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Topology of Beef Heart Cytochrome *c* Oxidase from Studies on Reconstituted Membranes†

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ABSTRACT: The orientation of purified beef heart cytochrome *c* oxidase, incorporated into vesicles by the cholate dialysis procedure [Carroll, R. C., & Racker, E. (1977) *J. Biol. Chem. 252*, 6981], has been investigated by functional and structural approaches. The level of heme reduction obtained by using cytochrome *c* along with the membrane-impermeant electron donor ascorbate was 78 ± 2% of that obtained with cytochrome *c* and the membrane-permeant reagent *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. Electron transfer from cytochrome *c* is known to occur exclusively from the outer surface of the mitochondrial inner membrane (C side), implying that at least 78% of the oxidase molecules are oriented in the same way in these vesicles as in the intact mitochondria. Trypsin, which cleaves subunit IV near its N terminus, modifies only 5–7% of this subunit in intact vesicles. This removal of the N-terminal residues has been shown to occur only in mitochondrial membranes with their inner side (M side) exposed. Diazobenzene[^{35}S]sulfonate ([^{35}S]DABS) likewise modifies subunit IV only in submitochondrial particles. Labeling of intact membranes with [^{35}S]DABS resulted in incorporation of only 4–8% of the total counts that could be incorporated into this subunit in membranes made leaky to the reagent by addition of 2% Triton X-100. Therefore, both the functional and structural data show that at least 80% and probably more of the cytochrome *c* oxidase molecules are oriented with their C domain outermost and M domains in the lumen of vesicles prepared by the cholate dialysis method. Labeling experiments with [^{35}S]DABS and proteinase digestions of intact membranes with trypsin and chymotrypsin were used to determine the topography of the subunits of cytochrome *c* oxidase. These studies confirm that subunits II and III are in the C domain and subunits IV and VII in the M domain and show for the first time that polypeptides b and c are on the C side of the mitochondrial inner membrane.

The exposure of the subunits of cytochrome *c* oxidase at different sides of the mitochondrial inner membrane has been probed by labeling with membrane-impermeant protein-modifying reagents such as diazobenzenesulfonate (DABS)[1] (Schneider et al., 1971; Eytan et al., 1975; Ludwig et al., 1979; Prochaska et al., 1980), by digestion of components with proteases (Malatesta et al., 1983a), and by reaction with antibodies raised to individual subunits of the enzyme (Chan & Tracy, 1978).

These studies, taken together, show cytochrome *c* oxidase as a transmembrane protein with most of the mass of subunit IV located on the matrix (M) side of the inner membrane, with most of subunit II on the cytoplasmic (C) side, and with subunits I, II, III, IV, and VII(s) [see Capaldi et al. (1983b) for nomenclature] each spanning the lipid bilayer at least once.

Recently, the sequences of all of the subunits of beef heart cytochrome *c* oxidase have been obtained either by direct procedures or by sequencing of mDNA [e.g., see Steffens et al. (1979), Tanaka et al. (1975), and Anderson et al. (1982)]. This makes possible more detailed topological studies at the level of the folding of individual polypeptides.

Detailed analysis of the folding of subunits in cytochrome *c* oxidase is difficult when the enzyme is labeled or digested by proteases in mitochondrial membranes because of the problems inherent in isolating modified proteins. For example, it requires purifying cytochrome *c* oxidase from large volumes of highly radioactive mitochondria in order to have sufficient enzyme with high enough specific activity for subunit isolation and fragmentation experiments. Also, chemically modified cytochrome *c* oxidase or enzyme partly digested by proteases may not purify in a manner identical with that of the native enzyme.

The folding of individual subunits of cytochrome *c* oxidase should be amenable to experimentation in vesicle preparations containing purified enzyme and phospholipid, provided that

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DCCD, dicyclohexylcarbodiimide; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; DABS, diazobenzenesulfonate; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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the protein is incorporated into these membranes in one orientation. In this paper, we have examined in detail, reconstituted vesicles prepared by the cholate dialysis procedure of Carroll and Racker (1977). We find that the enzyme is orientated predominantly with the C side outermost. Protease digestion studies and labeling with [^{35}S]DABS has been used to probe the folding of subunit III and to localize selected polypeptides in this oriented membrane preparation.

Experimental Procedures

Enzyme Preparation and Assays. Beef heart cytochrome *c* oxidase was prepared as described by Yonetani (1961). Electron transport activity was measured polarographically by the procedure of Vik & Capaldi (1977). Protein concentrations were determined according to Lowry et al. (1951).

Reconstitution and Assays of Cytochrome c Vesicles. Cytochrome *c* oxidase vesicles were prepared by using the procedure of Carroll & Racker (1977) with some modifications. Sodium cholate (80 mg) and asolectin (120 mg) were dissolved in 8 mL of medium containing 100 mM potassium chloride and 3 mM K-Hepes, pH 7.2, by a magnetic mixer and sonicated at 0 °C for 15 s by using a Bronston sonicator. Two milligrams of purified cytochrome *c* oxidase was added and sonicated in 15-s bursts with 3-min intervals between bursts until optically clear. Dialysis was performed for 24 h at 4 °C against 500 mL of the same buffer with four changes.

Respiratory control of cytochrome *c* oxidase vesicles was assayed as described by Hinkle et al. (1972). The orientation of reconstituted oxidase was determined by the spectroscopic procedure of Nicholls et al. (1980). Difference spectra were obtained at 25 °C on a Beckman DU-7 spectrophotometer.

Proteolytic Digestion. Trypsin cleavage of membranous cytochrome *c* oxidase was performed as follows: cytochrome *c* oxidase vesicles were suspended in 20 mM potassium phosphate, pH 8.2, and then incubated with trypsin (type III, Sigma) at ratios of 1:20 or 1:5 (w/w) for 2 h at room temperature in both the presence and absence of 2% Triton X-100. The reaction was terminated by adding PMSF (0.5 mM). Chymotrypsin (TLCK treated; Worthington) cleavage was carried out in the same manner.

Proteinase-digested oxidase was isolated by step sucrose gradient ultracentrifugation as described later.

Labeling of Membranous Cytochrome c Oxidase with Different Reagents. [^{35}S]DABS (5–9 Ci/mmol) was prepared from [^{35}S]sulfanilic acid (Amersham) according to Tinberg et al. (1974). Iodo[^{14}C]acetamide and [^{14}C]DCCD were purchased from New England Nuclear and Amersham, respectively. [^{3}H]adamantane diazirine was the kind gift of Dr. Jack Kyte, University of California, San Diego. The labeling of membranous cytochrome *c* oxidase by [^{35}S]DABS was done according to Ludwig et al. (1979) and labeling by [^{14}C]DCCD according to Prochaska et al. (1980). Membranes were labeled in a buffer containing 50 mM potassium phosphate (pH 8.0) in the presence or absence of 2% Triton X-100. Labeled membranes were separated from unreacted reagent, and the protein was separated from the large excess of phospholipid in a single sucrose density centrifugation procedure as follows:

The sample, concentrated 8-fold to 0.5 mL, was layered onto a discontinuous sucrose gradient (volume 4.2 mL), the upper layer of which contained 3% sucrose (0.5 mL), the middle layer 5% sucrose 2% Triton X-100, and 1% sodium cholate, pH 8.0 (3.2 mL), and the bottom layer 10% sucrose and 0.2 M sodium chloride. The gradient was centrifuged for 12 h at 200000*g* and the protein then collected as a tight pellet at the bottom of the centrifuge tube. This was dissolved in 10% $NaDodSO_4$, 2 mM β-mercaptoethanol, and 10 mM Tris-HCl (pH 7.5) for $NaDodSO_4$–polyacrylamide gel electrophoresis.

Labeling with [^{3}H]adamantane diazirine was conducted in a thin glass test tube. Enzyme (0.1–0.5 mg in 100 μL) was incubated with the reagent (100 000 cpm) added in ethanol (2% of final volume) for 2 h in the dark on ice. The sample was then irradiated under nitrogen with a low-intensity UV lamp (Mineralight) for 30 min at 0 °C. Samples labeled with [^{3}H]admantane diazirine were electrophoresed without prior separation of unbound reagent from the protein.

$NaDodSO_4$–polyacrylamide gel electrophoresis was conducted as described by Fuller et al. (1982). Staining with Coomassie Brilliant blue and destaining of gels suspended in this dye solution were performed according to Downer et al. (1976). Gels were scanned at 560 nm on a Gilford spectrophotometer with a linear scanning attachment and then sliced into 1-mm segments with a Mickle gel slicer. Radioactivity was measured by liquid scintillation counting in a Beckman Model LS 7000 liquid scintillation counter.

Results

Orientation of Cytochrome c Oxidase on the Basis of Heme Reduction. Beef heart cytochrome *c* oxidase, prepared according to Yonetani (1961) and incorporated in vesicles by the cholate dialysis procedure (Carroll & Racker, 1977), had a respiratory control ratio of 3.5–6.2 (two different enzyme preparations and seven different membrane preparations). As a first approach, the orientation of the cytochrome *c* oxidase complex in the reconstituted membranes was estimated by monitoring the reducibility of the hemes by membrane-impermeant reducing agents. The level of reduction of cytochrome *c* oxidase by such reagents, i.e., cytochrome *c* plus ascorbate, proved to be 78 ± 2% (seven different membrane preparations) of that obtained in the presence of the membrane-permeant electron donor *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. Experiments were done in the presence of cyanide to prevent reoxidation of the hemes. Thus, around 80% of the cytochrome *c* oxidase molecules in the reconstituted membranes must be arranged with their C domain and cytochrome *c* binding site outermost.

Trypsin Cleavage of Vesicles. Trypsin cleavage was used as a second approach to determining the orientation of cytochrome *c* oxidase in reconstituted vesicles. In recent studies, we have shown that trypsin cleaves subunit IV of beef heart cytochrome *c* oxidase to remove the N-terminal seven residues (when the reaction is done at pH 8.2). In addition, polypeptides b and c are degraded to smaller fragments (Malatesta et al., 1983a). Trypsin was found to cleave subunit IV from the matrix side, i.e., in submitochondrial particles. There was no effect of protease digestion in intact mitochondria or mitoplasts (right-side-out inner membrane preparations) (Malatesta et al., 1983a). Subunit IV is, therefore, oriented with its N terminus on the M side of the mitochondrial inner membrane.

The effect of trypsin on cytochrome *c* oxidase in reconstituted vesicles is shown in Figure 1. Incubation of intact vesicles with the protease (1:20 w/w trypsin:cytochrome *c* oxidase) resulted in cleavage of polypeptides b and c but only a small amount of cleavage of subunit IV. On the basis of density measurements of the Coomassie blue stained gels, the amount of clipped subunit IV ($M_{r,app}$ 16 000) was 5–7% of the total subunit ($M_{r,app}$ 16 000 + 17 000). In vesicles made leaky to trypsin by addition of 2% Triton X-100, much more of subunit IV was converted to the 16 000-dalton species. Incubation with higher levels of trypsin (1:5) did not significantly increase the amount of clipped subunit IV in intact vesicles but resulted in digestion of all of the subunit IV to the smaller

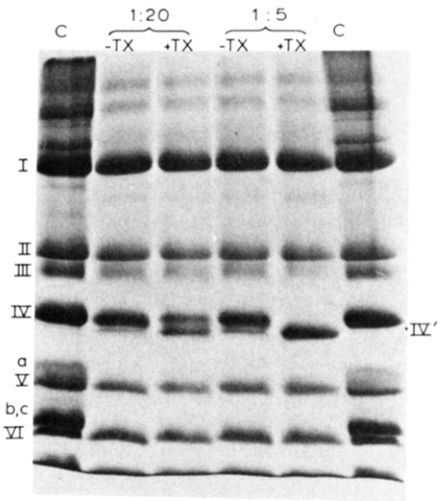

FIGURE 1: Effect of trypsin cleavage on the polypeptide composition of cytochrome *c* oxidase incorporated into membranes. Samples of cytochrome *c* oxidase in vesicles were incubated with trypsin (1:20 or 1:5) with or without prior treatment of the membranes with 2% Triton X-100 (TX). Protease digestion was stopped by addition of PMSF, samples were concentrated, and then protein was separated from the large excess of phospholipid by sucrose density gradient centrifugation as described under Experimental Procedures. Samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, and the gel was stained with Coomassie Brilliant blue. C = control of untreated cytochrome *c* oxidase not incorporated into membranes. IV′ marks the position on the gel of cleaved subunit IV.

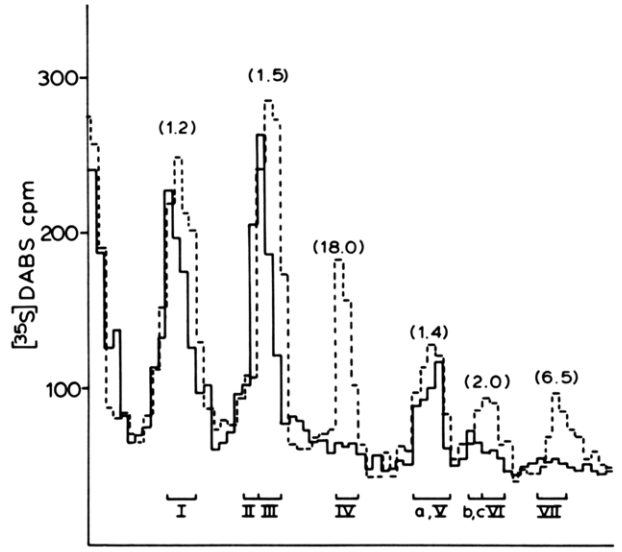

FIGURE 2: [^{35}S]DABS labeling of cytochrome *c* oxidase membranes. The reconstituted membranes were reacted with [^{35}S]DABS in the absence and in the presence of 2% Triton X-100. Samples were run on $NaDodSO_4$–polyacrylamide gels, the positions of subunits were identified from staining profiles with Coomassie Brilliant blue, and then the gels were sliced and counted. The solid line shows the labeling profile of intact vesicles; the dashed line is for sample that had included 2% Triton X-100 to disrupt the vesicles and make them leaky to DABS. The values in parentheses above each peak are the ratio of counts in intact membrane to those in Triton X-100-treated membranes.

molecular weight species in those vesicles that had been treated with Triton X-100. These results indicate that cytochrome *c* oxidase is oriented with the N terminus of subunit IV and therefore with its M domains inside the vesicle. The cleavage of components b and c in intact vesicles places each of these polypeptides on the C side of the mitochondrial inner membrane.

Labeling with [^{35}S]DABS. The reaction of [^{35}S]DABS with cytochrome *c* oxidase in reconstituted vesicles is shown in Figure 2. Also shown is the reaction of DABS with a second aliquot of the same vesicle preparation, to which 2% Triton X-100 had been added to make the membrane leaky to the chemical-modifying reagent. Subunit IV was very poorly labeled by DABS in the intact vesicles but relatively heavily labeled in the disrupted vesicles. This is consistent with previous studies on the enzyme in the mitochondrial inner membrane which showed labeling of subunit IV from the M side (now established as the inside of the reconstituted vesicles) but not from the C side (outer surface of the reconstituted vesicles) (Eytan et al., 1975; Ludwig et al., 1979). The ratio of labeling of subunit IV in the intact vesicles to Triton X-100 disrupted membranes was from 12:1 to 32:1 in different experiments (18:1 in the experiment shown in Figure 2). On the basis of these labeling studies, the proportion of cytochrome *c* oxidase oriented with the C domain outermost is from 92% to 96%. In addition to subunit IV, subunits VII (which comigrate in the gel system used here) were labeled more heavily in disrupted vesicles than in the intact vesicles. In the experiment shown in Figure 2, the ratio of labeling of these components was 6.5:1. For subunits II + III and V + a, the ratio of labeling in intact vesicles to Triton X-100 disrupted membranes was close to 1:1; for the combination of b, c, and VI, the value was closer to 2:1. The DABS labeling results indicate that with the exception of IV and VII, the major part of the other subunits is on the C side of the membrane. The labeling of subunit I by DABS in the above experiments is ambiguous because of the presence of a heavily labeled impurity comigrating with this component on gels. This impurity is also heavily labeled by iodoacetamide and by DCCD (see later). It is partly removed by cleaving cytochrome *c* oxidase preparations with trypsin as judged by the lower labeling in the subunit I region of the gels with all reagents after protease treatment [the protease does not cleave subunit I in highly purified enzyme preparations; see Ludwig et al. (1979)].

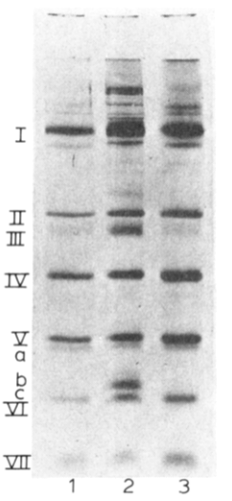

FIGURE 3: Effect of chymotrypsin cleavage on the polypeptide composition of cytochrome *c* oxidase in vesicles. Lane 1 of the $NaDodSO_4$–polyacrylamide gel is the polypeptide pattern of Triton X-100 treated membranes after chymotrypsin digestion; lane 3 shows the polypeptide pattern of intact membranes after chymotrypsin treatment; lane 2 shows control uncleaved enzyme.

Chymotrypsin Cleavage of Reconstituted Vesicles. Our recent studies have established that chymotrypsin cleaves subunit III and components b and c of detergent-dispersed cytochrome *c* oxidase at pH 8.0, without modifying other subunits and with very little loss of electron transfer activity (Malatesta et al., 1983b). Figure 3 shows the effect of chymotrypsin on reconstituted vesicles. Subunit III, as well as components b and c, is digested and removed from the gel

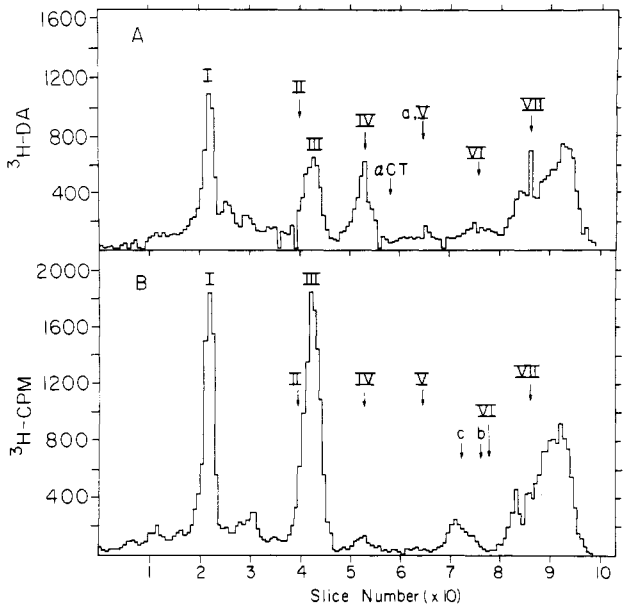

FIGURE 4: Effect of chymotrypsin cleavage on subunit III, monitored by using [^{3}H]adamantane diazirine labeled cytochrome *c* oxidase. The upper trace (A) shows the labeling profile after chymotrypsin treatment. The lower trace (B) shows a control sample before protease treatment. αCT is the position of migration of chymotrypsin (not removed before electrophoresis).

profile both in intact membranes and in Triton X-100 treated vesicles. The possibility that chymotrypsin cleavage disrupted the vesicles, thereby allowing the protease to attack from both sides of the membrane, was considered. Reconstituted vesicles were first treated with chymotrypsin to cleave subunits III and components b and c. These same vesicles were then treated with trypsin. There was no cleavage of subunit IV by trypsin until the membranes were disrupted by Triton X-100, establishing that the chymotrypsin cleavage was from the outer surface, i.e., from the C domain of cytochrome *c* oxidase.

Localization of Cleavage Sites in Subunit III. In initial experiments, cytochrome *c* oxidase was reacted with [^{3}H]-adamantane diazirine prior to chymotrypsin cleavage. This hydrophobic, photoactivated reagent was designed to react with the bilayer-intercalated parts of membrane proteins (Goldman et al., 1979; Bayley & Knowles, 1980). It reacts with cytochrome *c* oxidase predominantly in subunits I and III (Georgevich & Capaldi, 1982) (see Figure 4B). Cleavage of the adamantane diazirine labeled cytochrome *c* oxidase with chymotrypsin gave very similar results whether 2% Triton X-100 was present (result not shown) or absent (Figure 4A) from the reaction mixture. There was loss of the peak of ^{3}H-labeled subunit III and appearance of a major new band of radioactivity, not present in the control, migrating at the position of subunit IV ($M_{r,app}$ 17 000). This new peak was not derived from subunit I, which is not cleaved by chymotrypsin as shown in Figure 3. Therefore, it must be a fragment of subunit III, a conclusion supported by the DCCD labeling experiments detailed later (the gel in Figure 4A contained less protein that in Figure 4B, explaining the lower amount of label in subunits I and VII in the chymotrypsin-treated enzyme than in the control sample).

Examination of the sequence of subunit III as detailed in Capaldi et al. (1983a) shows six likely transmembrane helices (Figure 5) that are the potential sites of labeling with [^{3}H]-adamantane diazirine. These are distributed throughout the length of the polypeptide. Thus, the [^{3}H]adamantane diazirine labeled fragment ($M_{r,app}$ 17 000) could be derived from the N or C terminus and could possibly represent a mixture of

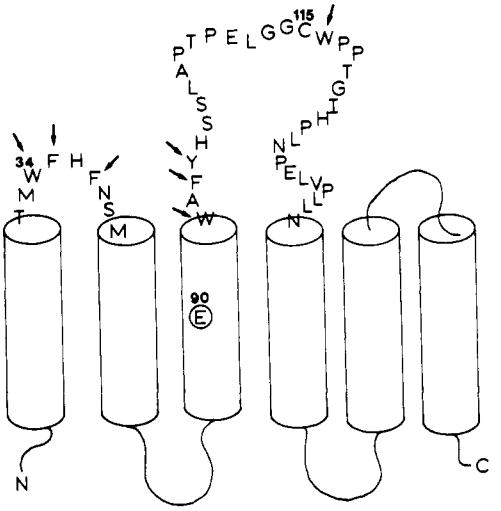

FIGURE 5: Schematic model of the folding of subunit III in the cytochrome *c* oxidase complex. Arrows next to residues show potential chymotrypsin cleavage sites.

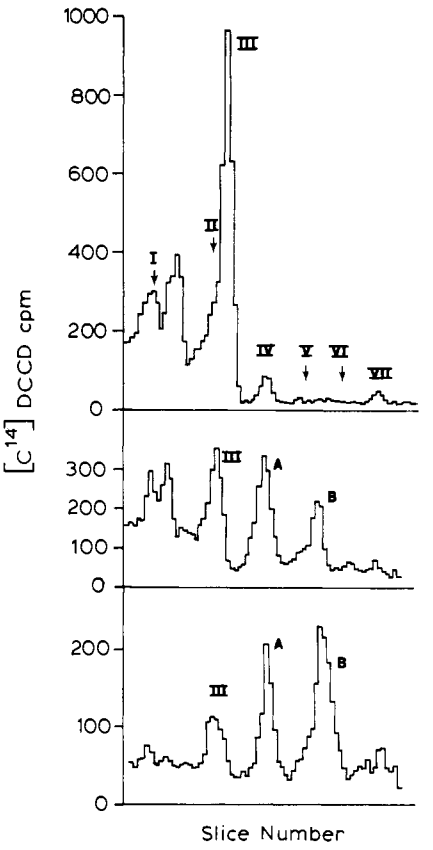

FIGURE 6: Effect of chymotrypsin cleavage on subunit III, monitored by using [^{14}C]DCCD-labeled cytochrome *c* oxidase. The upper trace shows the labeling by [^{14}C]DCCD of beef heart cytochrome *c* oxidase incorporated into vesicles by the cholate dialysis technique. The middle trace shows the labeling profile of an aliquot of vesicles that has been incubated with chymotrypsin as described under Results. The bottom trace is the labeling profile of a second aliquot of vesicles which had been treated with 2% Triton X-100 prior to incubation with chymotrypsin.

both ends if there were a single cleavage site close to the middle of this 33 000-dalton polypeptide.

To confirm that the chymotrypsin-generated fragment indeed came from subunit III and to localize it more specifically in the sequence, chymotrypsin experiments were conducted on [^{14}C]DCCD- and iodo[^{14}C]acetamide-labeled enzyme. DCCD has been shown to label subunit III specifically at Glu-90 (Prochaska et al., 1980) while iodoacetamide reacts

specifically with this subunit at Cys-115 (Malatesta & Capaldi, 1982).

The labeling of cytochrome *c* oxidase by [^{14}C]DCCD in reconstituted vesicles is shown in Figure 6. More than 75% of the radioactivity incorporated into the membranes is located in subunit III. Apart from a small amount of labeling of subunits II and IV, the rest is in high molecular weight impurities, one of which migrates close to subunit I in the gel system used here.

Chymotrypsin cleavage of DCCD-labeled membranes resulted in loss of the ^{14}C-labeled subunit III peak and appearance of a new, labeled band at the position of subunit IV, exactly as seen with cleavage of [^{3}H]adamantane diazirine labeled enzyme. These results taken together establish that the 17 000-dalton fragment derives from subunit III and the presence of DCCD in the peptide localizes it either to the N terminus or to the middle of the polypeptide.

In another set of experiments, cytochrome *c* oxidase vesicles were labeled with iodoacetamide prior to chymotrypsin cleavage. Iodoacetamide has been shown to label mainly subunit III (70% of the total counts incorporated), polypeptides a and c, and subunit(s) VII (Darley-Usmar et al., 1982). Chymotrypsin cleavage of iodoacetamide-labeled enzyme resulted in the disappearance of ^{14}C-labeled subunit III but without the appearance of any new peaks of radioactivity on the gel (result not shown). This can be explained by chymotrypsin cleaving subunit III just before and close after Cys-115, as indicated in Figure 5, to release a small fragment which is then lost from the gel during staining and destaining procedures. The important result is an absence of labeling of peak A by iodoacetamide, implying that the fragment is an N-terminal peptide generated by cleavage between Glu-90 and Cys-115. There are likely cleavage sites at Trp-99, Ala-100, Phe-101, and Tyr-102 which would yield a peptide of molecular weight around 11 000. Hydrophobic polypeptides are known to migrate anomalously in $NaDodSO_4$–polyacrylamide gel electrophoresis. [See, for example, the proteolipid protein of mitochondrial and bacterial ATP synthases (Sebald, 1977).] The N-terminal fragment 1–102 with two long stretches of hydrophobic residues could easily migrate with an apparent molecular weight larger than that determined from the sequence if it retains secondary structure in $NaDodSO_4$ or by becoming aggregated in the detergent (i.e., M_r 17 000).

Chymotrypsin cleavage of DCCD-labeled cytochrome *c* oxidase generated a second new ^{14}C-labeled peak (peak B in Figure 6) migrating between subunits V and VI. (This may correspond to the new, broad peak around subunit VI seen after digestion of the [^{3}H]adamantane diazirine labeled enzyme.) The fragment in peak B containing DCCD, and thus including Glu-90, must be derived from a further cleavage of the fragment in peak I at a site or sites toward the N terminus. The molecular weight calculated for fragment B from its migration on gels is 9000.

Figure 6 shows the labeling profile of DCCD-labeled cytochrome *c* oxidase both in experiments where chymotrypsin was added to intact vesicles and in experiments where the protease was added to membranes that had been disrupted with 2% Triton X-100. The two labeling profiles differ in that the amount of cleavage of subunit III is more when Triton X-100 is present. Also, there is more of peak B in relation to peak A and less of the DCCD-labeled high molecular weight impurities. Apparently, the presence of the detergent makes the protease cleavage more efficient, presumably by perturbing lipid–protein interactions. However, no new bands of radioactivity were obtained by allowing access of chymotrypsin to the interior as well as the exterior of the vesicles. This confirms that the major chymotrypsin cleavage sites are on the outer surface of the oriented vesicles, i.e., in the C domain of cytochrome *c* oxidase.

The orientation of subunit III in Figure 5 would place two loops from the N-terminal half of the polypeptide in the C domain. One of these loops contains the iodoacetamide-reactive site, Cys-115, which is rapidly cleaved away; the second loop involving residues 32–40 contains several potential chymotrypsin cleavage sites. A fragment derived by cleavage in this loop and cleavage around Cys-115, e.g., fragment 35–102, would have a molecular weight of 7500, in the same range as that derived for fragment B.

Discussion

Cytochrome *c* oxidase, the terminal member of the mitochondrial electron transfer chain, catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen and couples this to the movement of protons across the mitochondrial inner membrane [for recent reviews, see Azzi (1980), Wikstrom & Krab (1979), and Capaldi et al. (1983b)]. Thus, cytochrome *c* oxidase is a redox-linked proton pump.

Studies of structure–function relationships, particularly with respect to the coupling reaction, are better done in membranes reconstituted from purified cytochrome *c* oxidase and phospholipid than in mitochondrial preparations. Such vesicle preparations are also better systems than mitochondrial membranes in which to analyze in detail the folding of individual subunits in the cytochrome *c* oxidase complex. Membrane vesicles that retain the proton pumping function of cytochrome *c* oxidase have been obtained (Carroll & Racker, 1977; Wikstrom & Krab, 1979), but there is no consensus at present about the orientation of enzyme molecules in these vesicles. Several workers have claimed that 100% of the cytochrome *c* oxidase molecules are oriented with their C side out (Carroll & Racker, 1977; Eytan & Broza, 1978; Kornblatt et al., 1975); others have claimed a random (50%) orientation (Wrigglesworth & Nicholls, 1979; Nicholls et al., 1980). Recently, Casey et al. (1982) have presented evidence that 80–85% of the cytochrome *c* oxidase molecules are oriented C side out in vesicles prepared by cholate dialysis. This was based on heme reduction experiments with membrane-impermeant electron donors and on studies of the kinetics of the reduction of a spin-label covalently bound to the enzyme.

In this study, we confirm the results of Casey et al. (1982) by using heme reduction as one assay of the orientation of cytochrome *c* oxidase in our vesicle preparations. We have also used direct structural approaches to determine the orientation of the enzyme. Trypsin was found to cleave no more than 5–10% of the subunit IV in intact reconstituted vesicles, but 100% of this subunit in vesicles made leaky to the protease with Triton X-100. Previous studies have established that trypsin cleaves subunit IV close to the N terminus in submitochondrial particles and thus from the M side of the membrane (Malatesta et al., 1983a). The trypsin data indicate that only 5–10% of the cytochrome *c* oxidase molecules in the vesicles prepared by cholate dialysis are oriented with their M domains outermost. Labeling studies with [^{35}S]DABS, likewise, indicate that only a small fraction (less than 10%) of the oxidase molecules have their M domains exposed in these vesicles. Thus, all of the evidence in the present study and the results of Casey et al. (1982) are consistent. They indicate that at least 80% and probably more of the cytochrome *c* oxidase molecules in vesicles reconstituted by the cholate dialysis procedure are oriented with their C domain outermost. The trypsin digestion and [^{35}S]DABS labeling

experiments rule out the possibility that a large portion of the oxidase molecules remains unincorporated and instead lies on the outer surface of the vesicles. The fact that 80% of the oxidase molecules are readily reduced by the functional electron donor cytochrome *c* and recent studies on the stoichiometry and turnover rates in proton pumping by reconstituted vesicles (Penttila, 1983) argue against the denaturation of the protein during incorporation into membranes. In summary, cytochrome *c* oxidase containing vesicles reconstituted by cholate dialysis represent a highly oriented, functionally active preparation suitable for studies on the topology of the enzyme.

The protease digestion studies and chemical labeling experiments described here complement previous studies of the arrangement of subunits in beef heart cytochrome *c* oxidase [reviewed in Capaldi et al. (1983b)]. Both trypsin and chymotrypsin cleave polypeptides b and c in the intact reconstituted vesicles, establishing for the first time that these polypeptides are associated with the C domain of cytochrome *c* oxidase. Whether or not these are bona fide subunits with a function in cytochrome *c* oxidase activity remains a matter of debate [e.g., see Kadenbach & Merle (1981)]. The DABS labeling data obtained with reconstituted membranes are qualitatively similar to those seen with intact mitochondria (Ludwig et al., 1979). The relative labeling of subunits II, III, a + V (not resolved in the gel system used here), and b, c + VI (not resolved) was nearly the same in intact vesicles and in detergent-disrupted membranes. Subunits IV and VII(s) were proportionally less labeled in intact membranes compared with detergent-disrupted vesicles. Thus, in agreement with labeling studies on intact mitochondria, one or more of the small subunits, collectively called VII(s) here, appears to be exposed along with IV on the M side of the inner membrane.

Chymotrypsin cleavage experiments establish clearly that subunit III is part of the C domain. The pattern of proteolytic cleavage tentatively places two stretches of sequence in the C domain, one of which contains Cys-115. Localization of Cys-115 to the C domain is also indicated by the finding that this residue is the site of cross-linking of thionitrobenzoate-modified yeast cytochrome *c* bound at the high-affinity site for substrate (Malatesta & Capaldi, 1982). Further analysis of the folding of subunit III is now possible by determining the site(s) of labeling of this polypeptide by [^{35}S]DABS or other membrane-impermeant protein-modifying reagents in intact reconstituted vesicles. The detailed folding of other subunits can also be determined by such labeling studies, without recourse to modification on whole mitochondria or submitochondrial particles, and subsequent purification of the enzyme for further analysis.

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