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Arrangement of Subunit IV in Beef Heart Cytochrome *c* Oxidase Probed by Chemical Labeling and Protease Digestion Experiments[†]

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ABSTRACT: The arrangement of subunit IV in beef heart cytochrome *c* oxidase has been explored by chemical labeling and protease digestion studies. This subunit has been purified from four samples of cytochrome *c* oxidase that had been reacted with *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]sulfonate (NAP-taurine), diazobenzene[^{35}S]sulfonate, 1-myristoyl-2-[12-[(4-azido-2-nitrophenyl)amino]lauroyl]-*sn*-glycero-3-[^{14}C]phosphocholine (I), and 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine (II), respectively. The labeled polypeptide was then fragmented by cyanogen bromide, at arginyl side chains with trypsin (after maleylation), and the distribution of the labeling within the sequence was analyzed. The N-terminal part of subunit IV (residues 1–71) was shown to be heavily labeled by water-soluble, lipid-insoluble reagents but not by the phospholipid derivatives. These latter reagents labeled only in the region of residues 62–122, containing the long hydrophobic and putative membrane-spanning stretch. Trypsin cleavage of native cytochrome *c* oxidase complex at pH 8.2 was shown to clip the first seven amino acids from subunit IV. This cleavage was found to occur in submitochondrial particles but not in mitochondria or mitoplasts. These results are interpreted to show that subunit IV is oriented with its N terminus on the matrix side of the mitochondrial inner membrane and spans the membrane with the extended sequence of hydrophobic lipid residues 79–98 buried in the bilayer.

Cytochrome *c* oxidase, the terminal part of the electron-transport chain, is a redox-linked proton pump containing two heme moieties (*a* and a_3) and two copper atoms as electron acceptors (Azzi, 1980; Capaldi et al., 1983). The protein is Y shaped with two arms of the Y, called the M_1 and M_2 domains, each spanning the mitochondrial inner membrane (Deatherage et al., 1982; Fuller et al., 1979, Henderson et al., 1977).

Beef heart cytochrome *c* oxidase is isolated as a complex of at least 7 and up to 13 different polypeptides (Steffens & Buse, 1976; Downer et al., 1976; Kadenbach & Merle, 1981; Verheul et al., 1979), the three largest of which are coded for on mitochondrial DNA (mtDNA)[1] (Anderson et al., 1982). Labeling studies with diazobenzene[^{35}S]sulfonate (Ludwig et al., 1979; Prochaska et al., 1980) have established that all of the subunits are at least partly exposed to water in both the detergent-dispersed and the membrane-bound enzyme. Subunits II and III were found to be reactive to DABS in intact mitochondria, showing that these components are on the cytoplasmic side of the mitochondrial inner membrane and thus contribute to the stalk of the Y or C domain (Eytan et al., 1975; Ludwig et al., 1979). Subunits II, III, IV, and VII were reactive to DABS in inverted membranes or submitochondrial particles, and therefore, these components must be a part of the two M domains (Ludwig et al., 1979).

The arrangement of protein with respect to the lipid bilayer has also been examined by labeling with radioactive arylazidophospholipids (Bisson et al., 1979; Prochaska et al., 1980), iodonaphthyl azide (Cerletti & Schatz, 1979), and adamantane diazirine (Georgevich & Capaldi, 1982). Subunits I and III were heavily labeled by each of these hydrophobic reagents and must contribute the major portion of the protein in contact with lipid. Subunits II, IV, and VII were also labeled by the lipid analogues but not subunits V and VI. The above studies along with the results of cross-linking experiments (Briggs & Capaldi, 1977, 1978) have been used to derive a model for the arrangement of subunits in the cytochrome *c* oxidase complex (Capaldi et al., 1983). However, they do not locate the positions of the N and C termini of each polypeptide in the complex and do not establish which segments of the polypeptide are within the lipid bilayer. This information is critical to an understanding of the assembly of the protein, as well as to our understanding of how a membrane protein is stable when partly exposed to water and partly buried in a hydrocarbon environment.

Recently, the sequence of all of the subunits of beef heart cytochrome *c* oxidase has been obtained either from the protein

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DABS, diazobenzenesulfonate; mtDNA, mitochondrial DNA; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; TPCK, tosylphenylalanyl chloromethyl ketone; STI, soybean trypsin inhibitor; IgG, immunoglobulin G; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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directly [e.g., see Steffens et al. (1979), Sacher et al. (1979), and Tanaka et al. (1981)] or by sequencing of mtDNA [e.g., see Anderson et al. (1982) and Bibb et al. (1981)]. This has opened the way for studies on the topography of individual subunits in the cytochrome *c* oxidase complex. Here, we describe chemical labeling and protease digestion studies aimed at determining the arrangement of subunit IV, the largest of the cytoplasmically synthesised polypeptides in the cytochrome *c* oxidase complex.

Materials and Methods

Enzyme Preparation and Assays. Beef heart cytochrome *c* oxidase was prepared according to the method of Capaldi & Hayashi (1972). Electron-transport activity was measured polarographically by the procedure of Vik & Capaldi (1980). Mitochondria were isolated from beef hearts by the small-scale procedure of Smith (1967) employing Nagarse to disrupt the tissue. Rat liver mitochondria and mitoplasts were prepared from these liver mitochondria by using procedures described by Schnaitman & Greenawalt (1968). Submitochondrial particles were prepared from heart and liver mitochondria by the sonication procedure of Crane et al. (1956).

Preparation of Protein-Modifying Reagents. [^{35}S]DABS (5–9 Ci/mmol) was prepared from [^{35}S]sulfanilic acid (Amersham Searle) according to Tinberg et al. (1974). [^{35}S]-NAP-taurine (39 mCi/mmol) was synthesized from [^{35}S]-taurine by the method of Staros et al. (1975). The final product was 70% pure as judged by thin-layer chromatography on silica gel developed with chloroform–methanol. The major impurity (25%) was unreacted [^{35}S]taurine, as detected by 0.25% ninhydrin spray in butanol. There were also trace amounts of photolytic degradation products.

The preparation of 1-myristoyl-2-[12-[(4-azido-2-nitrophenyl)amino]lauroyl]-*sn*-glycero-3-[^{14}C]phosphocholine (I) and of 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine (II) has been described by Bisson & Montecucco (1981). The specific radioactivities of the two arylazidophospholipids were 177 and 3.9 Ci/mmol, respectively.

Labeling of Detergent-Dispersed Enzyme with [^{35}S]DABS and [^{35}S]NAP-taurine. Cytochrome *c* oxidase (0.5 mg/mL) was reacted with [^{35}S]DABS (20 μM) at room temperature for 10 min in a buffer containing 0.1% Tween 80 in 100 mM NaCl and 50 mM sodium phosphate (pH 7.5). The reaction was stopped by the addition of 10 mM histidine, and the mixture was centrifuged through 10% sucrose in 10 mM sodium phosphate for 12–16 h at 200000*g* to separate the protein from noncovalently bound protein-modifying reagent. The isolated enzyme (0.6 mg/mL) was also reacted with [^{35}S]-NAP-taurine (1.3 mM) in a buffer containing 0.1% Tween 80 with 100 mM NaCl and 50 mM sodium phosphate (pH 7.5). The reaction was initiated by illuminating the sample with high-intensity white light [(0.5–2.0) × 10^6 ergs/(cm^2 s)] that was filtered through a $CuSO_4$ solution to remove infrared light. Illumination was continued for 10 min at 20 °C in a water-jacketed cell. Cytochrome *c* oxidase was separated from noncovalently bound NAP-taurine by centrifugation through 10% sucrose in 10 mM sodium phosphate (pH 7.4) as in the DABS labeling experiments.

Labeling of Membranous Cytochrome c Oxidase with Arylazidophospholipids. Egg lecithin (Sigma) dissolved in $CHCl_3$–CH_3OH (2:1) was dried under a stream of nitrogen. The lipid was then suspended in a buffer of 10 mM sodium phosphate (pH 7.5) to a 0.4% solution by sonication using a Branson bath sonicator on the full setting. The sonication step was continued until the phospholipid solution was optically clear. Cytochrome *c* oxidase was incorporated into the egg lecithin vesicles by the method of Eytan & Broza (1978). Vesicles containing arylazidophospholipid were prepared according to Bisson et al. (1979). The ratios of arylazidophospholipid to egg lecithin used were 0.4% and 0.1% for azidophospholipids I and II, respectively. Reaction of cytochrome *c* oxidase and the azidophospholipids was initiated by illuminating the sample under UV light through glass [intensity (1–3) × 10^3 ergs/(cm^2 s)] for 30 min on ice. After vesicles were labeled by arylazidophospholipids, they were dissolved in 1% Triton X-100 and then centrifuged through sucrose (15%) for 12 h to separate the enzyme from nonbound labeling reagent and from excess phospholipid.

Trypsin Cleavage of Cytochrome c Oxidase. Purified cytochrome *c* oxidase dissolved in 0.15 M sodium phosphate, pH 8.2, with 1% Triton X-100 was incubated with trypsin (TPCK treated; Worthington) in the ratio 20:1 (w/w) for 2 h at room temperature. The reaction was stopped by adding a 5-fold molar excess (over trypsin) of soybean trypsin inhibitor (STI). The cleavage products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis or by gel filtration on Bio-Gel P100 in 2% $NaDodSO_4$.

Mitochondria, submitochondrial particles, or mitoplasts suspended in 0.15 M sucrose–10 mM sodium phosphate, pH 7.8, were reacted with trypsin in a 10:1 (w/w) ratio for 2 h on ice. The reaction was stopped by adding PMSF, and the membranes were dissociated in $NaDodSO_4$ for $NaDodSO_4$–polyacrylamide gel electrophoresis [see Fuller et al. (1981)].

Preparation of Subunit IV Antibody. Subunit IV (pure as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis) was dissolved in a buffer containing 6 M urea. The protein (2 mg) was mixed with an equal volume of Freunds complete adjuvant and injected into a rabbit at multiple sites on the back. Booster injections of subunit IV (1 mg; mixed with an equal volume of Freunds incomplete adjuvant) were also injected into the back of the rabbit. Samples of blood were collected from the ear vein, allowed to clot at room temperature, and then centrifuged at 15000 rpm for 15 min. The IgG fraction was partially purified by ammonium sulfate fractionation and stored at −70 °C in a 50 mM Tris-HCl buffer, pH 7.4.

Transfer of proteins to nitrocellulose paper was performed as described by Towbin et al. (1979). Antibody binding was performed as described by Millett et al. (1983).

Separation of Subunits of Cytochrome c Oxidase by Column Chromatography. Labeled cytochrome *c* oxidase (5 mg) reacted with [^{35}S]DABS, [^{35}S]NAP-taurine, or arylazidophospholipids I or II was mixed with unmodified enzyme (95 mg) and the total protein dissolved in 5% $NaDodSO_4$ and 0.2% β-mercaptoethanol. After 250 mg of sucrose was added, the sample was loaded onto a Bio-Gel P100 column (4.2 × 32 cm) with 2% $NaDodSO_4$ as the eluant. The flow rate was 15 mL/h, and 2-mL fractions were collected. Radioactivity was assayed by liquid scintillation counting and protein assayed by the absorption at 280 nm. Samples across each peak were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. The peak containing subunit IV was pooled and then lyophilized. This material was eluted through a Bio-Gel P4 column (5 × 35 cm) in 0.01% $NaDodSO_4$. Protein was detected by the absorbance at 280 nm. The peak fractions of subunit IV were pooled, and this solution was dialyzed against 10 volumes of 70% ethanol. The precipitated protein was collected by centrifugation and stored at 4 °C.

Cleavage of Subunit IV at Methionine. Purified and detergent-free subunit IV (1–2 nmol) was cleaved with CNBr as described by Sacher et al. (1979). Peptides were dissolved

in 70% acetic acid and then subjected to gel filtration on a Bio-Gel P10 (200–400 mesh) column (2.5 × 300 cm) in 70% acetic acid. The eluant was monitored for protein at 280 nm and for radioactivity by scintillation counting.

Cleavage of Subunit IV at Arginines. Subunit IV was maleylated to block all of the lysine residues as discussed by Sacher et al. (1979). The protein was then cleaved with trypsin (TPCK–trypsin; Merck) in 0.5% NH_4HCO_3, pH 8.2, in the presence of 0.1% $NaDodSO_4$. Arginyl fragments were separated on a Bio-Gel P30 column equilibrated in 70% acetic acid on a Sephadex LH 60 column in ethanol–88% formic acid (7:3).

Cleavage of Subunit IV at Lysines and Arginines. Subunit IV from control and trypsin-treated cytochrome *c* oxidase was concentrated under a stream of N_2 to 0.4–0.6 mg/mL in 0.1% $NaDodSO_4$, 0.1% octyl glucoside, and 80 mM $NaHCO_3$, pH 8.0. Trypsin (TPCK inhibited; Worthington) was added to give a final trypsin to protein ratio of 1:13 (w/w). After digestion, the protein was concentrated to 1.6 mg/mL. A sample (250 μL) was then injected into an Altex 334 high-performance liquid chromatograph equipped with a Brownlee RP300 column (4.6 × 250 mm). The eluant was monitored at 210 nm. The column was eluted for 20 min with 5 mM sodium phosphate, pH 7.0, prior to the application of a linear gradient of from 0 to 15% propanol in 5 mM sodium phosphate over a 90-min period (flow rate 0.8 mL/min). The peptides were identified by amino acid analysis.

Analytical Procedures. Amino acid analyses were performed on a Beckman Multichrome or a Dionex single-column amino acid analyzer equipped with a computing integrator. Samples were hydrolyzed in 6 N HCl in vacuo at 108 °C for 20 or 44 h. Radioactivity was measured by liquid scintillation counting on a Packard Tri Carb Model 3385 liquid scintillation counter.

Results

The arrangement of subunit IV in the beef heart cytochrome *c* oxidase complex was explored by labeling the enzyme with hydrophilic and hydrophobic protein-modifying reagents and by protease digestion studies. Thus, samples of enzyme were reacted with [^{35}S]DABS, [^{35}S]NAP-taurine, or the radioactive arylazidophospholipids; subunit IV was isolated by gel filtration in 2% $NaDodSO_4$ and fragmented by cleavage at methionine residues with CNBr in 70% formic acid.

Labeling with NAP-taurine. The CNBr fragments of NAP-taurine-labeled subunit IV could be partly resolved by gel filtration on a Bio-Gel P30 column in 70% acetic acid (Figure 1). Five peaks were separated, and these were identified by amino acid analysis. The void volume peak contained uncleaved subunit IV, concatamers of CB1 with other fragments, and CB1 alone. The other four peaks contained CB2+3, CB3, CB5, and CB2, respectively. The barograph shows the distribution of radioactivity. In the case of CB5, the peak of radioactivity was shifted slightly with respect to the peak of protein. Fragments copurifying in the void volume peak were pooled, lyophilized, resuspended in 70% acetic acid, and rechromatographed on a Bio-Gel P60 column. This resolved CB1 from pure subunit IV and large concatamers.

The incorporation of NAP-taurine into different fragments is listed in Table I as cpm per mole, the amount of protein having been determined from amino acid analysis. More than 80% of the reagent incorporated into subunit IV was located in CB1. There was a significant amount of labeling of both CB_2+CB3 and CB3 but only trace labeling of the C-terminal fragment CB5.

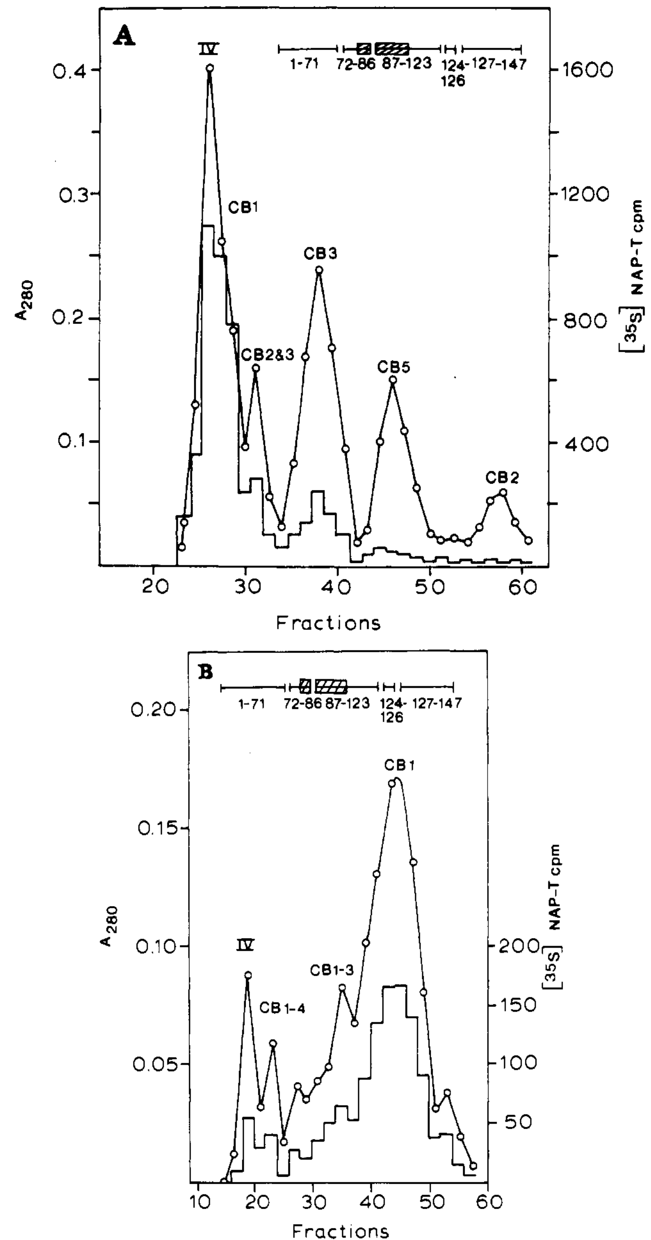

FIGURE 1: Labeling of subunit IV by NAP-taurine. (A) Cyanogen bromide fragments resolved by gel filtration on a Bio-Gel P10 (200–400 mesh) column (2.5 × 200 cm) in 70% acetic acid. The barograph shows the distribution of radioactivity in the fractions. (B) Chromatography of the void volume peak from the column shown in Figure 1A on a Bio-Gel P60 column (2.5 × 80 cm) in 70% acetic acid.

Table I: Labeling of Subunit IV by DABS and NAP-taurine

fragment	residues	DABS (cpm/nmol)	NAP-taurine (cpm/nmol)
CB1	1–71	725	230
CB2+3	72–123	70	45
CB3	86–123	45	30
CB5	127–147	15	5

Labeling with [^{35}S]DABS. The CNBr fragments of DABS-labeled subunit IV were resolved as described for NAP-taurine. The distribution of radioactivity is given in Table I. CB1 was by far the most heavily labeled part of subunit IV with more than 75% of the total amount of reagent incorporated in this fragment. CB2+3 contained around 10% of the DABS incorporated, most of this being in CB3 and thus in the hydrophilic stretch at the C-terminal side of the hy-

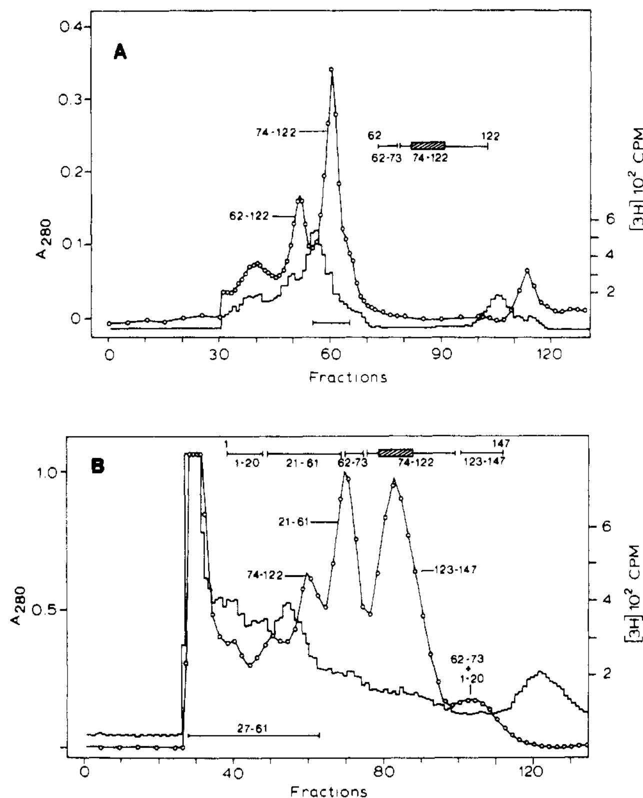

FIGURE 2: Labeling of subunit IV by arylazidophospholipid II. (A) Arginyl fragments separated on a Bio-Gel P30 column (2.5 × 300 cm) in 70% acetic acid. (B) Chromatography of the void volume and large molecular weight protein from the column in (A) (fractions 27–61) on a Sephadex LH60 column in ethanol–88% formic acid (7:3). The barograph shows the distribution of radioactivity in the fractions.

drophobic central sequence. The C-terminal end, CB5, was poorly labeled in relation to the number of DABS-reactive sites in this part of the sequence.

Labeling with [^3H]- *and* [^{14}C]*Arylazidophospholipids.* The distribution of arylazidophospholipids in the different CNBr fragments after labeling was analyzed initially on NaDodSO$_4$–polyacrylamide gels because of the high specific radioactivity of the lipid analogues. These experiments established that all of the [^{3}H]arylazidophospholipid I (with the reactive arylazido group close to the head group) as well as all of the [^{14}C]arylazidophospholipid II (with the reactive nitrene at the methyl terminus of one fatty acid chain) was incorporated into CB2+3 and CB3. However, the amount of radioactivity in the CNBr fragments was only a small fraction of that in the purified subunit IV before cleavage. This may be due to acid hydrolysis of the labile lipid phosphoester bond between the arylazido-containing fatty acid analogue and the radioactive head-group region of the phospholipid.

As an alternative approach, subunit IV was fragmented at arginine residues by trypsin cleavage after lysine groups were first blocked with maleimide. Fragments were separated by gel filtration on Bio-Gel P30 column in 70% acetic acid as before. Figure 2 shows the elution profile of trypsin-cleaved subunit IV that had been labeled with arylazidophospholipid I. Several major protein peaks were resolved, and these were identified by amino acid analysis. Radioactivity was associated with the void volume material and with fragments running close to the void volume. There was also a peak of radioactivity running ahead of the peak of fragment 74–122. Fragments with a phospholipid molecule attached have a molecular mass 750 daltons greater than that of the unmodified material and would be expected to elute from the column ahead of the

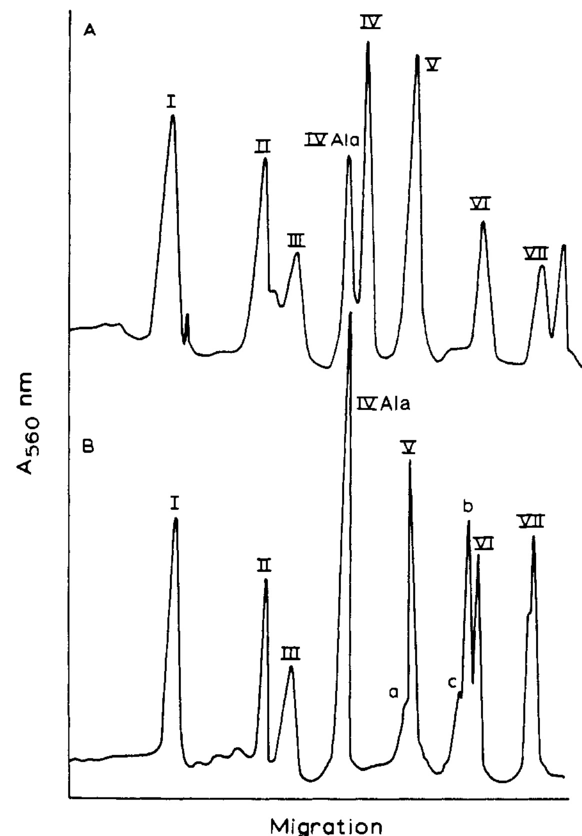

FIGURE 3: Trypsin digestion of purified cytochrome *c* oxidase. Purified beef heart cytochrome *c* oxidase was digested with trypsin (1:20 w/w) for 2 h in 1% Triton X-100, pH 8.2, at room temperature. The trypsin was inhibited by phenylmethanesulfonyl fluoride and dissociated in NaDodSO$_4$ and urea prior to NaDodSO$_4$–urea–polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Coomassie blue. Subunits are numbered according to Downer et al. (1976). (A) 40 μg of trypsin-treated cytochrome *c* oxidase. IVAla identifies the uncleaved subunit IV. (B) 40 μg of control cytochrome *c* oxidase.

unlabeled protein as seen in Figure 2. There were no peaks of radioactivity corresponding to fragments 21–61 and 123–147 or to the fractions containing 62–73 and 1–20. [The coelution of these last two fragments was indicated by amino acid analysis and confirmed by sequencing (results not shown).]

Hydrophobic polypeptides aggregate in 70% acetic acid, particularly after lyophilization in the absence of dispersing detergents. These aggregates would then run in the void volume of the Bio-Gel P30 column. Figure 2B shows the composition of the void volume material from the P30 column dissociated in 75% ethanol–25% formic acid and then chromatographed through a column of Sephadex LH 60 in the same solvent system. Three peaks of protein were resolved, the two major peaks containing fragments 62–122 and 74–122. There were peaks of radioactivity corresponding to both of these protein peaks.

Protease Digestion Studies on Isolated Cytochrome c Oxidase. Our previous studies have shown that incubation of beef heart cytochrome *c* oxidase with trypsin (1:20 w/w) at pH 7.4 results in cleavage of polypeptides b and c without an effect on the electron-transfer activity (Ludwig et al., 1979). More recent experiments indicate that the cleavage of beef heart cytochrome *c* oxidase by trypsin is pH sensitive and that at pH 8.0 or higher subunit IV is cleaved by the protease in addition to removal of polypeptides b and c from the gel profile. Figure 3 shows profiles of the trypsin-treated and control of untreated cytochrome *c* oxidase after incubation with the

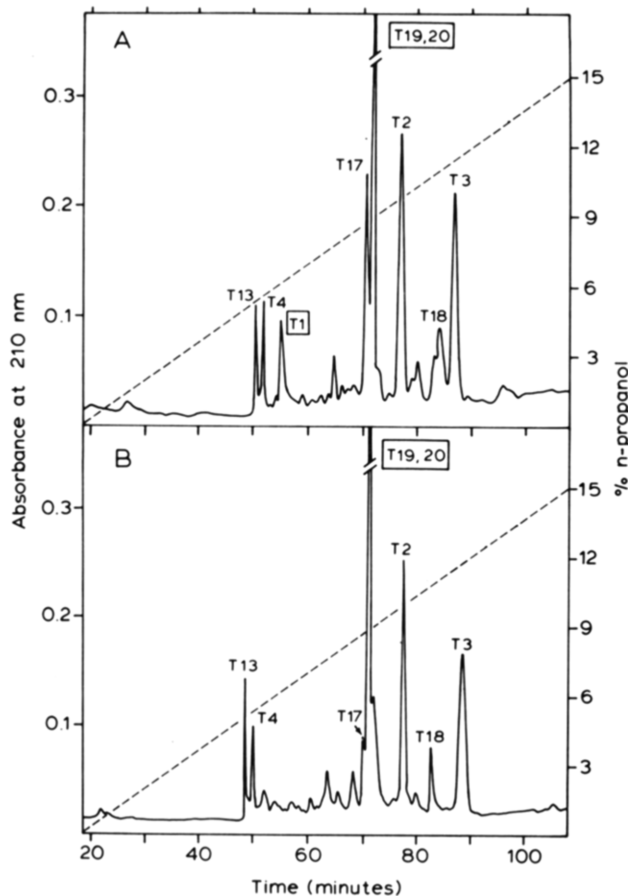

FIGURE 4: Separation of trypsin fragments of subunit IV by HPLC. (A) Elution profile of subunit IV from the control (untreated) cytochrome *c* oxidase resolved at low concentrations of 1-propanol (0–15%) in 5 mM sodium phosphate, pH 7.0; flow rate 0.8 mL/min on an RP-300 column. (B) Elution profile of subunit IV purified from trypsin-treated enzyme. Peptides were identified by amino acid analysis. The peak labeled T17 contains a mixture of trypsin fragment T17 and a concatamer. This concatamer was not present in a significant amount in the profile of the polypeptide from trypsin-cleaved enzyme.

protease (1:20 w/w) for 2 h at room temperature in a buffer (pH 8.2) containing 1% Triton X-100. Subunit IV is cleaved under these conditions to a polypeptide of approximately 16 000 daltons, corresponding to the loss of a fragment with a molecular weight of approximately 1000.

The site of cleavage of subunit IV was identified by comparing the trypsin fragments of subunit isolated from trypsin-treated cytochrome *c* oxidase with those from untreated enzyme. For this analysis, the fragments were separated by HPLC on a reverse-phase column as shown in Figure 4. The profiles in Figure 4 show those fragments of subunit IV eluting at low concentrations of the organic solvent 1-propanol. Individual fragments were identified by amino acid analysis and are numbered according to their order from the N terminus as deduced from the amino acid sequence (Sacher et al., 1979). It can be seen that fragment T1 is missing from subunit IV isolated from the trypsin-cleaved cytochrome *c* oxidase. Fragments T2 and T3 near the N terminus and T19 and T20 at the C terminus are all present in the same relative amounts in the digested and unmodified subunit. Thus, cleavage of subunit IV by trypsin in native cytochrome *c* oxidase occurs exclusively at the N terminus, with residues 1–7 being removed.

Trypsin Digestion of Cytochrome c Oxidase in the Mitochondrial Inner Membrane. The cleavage of the N terminus of subunit IV by trypsin provides an opportunity to determine the orientation of this subunit in the mitochondrial inner membrane.

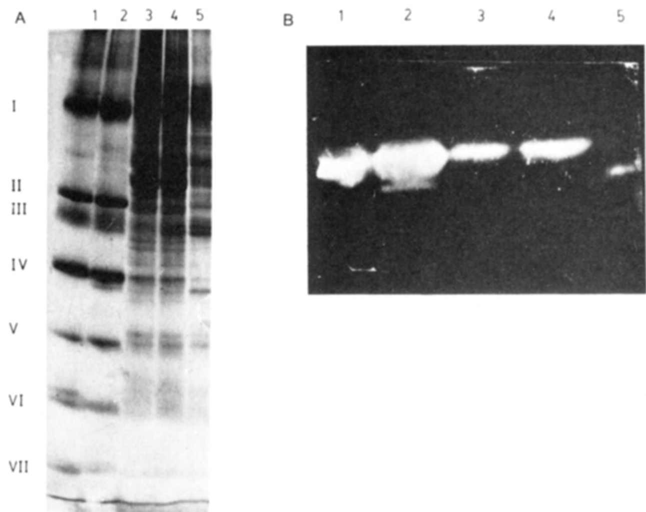

FIGURE 5: Trypsin digestion of beef heart mitochondria. Beef heart mitochondria and submitochondrial particles were digested with trypsin. (A) Coomassie blue stained $NaDodSO_4$–polyacrylamide gel of mitochondrial proteins: (lane 1) 40 μg of control cytochrome *c* oxidase; (lane 2) 40 μg of trypsin-treated cytochrome *c* oxidase; (lane 3) 100 μg of control beef heart mitochondria; (lane 4) 100 μg of trypsin-treated beef heart mitochondria; (lane 5) 100 μg of trypsin-treated submitochondrial particles. Cytochrome *c* oxidase subunits are numbered according to Downer et al. (1976). (B) Binding of subunit IV antibody. The section of the gel shown in (A) containing subunit IV was transferred to nitrocellulose paper and antibody binding performed. The numbering of the lanes is as described in (A).

Examination of the effect of trypsin on membrane-bound cytochrome *c* oxidase was facilitated by using the antibody blot technique devised by Towbin et al. (1979). With this method, it was possible to identify subunit IV of cytochrome *c* oxidase in gels loaded with whole mitochondria or submitochondrial particles. There was, therefore, no need to isolate cytochrome *c* oxidase after trypsin treatment of the membrane preparation in order to see if cleavage of subunit IV had occurred.

Figure 5A shows the Coomassie blue stained gel of trypsin-treated mitochondria (lane 4) and submitochondrial particles (lane 5). Control mitochondria (lane 3) purified cytochrome *c* oxidase (lane 1) and trypsin-treated purified cytochrome *c* oxidase (lane 2) are shown for comparison. The protein on the middle section of the gel containing subunit IV was transferred to nitrocellulose paper, and subunit IV specific antibody, was bound. The bound antibody was then visualized under UV light after binding fluorescein isothiocyanate labeled goat anti-rabbit antibody. Figure 5B shows that only in submitochondrial particles is subunit IV cleaved (lane 5). The cleavage results in the loss of the same size fragment as found in the control-purified enzyme (lane 2). Beef heart mitochondria treated with Nagarse lose some but not all of their outer membrane. The absence of an effect of trypsin from the C side could, therefore, reflect the fact that trypsin cannot reach the inner membrane. Mitoplasts in which the outer membrane is completely removed are difficult to prepare from beef heart mitochondria but are readily obtained from rat liver mitochondria. Subunit IV antibody was thus reacted with both mitoplasts and submitochondrial particles prepared from rat liver. Subunit IV was again only cleaved in submitochondrial particles but not in mitoplasts (results not shown).

The effect of trypsin cleavage on cytochrome *c* oxidase activity was measured in submitochondrial particles. Under

conditions in which essentially all of the subunit IV was cleaved to the smaller fragment (as judged by antibody blotting), the electron-transfer activity of the protease-treated cytochrome *c* oxidase was the same as that in the untreated membrane.

Discussion

Subunit IV, the largest of the cytoplasmically synthesized subunits of cytochrome *c* oxidase, contains 147 amino acids of which 48 are charged residues (Sacher et al., 1979). The polarity (Capaldi & Vanderkooi, 1972) of this subunit is 49.0, a value more typical of a nonmembrane or extrinsic membrane protein than of a transmembrane polypeptide. The sequence reveals a polypeptide with three distinct domains, a hydrophilic N terminus, a central hydrophobic domain, and a C-terminal hydrophilic domain.

The central hydrophobic domain, including residues 79–98, has a very favorable free energy for being in a hydrocarbon medium and out of water (Capaldi et al., 1982). Such long stretches of hydrophobic amino acids are not found even in the largest water-soluble proteins where, in principle, they can be buried in the interior of the protein (von Heinje, 1981; Capaldi, 1982). They are a common feature of intrinsic membrane proteins, and where structural data are available, as for glycophorin and bacteriorhodopsin, these sequences form transmembrane helices (von Heinje, 1981; Engelman & Steitz, 1981; Capaldi, 1982).

From the sequence then, it can be inferred that subunit IV is arranged in the cytochrome *c* oxidase complex with its N terminus on one side of the membrane, with its C terminus on the opposite side, and with the central hydrophobic domain in the lipid bilayer. The chemical labeling and protease digestion studies described here were conducted in order to test this prediction.

The hydrophilic labeling reagents DABS and NAP-taurine both labeled the N-terminal part of the subunit from residues 1–78 much more heavily than the rest of the polypeptide (by a factor of 5–10). This is consistent with the hydrophilic nature of this part of the polypeptide and indicates that the N-terminal segment is outside the lipid bilayer, in the aqueous phase and on the surface of the complex. The C-terminal domain was also labeled as evident by the incorporation of both reagents into CB3 (which has several DABS-reactive sites in the sequence 99–123) and into CB5. However, the C-terminal part from 127–147 was not as heavily labeled by DABS as expected from the number of His, Lys, and Tyr residues in this segment, suggesting that this part of the polypeptide is partly shielded by association with other parts of the protein.

It is important to note that the labeling of subunit IV by NAP-taurine is similar to that of DABS and not arylazidophospholipid I. NAP-taurine, being amphipathic, could concentrate at the membrane–water interface with the reactive nitrene within the bilayer and thus act as a hydrophobic probe. However, this does not appear to be the case in the present study.

The labeling of subunit IV by the two different arylazidophospholipids, one with the reactive nitrene near the head group and the other with the reactive group at the methyl terminus, was similar. Both reagents reacted exclusively with the sequence 73–122, as evidenced by CNBr cleavage experiments and by fragmentation of the subunit with arginyl fragments. This is consistent with the hydrophobic sequence 79–98 being in contact with the lipid molecules.

Our labeling studies, therefore, indicate that the N- and C-terminal regions are outside the bilayer and the central hydrophobic domain is among the lipid fatty acid chains.

The most likely structure is the one described already with the N and C termini on opposite sides of the membrane and with the sequence 79–98 spanning the membrane in a helical structure (α helix or $^3/_{10}$-helix). An alternative arrangement with both the N and C termini on the same side of the membrane is not ruled out but appears less likely. It would require that subunit IV spans the membrane twice. This is possible if the hydrophobic stretch is in a β structure (10 residues would span 35 Å in this structure) but would require a tight turn in the center of the sequence. Such turns generally contain a Pro or Gly (Chou & Fasman, 1979), two residues which are absent from the hydrophobic sequence. A third possibility is that the hydrophobic sequence runs parallel to the lipid bilayer with subunit IV located exclusively on one side of the membrane. This is unlikely given that long hydrophobic sequences, when present, are the transmembrane elements in other intrinsic membrane proteins studied so far.

Previous studies have established that subunit IV is labeled by DABS exclusively from the M side of the mitochondrial inner membrane (Eytan & Broza, 1978; Ludwig et al., 1979). The results presented here show that DABS labels the N-terminal part of subunit IV heavily but the C-terminal part hardly at all. This is indirect evidence that the N-terminal part of this subunit is on the M side of the membrane. Direct evidence for this orientation is provided by the protease cleavage experiments.

Subunit IV is cleaved in detergent-dispersed cytochrome *c* oxidase to a smaller polypeptide missing the N-terminal seven amino acids. The subunit is cleaved as a product of the same size, as judged by migration on gels, in submitochrondial particles with the matrix side of the inner membrane available for attack by the protease. There was no cleavage of subunit IV in mitochondria or mitoplasts which have the cytoplasmic side available for attack.

The possibility that trypsin cleaves subunit IV at a different site in the isolated enzyme (N terminus) and in submitochondrial particles (C terminus) seems remote. The membrane-bound form of an enzyme is generally more resistant to cleavage by proteases than the isolated detergent-dispersed form as is the case for the cytochrome c_1 in complex III of the respiratory chain (Li et al., 1981). With subunit IV, the results of labeling with NAP-taurine and DABS suggest that the C-terminal part may be mostly shielded from chemical reagents and thus presumably from proteases.

Our conclusions, then, are that subunit IV of cytochrome *c* oxidase is a transmembrane polypeptide with its N terminus on the matrix side of the mitochondrial inner membrane and with its C terminus integrated into the large C domain extending on the cytoplasmic side of the mitochondrial inner membrane. Subunit IV is made as a precursor with a molecular mass approximately 3000 daltons larger than the mature polypeptide (Schmelzer & Heinrich, 1980). This is processed to the mature form by a protease located in the mitochondrial matrix space (Bohni et al., 1980). The orientation of subunit IV in the inner membrane is consistent with the precursor entering the membrane N terminus first with an N-terminal extension then being removed during the processing and integration of the polypeptide into the cytochrome *c* oxidase complex.

Registry No. I, 71303-93-0; II, 71761-11-0; DABS, 55318-89-3; NAP-taurine, 57462-29-0; cytochrome *c* oxidase, 9001-16-5.

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