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Arrangement of Complex II (Succinate–Ubiquinone Reductase) in the Mitochondrial Inner Membrane†

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ABSTRACT: The arrangement of succinate–ubiquinone reductase (complex II) in the mitochondrial inner membrane was examined by a combination of antibody reactivity and chemical labeling methods. Antibodies were raised against the flavoprotein subunit of succinate dehydrogenase and against the holoenzyme. These cross-reacted with complex II, but not with complex III, cytochrome *c* oxidase, or oligomycin-sensitive ATPase. Antibody against the holoenzyme was a potent inhibitor of succinate dehydrogenase activity and other succinate-linked activities in submitochondrial particles but not in intact mitochondria. It did not affect the NADH oxidase activity of either of these preparations. The antibody to the flavoprotein subunit was not inhibitory to any of these enzyme activities. Reaction of purified complex II with [^{35}S]diazobenzenesulfonate labeled all polypeptide components, including the 70 000 and 27 000 molecular weight subunits of succinate dehydrogenase and two small polypeptides termed CII-3 and CII-4. When complex II was immunoprecipitated from [^{35}S]diazobenzenesulfonate-modified mitochondria, CII-3 was labeled significantly. When complex II was immunoprecipitated from [^{35}S]diazobenzenesulfonate-labeled submitochondrial particles, the flavoprotein subunit of succinate dehydrogenase was heavily labeled while CII-3 was unreacted. These results are consistent with the view that complex II spans the mitochondrial inner membrane with the succinate dehydrogenase flavoprotein clearly on the matrix side and with CII-3 on the cytoplasmic side.

Succinate dehydrogenase is one of the two major entry points for electrons into the mitochondrial electron transport chain. The enzyme can be isolated in water-soluble form and has been shown to consist of two dissimilar subunits, a peptide with a molecular weight of 70 000, carrying the flavin moiety (Davis & Hatefi, 1971) and the active site (Kenney et al., 1976), and a smaller subunit of 27 000 molecular weight. The isolated enzyme reacts with electron acceptors such as ferricyanide and phenazine methosulfate (PMS)[1] but is unable to transfer electrons to ubiquinone, the physiological electron acceptor in the normal membrane environment. The simplest unit capable of succinate–ubiquinone reductase activity is complex II, a fragment of the mitochondrial membrane that has recently been shown to contain, in addition to the two subunits of the soluble succinate dehydrogenase, two polypeptides with molecular weights of 13 500 and 7000, respectively (Capaldi et al., 1977). These small molecular weight components appear to be intrinsic to complex II, since they are not found in complex III (reduced ubiquinone–cytochrome *c* reductase), complex IV (cytochrome *c* oxidase), or complex V (ATPase).

There is evidence that succinate dehydrogenase is localized functionally on the matrix side of the mitchondrial inner membrane; thus, submitochondrial particles (everted vesicles, with the matrix side outermost) show electron transfer (antimycin insensitive) between succinate dehydrogenase and the membrane-impermeant anion ferricyanide, whereas intact mitochondria do not (Klingenberg, 1970).

Certain properties of the enzyme may be related to functional areas normally situated within the membrane rather than exposed to aqueous environment. For example, the extreme lability of the tetranuclear Fe_4–S_4 non-heme iron cluster (center 3 or HiPIP center) evident in the soluble enzyme is not observed in particulate preparations (Ohnishi et al., 1974; Beinert et al., 1977), where the center is protected against the deleterious effects of oxygen and prevented from interacting with added paramagnetic ions (Case et al., 1976). Also, the reaction site for ferricyanide characterized by a low K_m(apparent) (<200 μM) in the ferricyanide reductase activity of reconstitutively active soluble enzyme is not accessible in particulate preparations of the enzyme (Vinogradov et al., 1975).

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DABS, diazobenzenesulfonate; PMS, phenazine methosulfate; DCIP, dichlorophenolindophenol.

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In order to learn more about the organization of the enzyme in the membrane and how it is related to such enzymatic functions, we have prepared antibodies against succinate dehydrogenase holoenzyme and against the isolated flavoprotein subunit of the enzyme. These have been used in inhibition studies of succinate-driven reactions in mitochondria and submitochondrial particle preparations and to immunoprecipitate complex II from mitochondria and submitochondrial particles which had been labeled with the membrane impermeant reagent [^{35}S]diazobenzenesulfonate (DABS). The results provide a first insight into the arrangement of the peptides of complex II in the mitochondrial inner membrane.

Materials and Methods

Enzyme Preparations. The soluble form of cardiac succinate dehydrogenase was prepared from complex II either by the method of Davis & Hatefi (1971) or by that of Ackrell et al. (1977) and stored as ammonium sulfate pellets in liquid nitrogen before use. Subunits of the enzyme were isolated by dissociation of the purified enzyme with sodium dodecyl sulfate ($NaDodSO_4$) (Davis & Hatefi, 1971).

Complex II was prepared according to the method of Baginsky & Hatefi (1969) except that the deoxycholate-dispersed complex II was not subjected to the final steps of Sephadex chromatography and centrifugation but was dialyzed instead at 4 °C for 24 h under anaerobic conditions against 250 mM sucrose, 25 mM potassium phosphate, and 20 mM succinate, pH 7.5. After this treatment the preparation remained dispersed, indicating the presence of residual deoxycholate.

Mitochondria were isolated from beef hearts by the technique of Smith (1967), except that phosphate was substituted for Tris and EDTA (20 mM) was added in experiments where DABS was used. For labeling experiments, submitochondrial particles were generated by sonicating heavy-layer mitochondria (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 then centrifuged (10000g for 10 min) and the pellet was discarded. The supernatant was centrifuged at 78000*g* for 30 min, and the pellet of submitochondrial particles representing 10–15% of the initial mitochondrial suspension was resuspended in the SPMS buffer.

For activity measurements, submitochondrial particles were obtained by sonication of the mitochondrial suspension until full oxidase activity with exogenous NADH was expressed. These were separated from unbroken mitochondria as described above.

Enzyme Assays. Succinate–phenazine methosulfate (PMS) reductase activity was assayed spectrophotometrically at 38 °C with dichlorophenolindophenol (DCIP) as the terminal electron acceptor in 20 mM Tris-H_2SO_4, pH 7.6, containing 100 μM EDTA, 20 mM succinate, and the concentrations of PMS and DCIP recommended in the literature (Singer, 1974). KCN (1 mM) was included when particulate preparations of the enzyme were assayed. Succinate–ferricyanide reductase activity at the low-K_m site, which is taken to reflect the reconstitutive ability of the enzyme (Beinert et al., 1977), was measured at 38 °C in the same buffer system under the conditions described by Beinert et al. (1977). NADH–ferricyanide reductase activity was measured according to Singer (1974). Oxidase activities were measured polarographically at 30 °C in 70 mM sucrose, 220 mM mannitol, 500 μM EDTA, and 10 mM potassium phosphate, pH 7.6, with additions of substrates and inhibitors as required. Preparations of deactivated succinate dehydrogenase were activated by anaerobic incubation at 30 °C in the presence of 20 mM succinate prior to being assayed.

The histidylflavin content of enzyme preparations was determined according to Singer et al. (1971), and protein content was determined by the method of Lowry et al. (1951) or Gornall et al. (1949).

Preparation of Antibodies. Antibodies against succinate dehydrogenase holoenzyme and against the purified flavoprotein subunit of this enzyme were prepared by dissolving these antigens in Freund's complete adjuvant and then by injecting emulsions subcutaneously at multiple sites in the back 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, at 3 weeks after immunization and then at monthly 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. Ammonium sulfate was added to the serum up to 40% saturation; the pellet, dissolved in 0.9% NaCl and in 10 mM Tris-HCl, pH 7.6, was stored frozen. The IgG fraction was purified as described by Harboe & Inglid (1973) and stored in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.6, at −20 °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, and 10 mM Tris-HCl, pH 7.6. The plates were developed for 40 h at room temperture, washed with 1% Triton X-100, and stained with Coomassie brilliant blue R.

Rocket immunoelectrophoresis was carried out in 1% agarose containing 1% Triton X-100, 60 mM Tris, 38 mM glycine (pH 8.6), and 2 mL of antiserum. Antigen samples were applied in the same buffer. Electrophoresis was carried out for 18 h at 3 V/cm in a water-cooled LKB electrophoresis apparatus. The plates were stained for 15 min in the solution of Coomassie brilliant blue as described for the polyacrylamide gels.

Gel Electrophoresis Techniques. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by the procedure of Swank & Munkres (1971) using 15% acrylamide and 0.5% *N,N'*-methylenebis(acrylamide) and by the procedure of Weber & Osborn (1969) using 7.5 and 10% gels. Gels were stained with Coomassie brilliant blue R as described by Downer et al. (1976). Polyacrylamide gel electrophoresis under nondenaturing conditions (native gels) was performed according to system 1 of Maurer (1971) using 4% acrylamide. Isoelectric focusing on polyacrylamide gel was performed as described by Righetti & Drysdale (1971). Two-dimensional gel electrophoresis of the native gel and isoelectrofocusing gel was performed as described by O'Farrell (1975).

Labeling with [^{35}S]DABS. [^{35}S]diazobenzenesulfonate (50–9000 mCi/mmol) was prepared from [^{35}S]sulfanilic acid as described by Tinberg et al. (1974). Isolated complex II (1 mg/mL) was reacted with [^{35}S]DABS at room temperature for 30 min in phosphate buffer, pH 7.8. The reaction was stopped by addition of an equal volume of 60 mM histidine in 0.1 M Tris-HCl, pH 7.8.

Freshly prepared heavy-layer mitochondria were used in labeling experiments. These were labeled at 10 mg/mL in a buffer of 0.25 M sucrose and 10 mM sodium phosphate, pH 7.8, containing 40 or 60 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, 10 mM histidine, pH 7.8. The suspension was centrifuged at 15000*g* for 10 min, and the pellet was washed with the same solution of histine–Tris–sucrose. Submito-

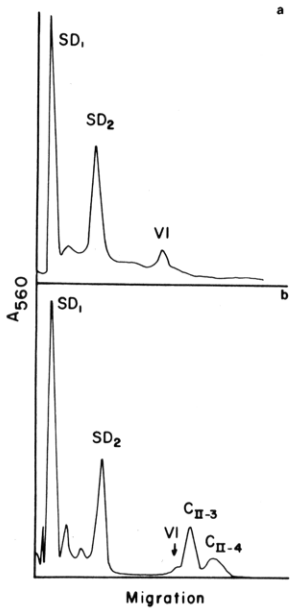

FIGURE 1: Peptide band patterns of purified succinate dehydrogenase (a) and complex II (b). SD_1 and SD_2 are the large and small subunits of succinate dehydrogenase, respectively; VI is the cytochrome c_1 associated polypeptide of complex III.

chondrial particles were labeled in the same way but were centrifuged at 78000*g* for 30 min to pellet the membranes.

Immunoprecipitation. Membrane preparations were dissolved in 2% Triton X-100, 1 M KCl, and 20 mM Tris-HCl, pH 7.4, for immunoprecipitation. All the solutions were centrifuged at 100000*g* for 40 min in order to remove any unsolubilized material before addition of the antiserum. After incubation with the antibody at 4 °C overnight, the tubes were centrifuged at 5000 rpm (Sorvall SS 34) for 10 min, and the pellet was washed in the incubation buffer used and then in water. The pellet was finally dissolved in 4% $NaDodSO_4$, 4% β-mercaptoethanol, and 8 M urea (final concentration 4 M urea) and heated for 1 min at 100 °C before being applied to the polyacrylamide gels. After staining, the gels were sliced to 1-mm thick slices with a Mickle gel slicer. These were dissolved in 1 mL of 15% H_2O_2 at 60–70 °C overnight; 7 mL of a solution of Omnifluor (New England Nuclear) (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. Solutions of labeled mitochondria and submitochondrial particles were also treated in parallel experiments with antibodies specific to cytochrome c_1, cytochrome *c* oxidase, and oligomycin-sensitive ATPase.

Results

Characteristics of Soluble Preparations of the Enzyme. Preparations of succinate dehydrogenase obtained by the method of Davis & Hatefi (1971) were used for generating rabbit antibody against the holoenzyme and in some of the immunoprecipitation experiments. These contained between 8.9 and 9.3 nmol of histidylflavin/mg of protein and showed two major and three minor bands when subjected to $NaDodSO_4$–polyacrylamide electrophoresis on 9% gels in the Swank–Munkres (1971) system (Figure 1a). The major components were the 70 000- and 27 000-dalton subunits of the enzyme; the minor bands were identified as components of complex III, namely, in order of decreasing molecular weights, core proteins I and II, peptides III and/or IV, and the so-called cytochrome c_1 associated polypeptide VI (see Capaldi et al., 1977). Enzyme isolated by the method of Ackrell et al. (1977) was used for study of the effects of

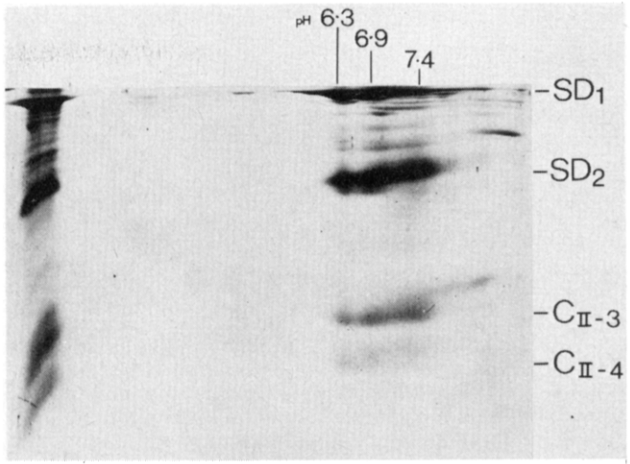

FIGURE 2: Isoelectric focusing of complex II. An aliquot of complex II was run on an isoelectric focusing gel as described by Righetti & Drysdale (1971). This gel was cemented onto a slab gel, and electrophoresis in the second dimension was performed on a 9% gel run in the Swank–Munkres buffer system (1971).

antibodies on enzymatic activities. It was essentially pure by gel electrophoresis and had high reconstitutive activity as judged by the criterion of high turnover number ($\simeq$11 000 at 38 °C) in the PMS and "low-K_m" ferricyanide assays (Ackrell et al., 1977).

Characteristics of Complex II. Preparations of complex II contained 4–5 nmol of histidylflavin/mg of protein and exhibited four major bands on $NaDodSO_4$–polyacrylamide gels (9%; Swank & Munkres, 1971) (Figure 1b). In addition to the succinate dehydrogenase subunits, two polypeptides of molecular weight 13 500 (designated CII-3) and 7000 (CII-4) were resolved (Capaldi et al., 1977). The complex III impurities seen in minor amounts in the soluble enzyme preparations were also present in slightly greater amounts in most complex II preparations (see Davis & Hatefi, 1971; Capaldi et al., 1977).

A point of importance for the antibody experiments described later was that complex II could be resolved into several bands by gel electrophoresis under nondenaturing conditions or by isoelectric focusing in the presence of nondenaturing detergents. At least three bands were obtained in native gels and five bands were seen after isoelectric focusing in the presence of Triton X-100. The components of each band were determined by two-dimensional gel electrophoresis using $NaDodSO_4$ as the denaturing detergent in the second dimension (O'Farrell, 1975). Both in native gels and in those obtained by isoelectric focusing (Figure 2), there was a band containing the four polypeptides of complex II, bands containing these same four polypeptides plus varying components of complex III, and bands containing only the complex III derived impurities found in complex II preparations. It is evident from these experiments that complex II is a heterogeneous preparation, containing particles of varying complexity.

Specificity of Antibody Preparations. Antibodies prepared against succinate dehydrogenase holoenzyme (SD antibody) and against the flavoprotein subunit alone (Fp antibody) were examined in double-diffusion studies (Ouchterlony & Nilsson, 1973) for immune reaction with different components of the mitochondrial membrane. The SD antibody precipitated both the flavoprotein subunit and the smaller subunit from detergent solution (the purified subunits were added in 0.1% $NaDodSO_4$ solution). The Fp antibody reacted with the flavoprotein subunit but did not cross-react with the smaller subunit of the enzyme. Both antibody preparations gave single precipitin lines

Table I: Effect of Antibody Preparation on the Oxidase Activities of Mitochondria[a]

	NADH [(μg atom of O/min)/mg]	malate–pyruvate [(μg atom of O/min)/mg]	RCI	succinate [(μg atom of O/min)/mg]	RCI
mitochondria	0.03	0.33	2.2	0.27	1.6
mitochondria + antibody	0.03	0.41	1.6	0.43	1.3
mitochondria + control serum	0.04	0.41	1.6	0.43	1.4
sonicated mitochondria + antibody	0.45			0.15	
sonicated mitochondria + control serum	0.45			0.30	

[a] Mitochondria and sonicated mitochondria (0.52 mg of protein) were incubated anaerobically with 100 μL of antiserum or control serum, as indicated, for 10 min at 30 °C. Succinate (20 mM) was included in the incubation of sonicated mitochondria to activate the enzyme at the same time. Mitochondria were activated by allowing succinate oxidation in the presence of rotenone (1 μM) to continue until linear rates were evident (1–2) before addition of ADP to elicit state 3 respiration. Oxidase activities were assayed at 30 °C with 5.5 mM pyruvate plus 2.8 mM L-malate, 20 mM succinate, and 1.3 mM NADH, as indicated, and 100 μM ADP. RCI represents the respiratory control index.

with the pure holoenzyme but formed two or three precipitin lines with complex II and submitochondrial particles in the presence of Triton X-100 (1%). The extra precipitin lines with complex II were attributed to the particle heterogeneity of this type of preparation (see above) which is presumably also the case with detergent-dispersed mitochondrial membrane. The antibody did not cross-react with cytochrome *c* oxidase or oligomycin-sensitive ATPase as judged by double-diffusion experiments. Cross-reaction with complex III (free of succinate dehydrogenase as judged by $NaDodSO_4$ gel electrophoresis) was examined by double-diffusion, immunoprecipitation, and rocket-immunoelectrophoresis experiments. No precipitin lines were produced against pure complex III in double-diffusion experiments, and the antibodies did not precipitate any complex III even at a 10-fold higher concentration than that completely precipitating complex II (shown for the SD antibody in Figure 3). Also, the antibody produced large rockets in rocket immunoelectrophoresis with succinate dehydrogenase subunits, complex II, and crude samples of complex III containing succinate dehydrogenase, but no rockets were formed with pure complex III (Figure 4). Thus, the minor amounts of complex III in the preparation of the soluble enzyme used as antigen gave rise to no detectable antibodies.

Inhibition of Succinate Dehydrogenase by Antibody. The antibody to the holoenzyme inhibited dehydrogenase activity, the inhibition being rapid but never complete. Thus, the inhibition of the fully reconstitutively active enzyme was fully expressed within 30 s after mixing but never exceeded 40–60% as monitored by the PMS or the low-K_m ferricyanide assay (Figure 5). This maximal level of inhibition was also noted for the PMS reductase activity of complex II, the inhibition being associated with a minor decrease in the K_m(apparent) for PMS (0.53–0.36 mM) but no change in the K_m(apparent) for succinate. The SD antibody inhibited succinate–PMS reductase and succinoxidase activities in submitochondrial particles up to 60% but did not inhibit succinoxidase activity in mitochondrial preparations; NADH oxidase activity was unaffected by the antibody (Table I). The Fp antibody, in contrast, did not inhibit activity of the soluble enzyme, complex II, or submitochondrial particles.

Polypeptide Composition of Immunoprecipitates. Both the SD and Fp antibodies were used to isolate succinate dehydrogenase from preparations of isolated enzyme, from complex II, and from submitochondrial particles, and the polypeptide composition of the immunoprecipitates was examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. Both subunits of succinate dehydrogenase were present in the

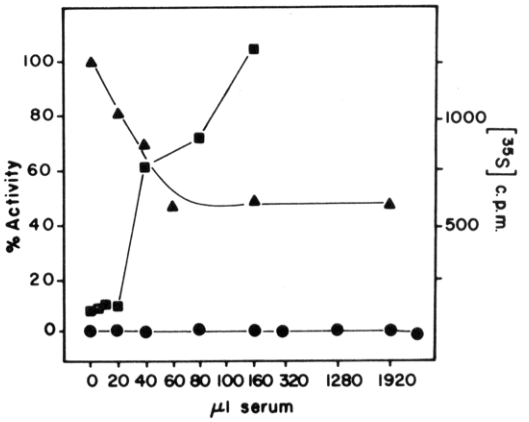

FIGURE 3: Immunoprecipitation of complexes II and III by the succinate dehydrogenase antibody. [^{35}S]DABS-labeled complex II (■) and [^{35}S]DABS-labeled complex III (●) were incubated with different levels of antiserum, and the amount of protein immunoprecipitated was measured by liquid scintillation counting. For inhibition studies, 10-μg aliquots of complex II, preactivated at 38 °C, were incubated for 10 min at 22 °C with different amounts of antiserum as indicated on the abscissa, and the residual succinate–PMS reductase activity was measured at 38 °C (▲).

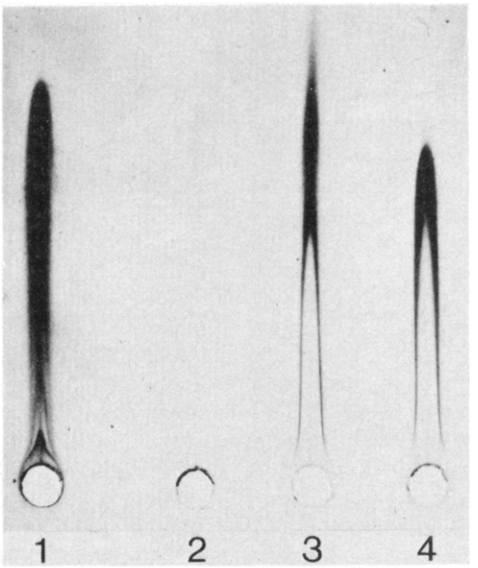

FIGURE 4: Rocket immunoelectrophoresis using the succinate dehydrogenase antibody. The wells contained the following antigens: (1) complex II, (2) pure complex III, (3) crude complex III, and (4) succinate dehydrogenase.

immunoprecipitates of purified enzyme. Four major bands were resolved in immunoprecipitates from isolated complex

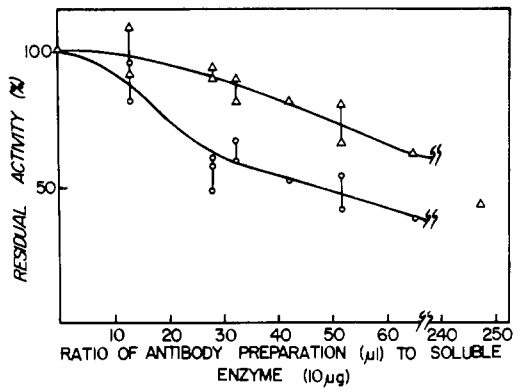

FIGURE 5: Inhibition of the PMS (Δ) and low ferricyanide reductase (O) activities of reconstitutively active succinate dehydrogenase. The antibody preparation was mixed with the enzyme at 22 °C in the presence of 20 mM succinate, 100 μM EDTA, and 20 mM Tris-H_2SO_4 buffer, pH 7.5, under an atmosphere of argon with glucose, glucose oxidase, and catalase added to ensure anaerobiosis. Residual activity is expressed as a percentage of the activity obtained with 0.9% NaCl substituted for antibody. Controls run with serum obtained from the rabbit before immunization were the same as those run with 0.9% NaCl. No loss of activity occurred in these control incubations.

II. These included the two subunits of succinate dehydrogenase along with CII-3 and CII-4. Components of complex III present in the complex II preparations were also immunoprecipitated in small amounts, although, as described above, neither antibody cross-reacted with complex III components (Figure 4).

When succinate dehydrogenase was immunoprecipitated from inner membrane fractions (submitochondrial particles), a much more complicated gel profile was obtained. In addition to the four bands seen in complex II preparations, there were major bands corresponding to complex III components and several minor bands of unidentified origin. This coprecipitation of complexes II and III was not only seen with the SD and Fp antibodies but also with antisera prepared against cytochrome c_1 (isolated from purified complex III) (see Bell et al., 1979). Preliminary experiments have been done in which the amount of histidylflavin and of c_1 heme in immunoprecipitates obtained with the Fp antibody has been measured. Values between 0.7 and 1.3 nmol/mg of protein for flavin and between 0.9 and 1.4 nmol/mg of protein for c_1 heme have been obtained (protein values include IgG subunits in pellets) (A. Merli, unpublished experiments).

The association of complexes II and III in these studies cannot be attributed to a generally incomplete solubilization of the membrane by Triton X-100, since the polypeptide profiles of cytochrome *c* oxidase and oligomycin-sensitive ATPase, when immunoprecipitated from the same batch of [^{35}S]DABS-labeled inner membranes, were similar to those of the purest preparations of these complexes (Ludwig et al., 1979; Ludwig and Capaldi, unpublished experiments).

Modification of Complex II with [^{35}S]DABS. Since certain data reported in the literature (see Table I in Grigolava & Konstantinov, 1977) indicated possible changes in permeability of the mitochondrial inner membrane as a result of DABS treatment, the effect of various levels of this protein-modifying reagent on succinate and NADH-linked enzymatic reactions was tested. Purified succinate dehydrogenase and succinate dehydrogenase in complex II retained full activity after treatment with 4 and 40 nmol of DABS/mg of protein. Enzymatic activities were inhibited more than 90%, however, after reaction of the protein with 200 nmol of DABS/mg of protein.

The effects of DABS on various activities of mitochondria and submitochondrial particles are summarized in Table II. Our mitochondria showed less than 5% of the NADH oxidase activity of extensively sonicated mitochondria. Thus there was very little breakage of these preparations. Treatment of the mitochondrial inner membrane on either the outer surface (mitochondria) or the inner surface (submitochondrial particles) with DABS at 4 nmol/mg of protein did not change the activities measured. The fact that both the respiratory control and NADH oxidase activity of mitochondria (before sonication) were unaltered is evidence that the membrane was not made leaky or otherwise adversely affected by this low level of protein-modifying reagent.

At 40 nmol of DABS/mg of protein, respiratory control was lost and both state 3 and state 4 respiration were very low, although some activity could be elicited with uncoupler (m-CCCP), i.e., about 60% of the activity of untreated mitochondria. Both succinate and NADH oxidase were more seriously affected by DABS reaction with the outer surface than with the inner surface of the membrane. This may be a consequence of modifying a component or components in the ubiquinone–cytochrome *c* reductase segment of the chain as first suggested by Tinberg et al. (1974) and later by Grigolava & Konstantinov (1977).

At still higher levels of DABS, i.e., 200 nmol/mg of protein, almost all activity was lost, in agreement with Tinberg et al. (1974). In summary, the effects of DABS are clearly multiple and complex, but, at low levels such as those used in the labeling studies reported here (4–6 nmol/mg of protein), the reagent does not appear to be deleterious to membrane integrity.

In other experiments, the effect of DABS modification on antibody reactivity was tested. Succinate dehydrogenase which had been reacted with 4 nmol of DABS/mg of protein and untreated enzyme were each titered against the SD and Fp antibodies. The antibody–antigen reaction was monitored by estimation of the amount of succinate dehydrogenase flavoprotein in immunoprecipitates (from $NaDodSO_4$–polyacrylamide gels of the pellets) and by measurement of the amount of succinate dehydrogenase activity left in the supernatant (for the Fp antibody). No effect of DABS reaction was detected in either assay.

Labeling Studies with [^{35}S]DABS. Both the flavoprotein and the 27 000-dalton subunit incorporated label when purified succinate dehydrogenase was reacted with [^{35}S]DABS. The two subunits of succinate dehydrogenase and CII-3 and CII-4 were all labeled in complex II preparations (Figure 6). Thus, each of these components must be exposed at the surface of the isolated and detergent-dispersed complex II.

Labeling of complex II in the mitochondrial inner membrane was investigated with both intact mitochondria and submitochondrial particles (Figures 6 and 7). After reaction with [^{35}S]DABS at a ratio of 4–6 nmol/mg of protein, the membrane was solubilized in Triton X-100, and succinate dehydrogenase containing particles were immunoprecipitated for examination by $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel conditions devised by Weber & Osborn (1969) were used to resolve the higher molecular weight subunits (Figure 6) while the Swank–Munkres (1971) gel conditions were used to separate low molecular weight polypeptides (Figure 7).

Mitochondria have the outer (cytoplasm) surface of the inner membrane available for reaction with DABS, and very few counts were incorporated into succinate dehydrogenase subunits in these preparations. There were, however, peaks of radioactivity at the positions of CII-3 and CII-4 on gels.

Table II: Effect of DABS on Enzymatic Activities of Mitochondria and Submitochondrial Particles

		DABS (nmol/mg of protein)							
		0		4		40		200	
expt[a]	act. measd:	mitochondria	SMP	mitochondria	SMP	mitochondria	SMP	mitochondria	SMP
pyruvate + malate → O_2[b]									
state 3		0.25		0.27		0.04		0.02	
state 4		0.10		0.12		0.04		0.02	
m-CCCP		0.49		0.49		0.29		0.02	
succinate → O_2[b] (+ rotenone)									
state 3		0.35		0.36		0.06		0.02	
state 4		0.18		0.17		0.06		0.02	
m-CCCP		0.42		0.43		0.27		0.02	
after sonication		0.42	0.92	0.48	0.93	0.24	0.83	0.02	0.14
succinate → PMS + DCIP[c] (+ antimycin + KCN)									
after sonication		1.37	1.85	1.37	1.83	0.98	1.87	1.10	0.39
NADH → O_2[b]		0.05		0.05		0.02		0.01	
after sonication		0.58	1.13	0.70	1.17	0.20	0.69	0.02	0.27
NADH → $K_3Fe(CN)_6$[d]			12.60		12.30		5.50		1.07

[a] Mitochondria or submitochondrial particles (SMP) at 7.7 mg of protein/mL ± DABS for 15 min at 22 °C in 250 mM sucrose and 10 mM phosphate buffer, pH 7.8, containing 1 mM succinate and 200 μM EDTA. The reaction was quenched by addition of Tris-histidine (final concentration, 40 mM and 0.5 mg/mL, respectively) in 250 mM sucrose and 10 mM phosphate buffer, pH 8.0, and aliquots of the mixture were assayed at 30 °C, with substrates and ADP added as in Table I, plus 1.1 μM m-CCCP, 1 mM KCN, and 1 nmol of antimycin per milligram of protein where indicated. Mitochondria and submitochondrial particles were activated as in Table I but without serum present. [b] Expressed as (μg atom of O/min)/mg. [c] Expressed as (μmol of succinate oxidized/min)/mg. [d] Expressed as (μmol of NADH oxidized/min)/mg.

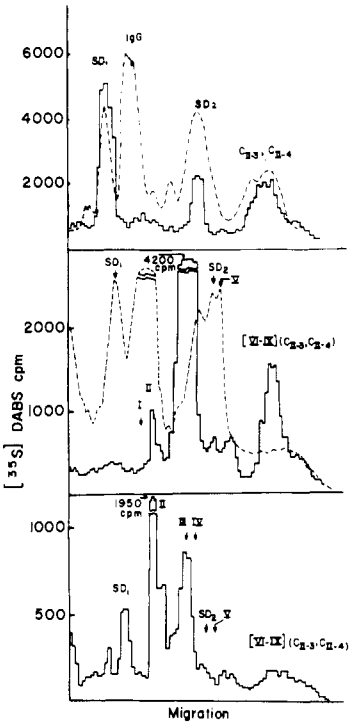

FIGURE 6: Labeling profiles of immunoprecipitates of purified complex II (top trace), mitochondria (middle trace), and submitochondrial particles (bottom trace) obtained with SD antibody. Each preparation was labeled with [^{35}S]DABS and then solubilized in detergent for immunoprecipitation as described under Materials and Methods. The gels are 7.5% acrylamide run in the Weber–Osborn (1969) buffer conditions. The bar graphs show the number of counts in each slice of the gel. The dashed lines are the Coomassie blue staining profiles of the gels.

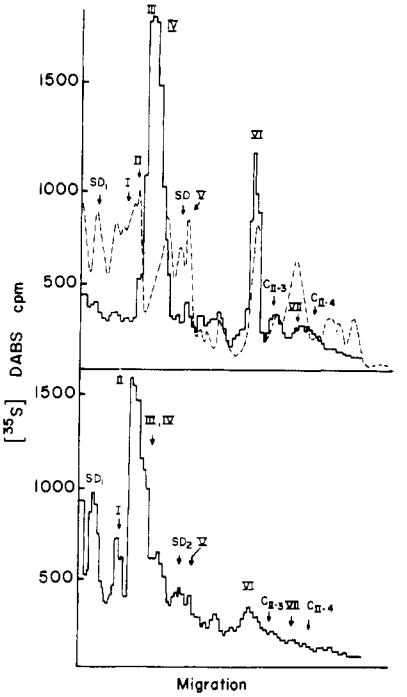

FIGURE 7: Labeling profiles of complex II immunoprecipitated from mitochondria (upper trace) and submitochondrial particles (lower trace) which had been labeled with [^{35}S]DABS. Gels were made with 15% acrylamide (1:30 cross-linker) and run in the Swank–Munkres (1971) buffer conditions.

Most of the counts in the immunoprecipitate from mitochondria were found in complex III components: cytochrome *b* (band III in Figures 6 and 7), cytochrome c_1 (IV), and the cytochrome c_1 associated polypeptide (VI) were all heavily labeled [see Bell et al. (1979) for a full analysis of the orientation of complex III].

Submitochondrial particles were used as a source of membranes in which the matrix side of the inner membrane is outermost. Reaction of these preparations with [^{35}S]DABS resulted in heavy labeling of the flavoprotein subunit of succinate dehydrogenase. There were also counts in the region of the smaller subunit of succinate dehydrogenase but no counts associated with CII-3 or CII-4. Most of the counts in the immunoprecipitate were once again in complex III

components, in this case in cytochrome *b* and one of the core proteins (II).

Discussion

In this paper we provide a detailed characterization of complex II preparations and describe the preparation and properties of rabbit antibody prepared against cardiac succinate dehydrogenase. Also, we report the use of these antibodies in combination with [^{35}S]DABS-labeling techniques to probe the microenvironment of the enzyme in the mitochondrial inner membrane and the orientation of succinate–ubiquinone reductase (complex II) across this membrane.

In recent studies, complex II has been shown to contain four different polypeptides, the two subunits of succinate dehydrogenase and two small molecular weight components (CII-3 and CII-4) of molecular weight 13 500 and 7000, respectively (Capaldi et al., 1977). The native-gel electrophoresis and isoelectric focusing gels described here confirm that these four components together constitute complex II. A functional role for CII-3 and CII-4 has not been conclusively determined. However, preliminary studies suggest that CII-3 may be necessary for electron transfer between succinate dehydrogenase and ubiquinone (B. A. C. Ackrell and E. B. Kearney, unpublished experiments). The relationship between this polypeptide and the polypeptide studied by Yu et al. (1978) and inferred to be a Q binding protein is not yet clear.

Antibodies were prepared against the succinate dehydrogenase holoenzyme and against the purified flavoprotein subunit of the enzyme. These antibodies immunoprecipitated both subunits of succinate dehydrogenase along with CII-3 and CII-4 from complex II preparations. The immunoprecipitate obtained by reaction with solubilized inner membrane was considerably more complex and contained polypeptides of complex III in addition to complex II components. Preliminary results suggest that the immunoprecipitate is a 1:1 aggregate of complexes II and III (see Results). The possibility that coprecipitation of complexes II and III was due to direct immune reaction between the antibody preparations and complex III was effectively ruled out. The immunoprecipitation data thus suggest that complex II plus complex III, i.e., succinate–cytochrome *c* reductase, is a stable structural unit in the mitochondrial inner membrane as advocated by King (1967).

The transmembrane arrangement of complex II components in the mitochondrial inner membrane was examined both by inhibition and labeling studies. Mitochondria were used as a source of membranes with the outer surface of the inner membrane available for antibody binding or chemical labeling. Several lines of evidence indicate that the mitochondria used were essentially intact. Firstly, they showed relatively good respiratory-control ratios for heart mitochondria with pyruvate and malate as substrate. Secondly, the rate of oxidation of NADH (to which the inner membrane is impermeable) was less than 5% of that of membranes made leaky to substrate by extensive sonication. Thirdly, there was very little labeling of F_1 ATPase which is known to be localized on the inner surface of the membrane (matrix side) [see Senior (1973) for a review].

Submitochondrial particles were used as a source of membranes with the matrix-facing surface available for reaction. Our preparations, obtained by mild sonication, were shown by antibody and labeling data to be mostly of opposite orientation to mitochondria. Firstly, antibody against F_1 ATPase brought down most of the submitochondrial particles under conditions where antibodies against cytochrome c_1 (known to be on the cytoplasmic face of the inner membrane) brought down less than 15% of the vesicles (Ludwig et al., 1979). Secondly, subunit VI of complex III, which was heavily labeled by DABS in mitochondria, was only poorly labeled in submitochondrial particles. Similarly, several components of cytochrome *c* oxidase and oligomycin-sensitive ATPase which were labeled in mitochondria were not labeled in submitochondrial particles and vice versa.

Evidence that succinate dehydrogenase is located on the matrix side of the mitochondrial inner membrane was first provided by Klingenberg (1970), who showed that submitochondrial particles but not intact mitochondria exhibited antimycin-insensitive succinate–ferricyanide reductase activity. As this electron acceptor is membrane impermeant (Mitchell & Moyle, 1969), this finding implies that the catalytic side, and hence part of the flavoprotein subunit on which this site is located (Kenney et al., 1976), is exposed on the matrix side.

Our antibody binding and chemical modification studies confirm the localization of this enzyme on the matrix surface of the inner membrane. Antibodies against succinate dehydrogenase holoenzyme inhibited succinate-linked activities in submitochondrial particles. Reaction of submitochondrial particles with 200 nmol of DABS/mg of protein inhibited succinate–PMS and succinate–ferricyanide activities while reaction of intact mitochondria with this high level of protein-modifying reagent had relatively little effect on these activities. We can therefore conclude that succinate dehydrogenase is at least functionally located in the matrix side.

As an alternative approach to determination of the location of subunits of complex II, mitochondria and submitochondrial particles were reacted with [^{35}S]DABS, and the extent of labeling of all of the subunits of complex II was determined from NaDodSO$_4$–polyacrylamide gels. In designing these labeling experiments, it was important to use concentrations of DABS which did not render the inner membrane permeable to the protein-modifying reagent. DABS at the level of 4 nmol/mg of protein appeared to meet this requirement as judged by the lack of effect on any of the mitochondrial activities monitored, including respiratory control (Table II). With 40 nmol of DABS/mg of protein, however, respiratory control was lost and oxidase activities were significantly lower. At 200 nmol of DABS/mg of protein, respiratory control was gone and most oxidase activities were completely destroyed. These higher levels of DABS have been used in several studies of the topology of the mitochondrial inner membrane (Schneider et al., 1972; Tinberg et al., 1974; Grigoliva & Konstantinov, 1977).

Our labeling studies then were done with between 4 and 6 nmol of DABS/mg of protein. These data show unambiguously that the flavoprotein subunit of succinate dehydrogenase is localized on the matrix side of the mitochondrial inner membrane and that CII-3 is exposed on the cytoplasmic side of the inner membrane. The locations of the smaller subunit of succinate dehydrogenase and CII-4 were not as clearly defined because neither was very heavily labeled and because it was difficult to be certain that the few counts which were present were actually associated with the complex II components and not with complex III subunits or with other polypeptides immunoprecipitated by the succinate dehydrogenase antibody. It is reasonable to assume that a portion of the smaller subunit of succinate dehydrogenase associates with the flavoprotein subunit at the matrix side of the mitochondrial inner membrane. Also, CII-3 and CII-4 probably form the major part of the bilayer intercalated part of complex II and are thus available for interaction with the lipophilic ubiquinone molecules. Recently, radioactively la-

beled arylazidophospholipids have been synthesized which can be used to tag those polypeptides extending into the interior of the membrane (R. Bisson, unpublished experiments). Such studies are now in progress in our laboratory, and, when taken together with the surface-labeling results presented here, they will allow a more complete picture of the structure of the succinate–ubiquinone reductase portion of the mitochondrial inner membrane.

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