Labeling of Cytochrome *c* Oxidase with [^{35}S]Diazobenzenesulfonate. Orientation of This Electron Transfer Complex in the Inner Mitochondrial Membrane†

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ABSTRACT: Isolated cytochrome *c* oxidase was fractionated by native-gel electrophoresis in Triton X-100, and a preparation of enzyme almost completely free of the usual impurities was recovered. This fraction was used to generate antibodies specific to cytochrome *c* oxidase. These antibodies inhibited cytochrome *c* oxidase activity rapidly and completely and immunoprecipitated an enzyme containing seven different subunits from detergent-solubilized mitochondria or submitochondrial particles. Reaction of detergent-solubilized cytochrome *c* oxidase with [^{35}S]diazobenzenesulfonate labeled all seven subunits although I and VI were much less reactive than the other five components. When cytochrome *c* oxidase was immunoprecipitated from mitochondria which had been reacted with [^{35}S]DABS, subunits II and III were the only components labeled. When the complex was immunoprecipitated from labeled submitochondrial particles, II, III, IV, V, and VII were all labeled. Polypeptides I and VI were not labeled from either side of the membrane. These results confirm earlier studies which showed that cytochrome *c* oxidase spans the mitochondrial inner membrane and is asymmetrically arranged across this permeability barrier.

Cytochrome *c* oxidase is a multipeptide complex containing hemes and copper atoms. The enzyme catalyzes the oxidation of cytochrome *c* by molecular oxygen and conserves the energy liberated in this redox reaction for the synthesis of ATP.

Recently, considerable progress has been made toward understanding the structure of cytochrome *c* oxidase. Two-dimensional arrays of the enzyme have been examined, and these give a first indication of the size and shape of the complex (Henderson et al., 1977; S. F. Fuller, R. A. Capaldi, and R. Henderson, unpublished experiments). Cross-linking studies of the isolated enzyme have provided data on the proximities of subunits within the complex (Briggs & Capaldi, 1977a). Cross-linking of cytochrome *c* to cytochrome *c* oxidase has localized the binding site for this substrate to subunit II in the beef heart enzyme (Briggs & Capaldi, 1977b; Bisson et al., 1977) and to subunit II (Bisson et al., 1978) and subunit III (Birchmeier et al., 1976) in the yeast enzyme.

There is good evidence that cytochrome *c* oxidase spans the mitochondrial inner membrane (Schneider et al., 1972; Hackenbrock & Hammon, 1975; Eytan et al., 1975), and this raises the question of how the individual components of the complex (hemes, copper, and subunit polypeptides) are distributed across the membrane. In this study we have used the membrane-impermeable protein-modifying reagent [^{35}S]-diazobenzenesulfonate to label those polypeptides exposed in intact mitochondria (with only the cytoplasm-facing side of the inner membrane available for reaction) and in submitochondrial particles which have the opposite orientation (i.e., the matrix side exposed). Our results confirm the transmembrane location of cytochrome *c* oxidase and show an asymmetric distribution of components across the membrane.

Experimental Procedures

Enzyme Preparations. Cytochrome *c* oxidase was prepared according to Capaldi & Hayashi (1972), Yonetani (1961), or Sun et al. (1968). Enzyme prepared as described by Kuboyama et al. (1972) was the kind gift of Dr. P. Jost, University of Oregon. Mitochondria were isolated from beef hearts according to Smith (1967), except that phosphate was substituted for Tris, and EDTA (20 mM) was added in experiments where DABS[1] was used. Only freshly prepared mitochondria were used for labeling experiments. Submitochondrial particles were obtained by sonication of the mitochondrial suspension (20 mg/mL) in SPMS buffer (0.25 M sucrose, 10 mM phosphate, 1 mM $MgCl_2$, and 1 mM succinate, pH 7.8) three times for 15 s at full setting in an MSE sonicator. The suspension was centrifuged at 10000*g* for 10 min in a Sorvall SS34, and the pellet was discarded. The supernatant was centrifuged at 78000*g* for 30 min, and the pellet was resuspended in SPMS buffer.

Labeling Procedures. [^{35}S]Diazobenzenesulfonate (50–9000 mCi/mol) was prepared from [^{35}S]sulfanilic acid (Amersham Searle) as described by Tinberg et al. (1974).

Isolated cytochrome *c* oxidase (1 mg/mL) was reacted with [^{35}S]DABS at room temperature for 30 min either in 0.1% Tween 80 and 20 mM sodium phosphate, pH 7.8, or in 1% $NaDodSO_4$ and 20 mM sodium phosphate, pH 7.8, after preincubation at 37 °C for 20 min in the same buffer. The reaction was stopped by addition of an equal volume of 10 mM histidine. The mixture was then dialyzed repeatedly against 1% $NaDodSO_4$, 20 M Tris-HCl, and 5 mM histidine, pH 7.8.

Mitochondrial membranes were labeled at 10 mg/mL in a buffer of 0.25 M sucrose and 10 mM sodium phosphate, pH 7.8, containing 40 μM DABS (5–9 Ci/mmol) for 20 min at room temperature. The reaction was stopped by addition of an equal volume of buffer containing 0.25 M sucrose, 20 mM Tris-HCl, and 10 mM histidine, pH 7.8. Membranes were washed by centrifugation in the quenching buffer and then stored at −70 °C in small aliquots suspended to 20 mg/mL in the same buffer.

Preparation and Characterization of Antibodies. Cytochrome *c* oxidase (1 mg) in Freund's complete adjuvant was injected at multiple sites into the backs of rabbits. Booster injections were administered intravenously in the ear using 0.5 mg of enzyme in 0.9% NaCl and in 10 mM Tris-HCl, pH 7.6,

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DABS, diazobenzenesulfonate; IgG, immunoglobulin.

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at 3 weeks after immunization and then at regular intervals. Blood was collected from the ear vein, allowed to clot at room temperature for 1 h, and then centrifuged for 10 min at 15000 rpm. The IgG fraction was purified as described by Harboe & Inglid (1973) and stored in a sucrose–Tris buffer at −70 °C.

Double-diffusion analysis (Ouchterlony & Nilsson, 1973) was carried out on plates prepared with 0.7% agarose containing 1% Triton X-100, 0.1 M KCl, 0.05 M Tris-HCl, and 0.05% sodium azide, pH 7.4. The plates were developed for 40 h at room temperature, washed with the Triton buffer, and stained with Coomassie brilliant blue R.

Membrane Solubilization. Membranes were incubated at 10 mg/mL in 2% Triton X-100, 1 M KCl, and 20 mM Tris-HCl, pH 7.4, for 10 min, then diluted to 1 mg/mL in 2% Triton X-100, 0.2 M KCl, 20 mM Tris-HCl, and 0.05% sodium azide, pH 7.4, and centrifuged at 100000*g* for 40 min. Approximately 95% of the protein was solubilized and recovered in the supernatant. Aliquots of the supernatant were incubated overnight with antiserum dissolved in the same buffer–detergent solution. Immunoprecipitates were centrifuged at 5000 rpm (Sorvall SS34) for 15 min, washed once with 0.1% cholate and 20 mM Tris-HCl, pH 7.4, and then with distilled water before solubilization in 4% $NaDodSO_4$, 8 M urea, and 20 mM Tris-HCl, pH 7.0, for $NaDodSO_4$–polyacrylamide gel electrophoresis.

Fractionation of Submitochondrial Particles with F_1 Antibody. Freshly labeled submitochondrial particles were centrifuged at 10000 rpm (Sorvall SS34) to separate large aggregates, and the supernatant from this spin was incubated overnight at 2 mg/mL with optimal amounts of an antiserum against mitochondrial F_1 ATPase (the preparation of this antibody will be reported elsewhere) in SPMS buffer. Membranes aggregated by antibody cross-linking were centrifuged down at 2000 rpm for 10 min and washed once in the SPMS buffer. Typically, the yield of aggregated membranes was 65–80% of the total submitochondrial particles. The amount of unspecific aggregation, estimated by analogous incubation with blank serum followed by centrifugation, was 5–10% of the starting material.

Gel Techniques. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the procedure of Swank & Munkres (1971) using 15% acrylamide and *N,N'*-methylenebis(acrylamide) (1:30) or 12.5% acrylamide and bis(acrylamide) (1:10).

Gels were stained and destained as described by Downer et al. (1976). Polyacrylamide gel electrophoresis under nondenaturing conditions was performed according to system 1 of Maurer (1971) using 5% acrylamide.

Radioactive gels were sliced into 1-mm thick slices with a Mickle gel slicer. These were dissolved in 1 mL of 15% H_2O_2 at 80 °C overnight, 6 mL of a solution of Omnifluor (NEN) 2.66 g/L) in toluene–Triton X-100 (2:1) was added to each vial, and the radioactivity was measured in a Packard liquid scintillation counter.

Other Methods. Protein was estimated as described by Lowry et al. (1951). Heme *a* determinations were done according to Williams (1964). Cytochrome *c* oxidase activity was measured polarographically as described by Vik & Capaldi (1977) or spectrophotometrically following the procedure of Vanneste et al. (1974) as modified by Vik & Capaldi (1977).

Triton X-100 and cytochrome *c* were obtained from Sigma; trypsin and trypsin inhibitor were obtained from Worthington Biochemicals.

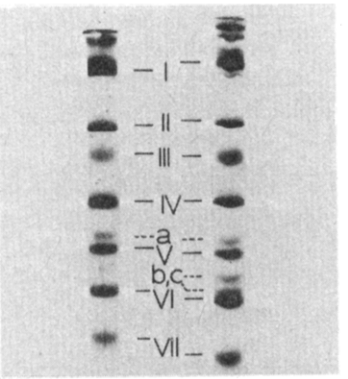

FIGURE 1: Effect of mild proteolysis on the peptide composition of cytochrome *c* oxidase. Cytochrome *c* oxidase was incubated with trypsin (50:1) at room temperature for 30 min in a buffer containing Triton X-100 (5 mg/mg of protein) and 50 mM sodium phosphate, pH 7.6; the reaction was stopped by addition of an excess of trypsin inhibitor. After gel filtration on Sepharose 6B in 20 mM sodium phosphate, pH 7.6, 0.1% Triton X-100, and 0.09 M NaCl as eluant, 80 μg of the peak fraction of the enzyme (left) and 80 μg of the untreated stock enzyme (right) were electrophoresed on 12.5% $NaDodSO_4$–urea gels as described under Experimental Procedures.

Results

Polypeptide Composition of Cytochrome c Oxidase. Our previous studies have shown that, under optimal conditions of $NaDodSO_4$–polyacrylamide gel electrophoresis, cytochrome *c* oxidase can be resolved into seven major components which are present in constant proportions in several different preparations of the beef heart enzyme (Briggs et al., 1975; Downer et al., 1976) and which are probably analogous to the seven subunits of the yeast and neurospora enzyme (Poyton & Schatz, 1975; Sebald et al., 1973). Additionally, several minor components including bands labeled a, b, and c in Figure 1 and small molecular weight polypeptides migrating close to VII are seen in all preparations but usually in small and variable amounts (Downer et al., 1976; Capaldi et al., 1977). It seemed desirable to use a cytochrome *c* oxidase preparation devoid of as many of these minor components as possible as an antigen against which to raise an antibody for the labeling experiments described here. Therefore, several different approaches were tested for removal of these impurities. The effect of mild proteolysis on cytochrome *c* oxidase is shown in Figure 1. The enzyme was incubated with trypsin as described in the figure legend and then the mixture was eluted through a column of Sepharose 4B equilibrated with Triton X-100. This gel filtration step removed the cleaved fragments, along with trypsin and with the trypsin inhibitor added to stop the reaction. The enzyme eluting from the column contained the seven major components (I–VII) apparently untouched by the protease while both impurities b and c were completely missing and the amount of impurity a was somewhat diminished. The activity of the protease-treated enzyme was similar to that of untreated enzyme which had been eluted through the same column. Antibodies were raised against the trypsin-treated enzyme, but they showed only a very poor titer against isolated cytochrome *c* oxidase and were not suitable for the labeling experiments planned.

A second approach tested for removing minor components from cytochrome *c* oxidase was to use native-gel electrophoresis in Triton X-100 (Maurer, 1971). One major broad band (fraction A), two major sharp bands (fractions B and C), and several minor bands were resolved on these gels. The polypeptide composition of each fraction was analyzed by two-dimensional electrophoresis in which bands were electrophoresed out of the native gel and into a slab gel containing

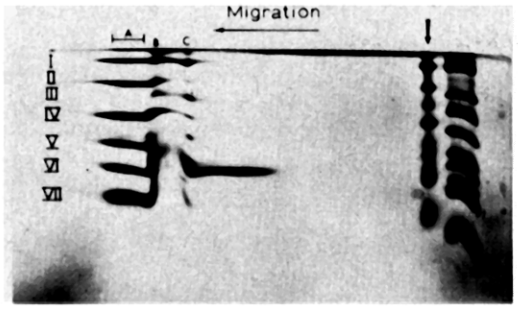

FIGURE 2: Electrophoresis of cytochrome *c* oxidase in nonionic detergent. Cytochrome *c* oxidase (300 μg) was electrophoresed on a cylindrical gel under nondissociating conditions in a system similar to that described by Maurer (1971). Gel system 1 was used with the following modifications: separating gel was 5% acrylamide with 0.1% Triton X-100 and enzyme was applied as overlay in spacer gel buffer supplemented with Triton X-100 (5 mg/mg of protein). Electrophoresis was carried out for twice the amount of time required for the tracking dye to reach the bottom of the gel. The cylindrical gel was incubated for 30 min at 37 °C in 4% $NaDodSO_4$, 100 mM sodium phosphate, and 1% β-mercaptoethanol, pH 7.0, and then fused to a slab gel of 15% acrylamide in $NaDodSO_4$–8 M urea (see Experimental Procedures). A sample of stock cytochrome *c* oxidase was run on the right-hand side of the slab. ↓, material that did not enter the first-dimension gel.

both $NaDodSO_4$ and urea (Figure 2). Fraction A was seen to contain subunits I, II, and IV–VII but was missing III. It was free of impurities b and c and the amount of impurity a present was less than in the stock enzyme. This fraction was eluted from the native gel and used as an antigen against which to raise cytochrome *c* oxidase specific antibodies. Bands B and C on the native gel each contained all of the subunits and associated impurities usually seen in cytochrome *c* oxidase preparations. Fraction C was enriched in polypeptides I and III as well as impurities b and c. Further studies are in progress to determine the functional characteristics of fractions A–C.

Antibody Characterization. Antibodies raised against fraction A cross-reacted in double-diffusion experiments with isolated cytochrome *c* oxidase and with detergent-solubilized submitochondrial particles but did not react with complex III, oligomycin-sensitive ATPase, or succinate dehydrogenase (Figure 3a). They gave complete and rapid inhibition of cytochrome *c* oxidase activity in solubilized preparations of enzyme (Figure 3b). These antibodies coprecipitated all seven major polypeptides from isolated cytochrome *c* oxidase dissolved in 1% Triton X-100 or 1% DOC. They brought down cytochrome *c* oxidase from detergent-solubilized mitochondria or detergent-solubilized submitochondrial particles. The enzyme precipitated from membranes dissolved in a combination of 2% Triton X-100 and 1 M KCl contained all seven subunits (Figure 4) in the same relative amounts found in the Capaldi and Hayashi preparation (as judged by the staining intensities of bands on the gel). This pellet contained lower amounts of impurities a–c than found in enzyme preparations obtained by conventional detergent solubilization and ammonium sulfate fractionation methods.

When lower levels of detergent were used to solubilize membranes (1% Triton X-100 or less), impurities of molecular weight 30 000 and higher were present in the cytochrome *c* oxidase immunoprecipitates. After we used high levels of detergent (3% Triton X-100 or more), the antibody immunoprecipitated a cytochrome *c* oxidase which contained low amounts of III and VI. This interesting finding is currently the subject of further study.

Labeling of the Isolated Enzyme with [^{35}S]*DABS.* Purified cytochrome *c* oxidase was reacted with [^{35}S]DABS in order

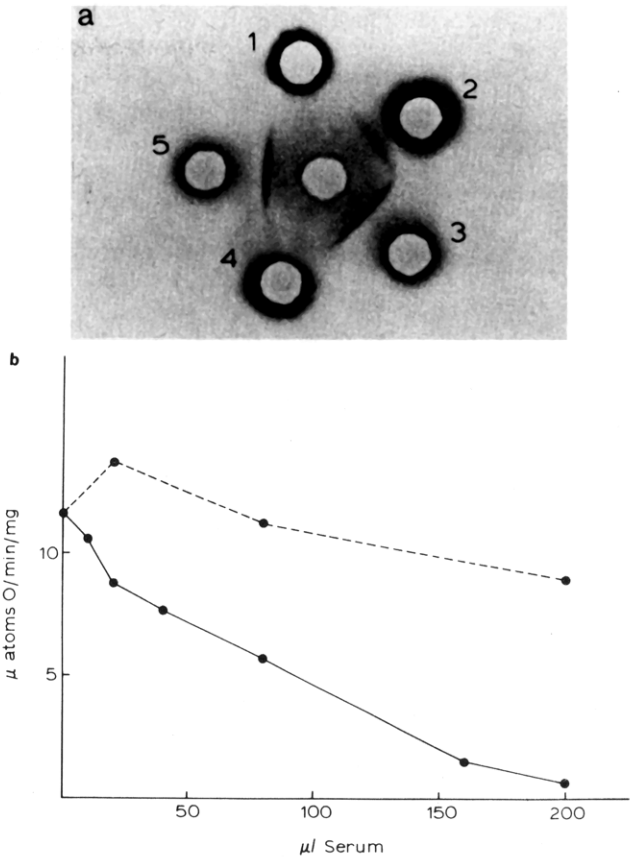

FIGURE 3: Characterization of the cytochrome *c* oxidase antibody. Double diffusion was performed on agarose plates as described under Experimental Procedures. Thirty microliters was applied to each well: (center) cytochrome *c* oxidase antiserum; (1) complex III, 1 mg/mL; (2) submitochondrial particles, 1 mg/mL, solubilized in 1% Triton X-100, 0.1 M KCl; (3) cytochrome *c* oxidase, 1 mg/mL; (4) F_1 ATPase, 1 mg/mL; (5) cytochrome *c* oxidase, 0.5 mg/mL. (b) Isolated cytochrome *c* oxidase (20 μg) was incubated with increasing amounts of serum and blank serum in 2 mL of the assay mixture for polarographic activity determination (see Experimental Procedures). After 5 min, cytochrome *c* was added and the rate of oxygen consumption followed. (—) Cytochrome *c* oxidase antiserum; (---) blank serum.

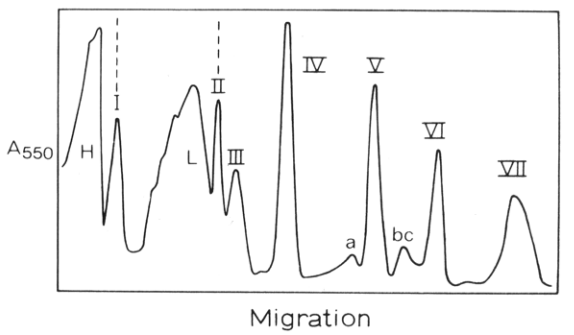

FIGURE 4: Polypeptide composition of cytochrome *c* oxidase immunoprecipitated from membranes. Gel scan of an immunoprecipitate from submitochondrial particles, run on 15% $NaDodSO_4$–urea gels. H, L: heavy and light subunit of IgG.

to determine the relative exposure of component polypeptides in the complex. Enzyme prepared by the method of Capaldi & Hayashi (1972) was dissolved in Tween 80 (native complex) for labeling. The labeling profile of enzyme run on 12.5% gels (Figure 5A) showed that polypeptides II–VII were each reactive to DABS as were impurities a–c. Subunit VI picked up relatively few counts compared with these other subunits. This was most evident when the trypsin-treated enzyme

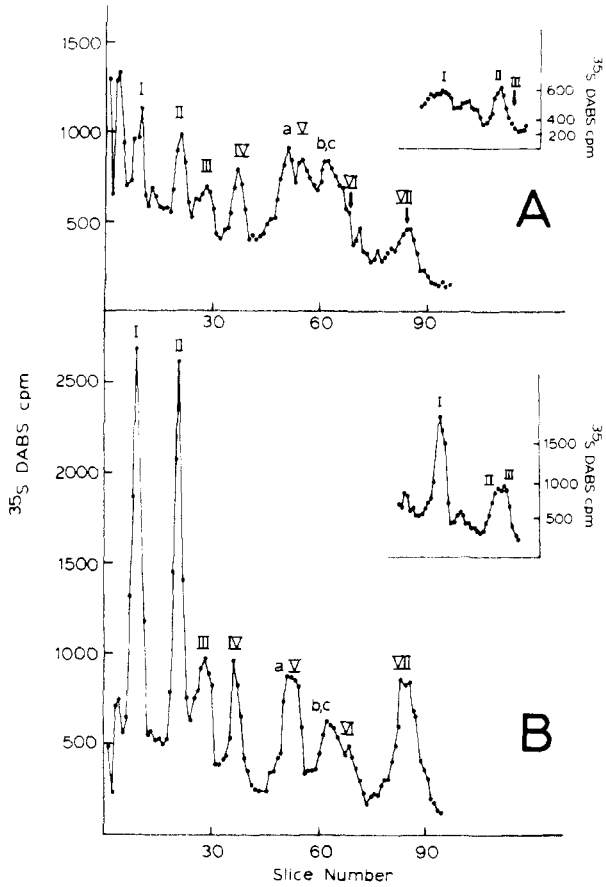

FIGURE 5: [^{35}S]DABS labeling of the isolated enzyme. Cytochrome *c* oxidase (50 μg) in Tween 80 (A) or in $NaDodSO_4$ (B) was labeled with [^{35}S]DABS (13 mCi/mmol) at a ratio of 50 nmol of reagent/mg of protein; the reaction was quenched, and samples were dialyzed as described under Experimental Procedures. Main traces, electrophoresis was carried out on 12.5% $NaDodSO_4$–urea gels. Inserts, labeled cytochrome *c* oxidase run on 15% $NaDodSO_4$–urea gels, which provide better resolution for subunit I (but do not completely separate subunits II and III).

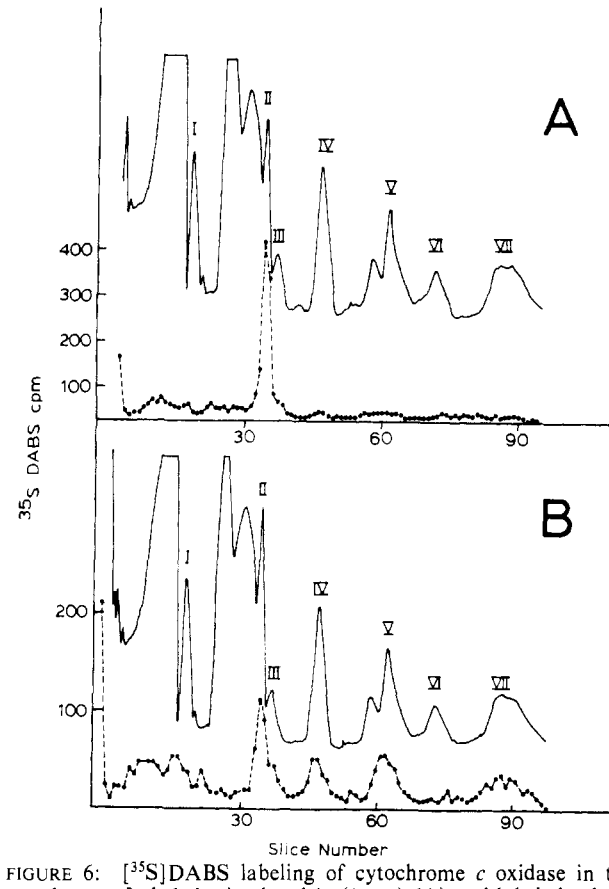

FIGURE 6: [^{35}S]DABS labeling of cytochrome *c* oxidase in the membrane. Labeled mitochondria (1 mg) (A) and labeled submitochondrial particles (0.5 mg) (B) were solubilized in Triton X-100–KCl (for details, see Experimental Procedures) and incubated with optimal amounts of the cytochrome *c* oxidase antiserum. The washed immunoprecipitates were dissociated in 4% $NaDodSO_4$, 8 M urea, 20 mM Tris–phosphate, pH 6.8, and 2% β-mercaptoethanol for 1 h at 37 °C and subjected to electrophoresis on 15% $NaDodSO_4$–urea gels. After staining, and before slicing, subunit bands were marked with black ink. Dashed line, radioactivity recovered after slicing (Experimental Procedures); solid line, gel scan at 550 nm. Under the dissociation conditions used, the light chain of the IgG occasionally consisted of two staining peaks. Also there was some loss of subunit III from the gel scan.

(missing impurities b and c) was examined (data not shown). On 12.5% gels there was a peak of radioactivity comigrating with I and close to the top of the gel. On 15% gels (inset) these counts were found to be spread out over the gel in the molecular weight range 40 000–30 000, and subunit I is evidently only poorly labeled compared to the smaller components (except VI).

Figure 5B shows the labeling profile of $NaDodSO_4$-denatured and -dissociated cytochrome *c* oxidase. All of the polypeptides were heavily labeled under these conditions, including I and VI.

Labeling of Cytochrome c Oxidase in Membranes. Freshly isolated mitochondria were used as a source of membranes with the outer (cytoplasm) side of the inner membrane exposed for labeling while submitochondrial particles were used as a preparation with the inner surface (matrix side) outermost. These membrane preparations were labeled with 4 nmol of DABS/mg of protein and then cytochrome *c* oxidase, oligomycin-sensitive ATPase, and succinate cytochrome *c* reductase were each immunoprecipitated from the same batches of membranes.

Figure 6A shows the distribution of counts in the subunits of cytochrome *c* oxidase after labeling of intact mitochondria. Component II was found to be by far the most reactive polypeptide. Component III was also labeled, and there was a very small peak of radioactivity running close to but just before IV. Polypeptides I, V, VI, and VII were not significantly labeled in these experiments. Also there were very few counts in any impurities on the gel.

The labeling profile of cytochrome *c* oxidase immunoprecipitated from [^{35}S]DABS-labeled submitochondrial particles is shown in Figure 6B. Subunits II–V and VII, along with impurity a, were all labeled, and there was incorporation of counts into several minor contaminants of high molecular weight. Polypeptide I was only labeled to a small extent while VI was unreactive to DABS in these preparations.

The labeling of subunit II in submitochondrial particles could have been due to incorporation of counts in the small number of vesicles of mitochondrial orientation in preparations, particularly as this component picks up almost all of the counts incorporated into cytochrome *c* oxidase from the cytoplasmic side. In order to test this possibility, antibodies against F_1 ATPase were used to separate membranes with their matrix side exposed from those retaining the mitochondrial orientation, and the labeling of cytochrome *c* oxidase in these resolved membranes was examined. Submitochondrial particles were incubated with antibodies against F_1, and the aggregated material generated by cross-linking of vesicles with the antibody was centrifuged down. The immunoprecipitate was solubilized in Triton X-100, insoluble material was removed by centrifugation (mainly cross-linked ATPase), and

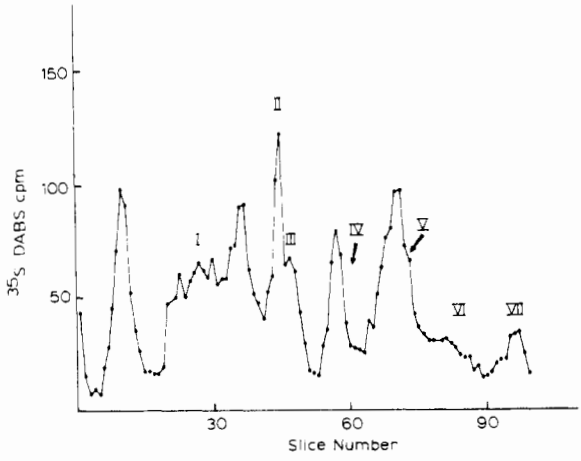

FIGURE 7: Cytochrome *c* oxidase labeling in resolved submitochondrial particles. Labeled submitochondrial particles were incubated with an antiserum against F_1 ATPase to separate out particles with the F_1 ATPase on the outside of the membrane (see Experimental Procedures). Antibody-aggregated vesicles were separated by low-speed centrifugation and solubilized, and the cytochrome *c* oxidase was immunoprecipitated and electrophoresed on 15% $NaDodSO_4$–urea gels.

cytochrome *c* oxidase was immunoprecipitated for analysis. The labeling profile of such a sample of cytochrome *c* oxidase is shown in Figure 7. The labeling pattern is qualitatively similar to that of unresolved submitochondrial particles with II still incorporating significant amounts of counts and with III, IV, impurity a, V, and VII all labeled.

Discussion

Cytochrome *c* oxidase can be isolated from several different sources as a complex containing two hemes (*a* and a_3), two copper atoms, and multiple polypeptides. The question of how many different subunits are present and their stoichiometry in the complex has proved difficult to answer. For beef heart cytochrome *c* oxidase, anywhere from one (Orii et al., 1977) to ten (Bucher & Penniall, 1975) different component polypeptides have been reported. Similarly, variable numbers of subunits have been resolved in both yeast and neurospora cytochrome *c* oxidase (Sebald et al., 1973; Werner, 1977; Poyton & Schatz, 1975; Phan & Mahler, 1976). This variability is at least in part a consequence of the limitations in resolution as well as artifacts inherent in the procedures of $NaDodSO_4$ gel electrophoresis used in these subunit composition studies [for a review, see Capaldi et al. (1977)].

Our recent studies have shown that, under optimal conditions of $NaDodSO_4$ gel electrophoresis, beef heart cytochrome *c* oxidase can be resolved into seven major component polypeptides (Downer et al., 1976) which are labeled I–VII in order of decreasing molecular weight (in Figure 1). In addition, there are several components in all preparations which are present in variable and always in substoichiometric amounts. These include bands a–c in Figure 1 as well as components running close to VII which are best resolved on two-dimensional gels (see Capaldi et al., 1977). The results presented here show that bands a–c are present in only small amounts in cytochrome *c* oxidase immunoprecipitated from mitochondria. Also, these components can be removed (for bands b and c) or diminished (for band a) by mild proteolytic digestion without affecting electron transfer activity of cytochrome *c* oxidase. We conclude, therefore, that these are impurities in enzyme preparations.

Several laboratories have recently confirmed that the beef heart enzyme contains seven major component polypeptides and have adopted the same numbering system for subunits employed here [McGeer et al. (1977), Rosen (1977), Bisson et al. (1977), and Chan & Tracy (1978)]. Steffens & Buse (1976) resolve the same bands as shown in Figure 1 but they label them I, II, III, IV, V, VI a, b, and c (our a, b, and c), VII (equivalent to our VI), and VIII a, b, and c (our VII and associated impurities). A different nomenclature is also used by Yu & Yu (1977). In their recent paper, these workers claim to have resolved beef heart cytochrome *c* oxidase into seven subunits present in stoichiometric amounts. However, they used Weber–Osborn gels which do not separate all of the polypeptides of the enzyme (see Downer et al., 1976; Capaldi et al., 1977), and subunit II of Yu and Yu is equivalent to II and III in our terminology (for confirmation of this see also Chan & Tracy, 1978), their III is our IV, their IV is a in our system, and V, VI, and VII in their gel includes V, b, c, VI, and VII in our numbering system.

Our working hypothesis remains that the seven-subunit enzyme is the structural unit in the membrane. As shown here, the seven components (labeled I–VII) are immunoprecipitated together from mitochondrial membranes under mild conditions of membrane solubilization. Similarly, a seven-subunit enzyme is immunoprecipitated from yeast mitochondria by antibodies raised against any single subunit of the enzyme (Mason & Schatz, 1973). This does not preclude that a preparation of cytochrome *c* oxidase missing one or more subunits might retain electron transfer activity. Indeed, antibody immunoprecipitation and "native-gel" electrophoresis of cytochrome *c* oxidase, which had been treated with a combination of high levels of detergent and high salt or high pH, yielded fractions of enzyme which were diminished in if not completely devoid of subunits III and/or VI. It is not known whether such fractions are functionally active, but these findings point to the labile nature of the cytochrome *c* oxidase complex.

We can calculate a minimum molecular weight of 125 000 for the two heme, two copper unit by summing the apparent molecular weights of the seven subunits (assuming one copy of each component). The purest preparations of enzyme described have a heme *a* content of 14–15 nmol/mg of protein (Hartzell et al., 1978) from which a molecular weight of 140 000 for the two-heme complex can be calculated. Attempts to measure the molecular weight of isolated cytochrome *c* oxidase directly have given variable results, possibly because of the failure of most workers to account adequately for the bound detergent and bound lipid in preparations of enzyme (Love et al., 1970; Wainio et al., 1973). We have used sedimentation equilibrium studies to estimate the molecular weight of the beef heart enzyme dissolved in Triton X-100 and dissolved in deoxycholate (Robinson & Capaldi, 1977). Corrections were made for bound amphiphile and bound lipid by use of the approach first described by Tanford et al. (1974). In the nonionic detergent, cytochrome *c* oxidase was found to be a dimer (four-heme complex) of molecular weight 324 000. In bile salts the dimer was dissociated into monomers.

Beef heart cytochrome *c* oxidase can be isolated in two different two-dimensional arrays: one is obtained with enzyme dissolved in deoxycholate (deoxycholate lattice) and in this array the protein is monomeric (or two-heme complex); the other is obtained in Triton X-100 (TX lattice) and the protein is dimeric (a four-heme complex). Electron microscopy and image reconstitution studies of these arrays have established that the cytochrome *c* oxidase (two heme) is approximately 120-Å long and Y shaped, extending about 60 Å from one side of the bilayer and 10–20 Å from the other (Henderson et al., 1977; S. F. Fuller, R. A. Capaldi, and R. Henderson, un-

published experiments). Recently, Fry et al. (1978) have examined the orientation of cytochrome *c* oxidase in the collapsed vesicles which characterize the TX lattice, and they have concluded that it is the matrix-facing surface of the protein which is outermost in these vesicles.

The labeling experiments described here were undertaken to explore the arrangement of the different polypeptides in the isolated complex and as the enzyme sits in the mitochondrial inner membrane. Labeling of isolated cytochrome *c* oxidase with [^{35}S]DABS was conducted in order to assess the exposure of the different polypeptides to the aqueous medium. Polypeptides II–V and VII were all significantly labeled by DABS in the solubilized enzyme, while I and VI picked up very few counts under the conditions of labeling chosen. Eytan et al. (1975) have used a similar approach to look at the surface exposure of the polypeptides in both beef heart and yeast cytochrome *c* oxidase. They find polypeptide I plus one of the smaller components shielded from solvent in the beef heart enzyme. Their studies were done by use of Weber–Osborn gels which, as discussed already, do not adequately resolve the smaller molecular weight subunits V–VII from each other or from associated impurities a–c. Thus, it is difficult to compare our results with theirs directly, and we cannot be certain that VI (in our terminology) was not labeled in their experiments. Chan & Tracy (1978) have also looked at the surface location of subunits but using antibodies made specifically against each component of cytochrome *c* oxidase. They find that antibodies made against subunits II and III together and against IV, V, VI, or VII, but not antibodies to subunit I, cross-react with the detergent-solubilized enzyme. In our experiments, all seven components were heavily labeled when the complex was dissociated with $NaDodSO_4$ prior to reaction with DABS. Similar results have been obtained by Eytan et al. (1975).

The major goal of the present study was to determine the distribution of components of cytochrome *c* oxidase across the mitochondrial inner membrane. Therefore, intact mitochondria with their cytoplasmic-facing surface available for labeling and submitochondrial particles (matrix face outmost) were reacted with [^{35}S]DABS. Several critical factors were considered in designing these experiments. It was important to establish that the mitochondria being used were intact and that the levels of DABS used did not modify the inner membrane in a way which made it leaky to the reagent. These possibilities were examined by a comprehensive analysis of NADH- and succinate-linked activities both before and after reaction with different levels of DABS. These experiments are described in detail in the preceding paper by Merli et al. (1979). Briefly, our mitochondrial preparations proved to be mostly intact (95% or more unbroken mitochondria), and the levels of DABS chosen for this study (4–6 nmol/mg of protein) were levels found to have no effect on respiratory control or other respiratory activities of the inner membrane.

Cytochrome *c* oxidase was isolated from these preparations by immunoprecipitation, and the labeling of individual subunits was examined. Polypeptide II was by far the most heavily labeled polypeptide of cytochrome *c* oxidase in mitochondria. There is now good evidence from cross-linking of a cytochrome *c* oxidase complex with DSP (Briggs & Capaldi, 1977b) and from cross-linking of arylazidocytochrome *c* to cytochrome *c* oxidase (Bisson et al., 1977) that subunit II of the beef heart enzyme is at or close to (within 11 Å) the cytochrome *c* binding site. This is consistent with a location of II on the cytoplasmic side. Polypeptide III was also labeled in mitochondria. Williams and associates (Kornblatt et al., 1975; McGeer et al., 1977) have labeled III selectively with a maleimide spin

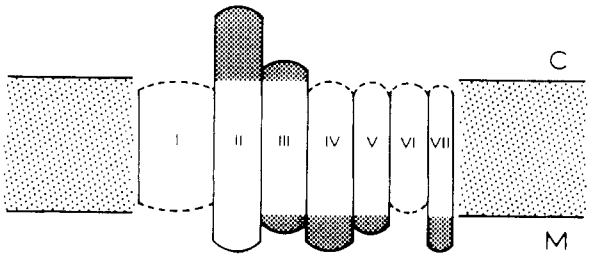

FIGURE 8: Schematic model of the relative exposure of the subunits of cytochrome *c* oxidase in the mitochondrial inner membrane. Lightly shaded area, membrane bilayer; C, cristal side; M, matrix side of the membrane. (The shape of subunits and their relative position to each other is arbitrary. VII may represent several copies of this subunit; see Discussion.)

label. The electron spin resonance signal of this probe was quenched by membrane-impermeable paramagnetic ions added to intact mitochondria, but the signal was not affected by addition of the quenching reagent to submitochondrial particles. In yeast cytochrome *c* oxidase it has been shown that III is close to the cytochrome *c* binding site (Birchmeier et al., 1976). These results support the location of III at the cytoplasmic surface of the mitochondrial inner membrane.

Polypeptides II–V and VII were all labeled in submitochondrial particles. Labeling of II but not that of other components could have reflected the presence of small amounts of vesicles of mitochondrial orientation (in which II is heavily labeled compared with other subunits) in submitochondrial particle preparations. However, this is unlikely because subunit II was still labeled significantly in membranes immunoprecipitated with antibody to F_1 ATPase, a step which should have selected out for further examination only vesicles with their matrix side exposed. One other explanation for the labeling of II in submitochondrial particles is that sonication leads to some scrambling of the cytochrome *c* oxidase molecule in otherwise sealed vesicles (Eytan et al., 1975). We cannot rule out this possibility at present.

Our results show some agreement with the study of Eytan et al. (1975) who also used [^{35}S]DABS to label cytochrome *c* oxidase subunits in beef heart mitochondria and submitochondrial particles. In both sets of experiments, II [really II and III in the gels of Eytan et al. (1975)] was labeled from the cytoplasmic-facing surface of the inner membrane and IV [numbered III by Eytan et al. (1975)] was labeled from the matrix side while I was not labeled from either side. The major points of difference relate to the positioning of the smaller components which migrated close to one another and to several impurities in the gels used by Eytan et al. (1975). Our labeling data are in reasonable agreement with the recent studies of Chan & Tracy, 1978. They found that antibodies against a mixture of II and III or against V or VII cross-reacted with cytochrome *c* oxidase in mitochondria while antibodies against IV, V, or VII cross-reacted with the enzyme in submitochondrial particles.

A summary of our results is presented in Figure 8. Polypeptides II and III are drawn as spanning the membrane. Polypeptides IV, V, and VII are shown as extending a small way from the matrix side while I and VI are shown as more buried in the complex. The possibility that V and VII span the membrane as suggested by the antibody binding studies of Chan & Tracy (1978) has not been ruled out. The fact that these polypeptides were not labeled with DABS in mitochondria cannot be taken as absolute proof that they do not extend to the outer surface of the inner membrane but only indicates that they do not have any Lys, His, or Tyr residues available for reaction on this side of the membrane. Based

on the same argument, I and VI could be exposed at one side of the membrane or the other, although antibody and chemical activity studies both suggest that these subunits are shielded from solvent. In order to elaborate on Figure 8, therefore, further experimentation is needed using surface probes with a broader reactivity than DABS. One such reagent which could be used is *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-taurine) (Staros et al., 1975). This protein-modifying reagent has been used successfully to explore the topology of the red cell membrane (Staros et al., 1975) but so far has not been used in mitochondrial studies.

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