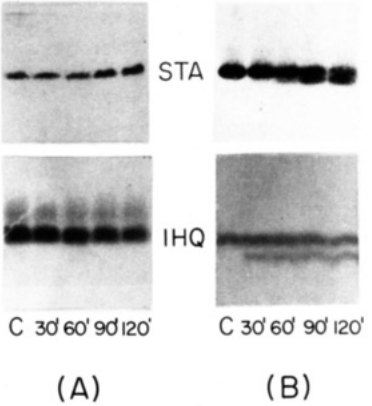

FIGURE 6: Time course of proteolytic digestion of subunits STA and IHQ in reconstituted cytochrome *c* oxidase vesicles (A) and in submitochondrial particles (B). The data of subunit STA were from proteinase K cleavage experiments (1:50 w/w), while the data of subunit IHQ were obtained with trypsin as the protease (1:20 w/w). After the proteins were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis, subunits STA, and IHQ and their major cleavage fragment were immunodetected by Western blotting using specific antibodies, as described in Figure 3. C = control, without proteolytic treatment.

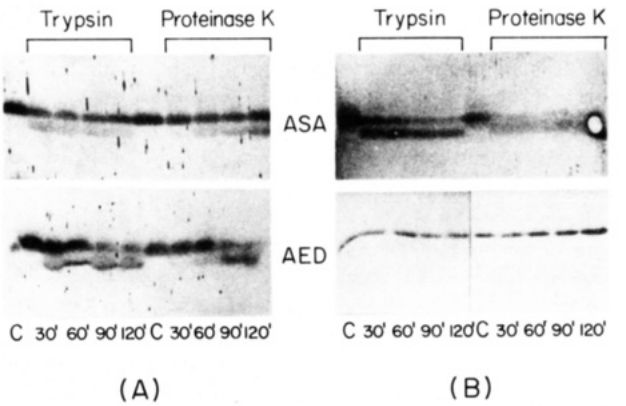

FIGURE 7: Time course of trypsin and proteinase K cleavage of subunits ASA and AED in cytochrome *c* oxidase containing vesicles monitored by antibody binding (A) and in submitochondrial particles (B). The experimental conditions are the same as in Figure 3.

neither is cleaved in significant amounts in reconstituted vesicles. The results for subunits AED and ASA are given in Figure 7. Polypeptide ASA was cleaved at both sides of the membrane. AED was rapidly degraded in reconstituted vesicles but not modified in submitochondrial particles.

The cleavage products of IHQ, STA, and AED were each identical with those obtained by protease digestion of the detergent-dispersed enzyme, indicating that cleavage was from one end of each polypeptide only. The size of the fragment of ASA, obtained in either reconstituted vesicles or submitochondrial particles, was larger than that obtained when the proteases were reacted with detergent-soluble enzyme, where cleavage could occur from the C and M domains simultaneously (Figure 8).

The sequences of the cleaved products of IHQ, STA, and AED were determined. All of these polypeptides were cleaved from the N-terminus (Table I).

Discussion

Cytochrome *c* oxidase is a Y-shaped protein. It is oriented at the mitochondrial inner membrane with a large domain, the C domain (stalk of the Y), extending into the intracristal space. The two other domains, M_1 and M_2 (the arms of the Y), each traverse the membrane and extend a small amount into the matrix space (Fuller et al., 1979; Deatherage et al.,

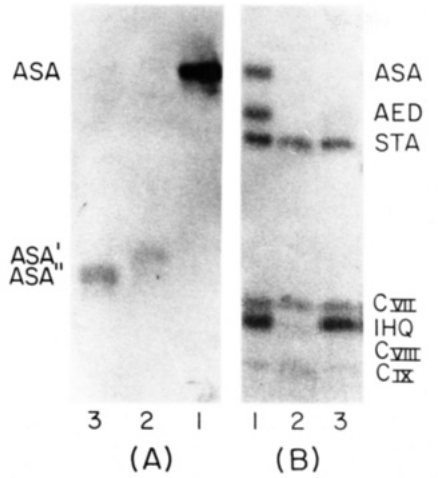

FIGURE 8: Identification of the protease-derived fragments of subunit ASA. Detergent-dispersed cytochrome *c* oxidase (2 mg/mL in 0.1% Triton X-100 and 25 mM potassium phosphate, pH 8.2) was incubated with trypsin (1:20 w/w, lane 2) and chymotrypsin (1:20 w/w, lane 3), respectively. After protease digestion was terminated by addition of 1 mM PMSF, subunit ASA and its cleavage products were electrophoretically separated by a $NaDodSO_4$–polyacrylamide gel (19% acrylamide, 25 cm long, 20 μg of protein was loaded in each lane). A part of the gel was stained with Coomassie blue (B). An immunoblot from another identical part of the gel was reacted with antibody against ASA, and the immunoreactive proteins were detected by peroxidase-conjugated goat anti-rabbit IgG antibodies. Lane 1 in both (A) and (B) shows the control of untreated enzyme. The experimental conditions are as described under Experimental Procedures.

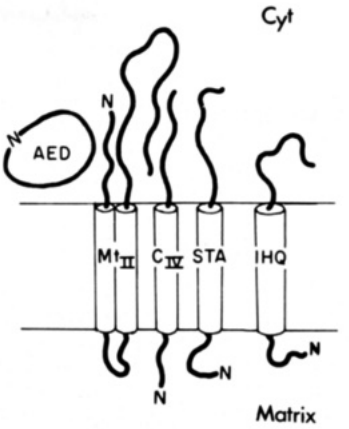

FIGURE 9: Schematic of the folding of polypeptides in the beef heart mitochondrial cytochrome *c* oxidase complex.

1982a,b; Frey et al., 1982, 1985).

Recent studies in this laboratory have been directed toward determining the arrangement of component polypeptides, associated prosthetic groups, and substrate binding sites within the low-resolution structure (Malatesta et al., 1983; Zhang et al., 1984; Millett et al., 1983; Malatesta & Capaldi, 1982). Our experiments to date have focused on the core polypeptides of the cytochrome *c* oxidase complex, i.e., those subunits not easily removed by detergent treatment (Zhang, 1987). These are the subunits that are found in cytochrome *c* oxidase from lower eukaryotes (represented by yeast) and in higher eukaryotes (as represented by the beef heart enzyme). It is now clear that the mammalian cytochrome *c* oxidase contains four additional polypeptides, not present in yeast (Capaldi et al., 1986), and identified here by their N-terminal amino acid sequences. The topology experiments described here help define the orientation of three of the components, AED, STA, and IHQ.

Protease digestion was used to determine the topology of these polypeptides. The enzyme was reacted with either trypsin or proteinase K in oriented vesicles, using mitoplasts or vesicles reconstituted with isolated enzyme as populations with the C side outermost, and submitochondrial particles as a population with the M side exposed. In our hands, reconstituted vesicles were better oriented than mitoplasts and were used predominantly, although the same conclusion about the topology of each subunit could be drawn by using either preparation.

The cleavage of different polypeptides was done as a time course experiment, using the cleavage of subunit C_{IV} as a standard. It was necessary to confirm our previous results, that C_{IV} is oriented with its N-terminus on the M side and that the polypeptide is clipped only from the M side (Malatesta et al., 1983), because of a report by Buge and Kadenbach (1986) to the contrary. The results presented here establish unambiguously that C_{IV} is cleaved only from the N-terminus by trypsin and proteinase K, and only from the M side of the inner membrane. The large amount of cleavage of C_{IV} from the C side in reconstituted vesicles reported by Buge and Kadenbach (1986) must be due to scrambling of the orientation of the enzyme in their vesicle preparations.

In the experiments described here, polypeptide AED was cleaved in reconstituted vesicles, i.e., from the C side. This cleavage was localized by sequencing of the protease-derived fragment to the N-terminus and after Lys_7 in the intact sequence. AED has the characteristics of an extrinsic protein. Its amino acid sequence is hydrophilic throughout the length of the polypeptide (Capaldi et al., 1986) and it is readily extracted from the cytochrome *c* oxidase complex in a water-soluble form (Zhang, 1987). Therefore, the entire mass of AED is probably on the C side as shown in Figure 9 and in the large C domain of the enzyme.

Polypeptides STA and IHQ were cleaved in submitochondrial particles, i.e., from the M side. The cleavage of both of these polypeptides was in the N-terminus between Leu_4 and Ala_5 in STA and after Lys_4 and Arg_5 in IHQ. Both STA and IHQ have sequences typical of intrinsic membrane proteins with their hydrophilic N-terminus parts being followed by a likely transmembrane stretch of around 20 hydrophobic amino acids [reviewed in Capaldi et al. (1986)].

We propose that STA, IHQ, and C_{IV} are all arranged in the cytochrome *c* oxidase complex in a similar way with their N-terminal parts in the M domains and C-termini into the C domain (Figure 9). The alternative, that any or all of these subunits are localized exclusively on the M side, with their long hydrophobic stretch of sequence outside the bilayer or at the lipid/aqueous interface, cannot be ruled out completely. However, this seems unlikely given that C_{IV} has been found to label with arylazidophospholipids in the central hydrophobic sequence (Malatesta et al., 1983) and C_{IV}, STA, and IHQ are each labeled by 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine ([^{125}I]TIDS) (Y.-Z. Zhang and R. A. Capaldi, unpublished results), a reagent which partitions to the hydrophobic interior of the membrane.

Polypeptides C_{IV}, STA, and IHQ, derived by protease digestion in submitochondrial particles, were in each case the same size as obtained when the membranes were made leaky to protease, or when the complex was dissolved in detergent. Only polypeptide ASA was unequivocally clipped from each side of the membrane, being cleaved significantly in reconstituted vesicles and in submitochondrial particles. Importantly, the size of the fragment of ASA obtained by protease digestion of either vesicle orientation was larger than that obtained in leaky membranes or in detergent-dissolved enzyme. This is taken to show that ASA is cleaved from different ends from the C and M side. ASA has a sequence typical of a

transmembrane polypeptide with hydrophilic N- and C-termini separated by a long (19-residue) hydrophobic sequence of amino acids. Unfortunately, the fragments of ASA could not be separated for sequencing, and the orientation of this polypeptide remains unknown.

Polypeptides C_{IV}, AED, STA, and IHQ are synthesized on cytoplasmic ribosomes (Schatz & Butow, 1983; Hurt & van Loon, 1986). Subunit C_{IV} is the best characterized of these. It is made as a precursor in yeast (Koerner et al., 1985) and in mammals (Lomax et al., 1984) with an N-terminal leader extension thought to be responsible for directing this subunit to the mitochondrion. The leader sequence along with the mature protein sequence together must specify the topology. In the case of C_{IV} and presumably STA and IHQ as well, the leader sequence carries the N-terminus to the M side, where it is cleaved by the leader protease. Insertion across the membrane is via the hydrophobic central segment, acting as a stop transfer sequence. The location of AED to the C side of the mitochondrial inner membrane in the C domain of cytochrome *c* oxidase must occur via a different mechanism.

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

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