# Interaction of Succinate–Ubiquinone Reductase (Complex II) with (Arylazido)phospholipids†

John Girdlestone, Roberto Bisson,‡ and Roderick A. Capaldi*

ABSTRACT: The interaction of purified succinate dehydrogenase and succinate–ubiquinone reductase (complex II) with lipids was explored by using two (arylazido)phospholipids, one with the reactive nitrene in the head-group region of the bilayer [1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[$^3$H]phosphocholine (PLII)] and one with the nitrene on the methyl terminus of one of the fatty acid chains [1-myristoyl-2-[12-[(2-azido-4-nitrophenyl)amino]lauroyl]-*sn*-glycero-3-[$^{14}$C]phosphocholine (PLI)]. Protein was reacted with vesicles of egg lecithin containing radioactive (arylazido)-phospholipids and the covalent cross-linking of lipid and protein induced by irradiation under UV light. Purified succinate dehydrogenase was found to bind to lipid vesicles through both subunits as both were labeled by PLII. The smaller subunit was inserted into the interior of the bilayer and labeled by PLI. Complex II was found to interact with lipid vesicles, with the smaller subunit of succinate dehydrogenase, CII-3, and CII-4 all inserted into the interior of the bilayer. The large subunit of succinate dehydrogenase was found to be held above the bilayer in complex II and not labeled by either probe. Results are used to derive a picture of the arrangement of subunits in complex II and to evaluate the utility of (arylazido)-phospholipids in membrane studies.

Succinate dehydrogenase is one of two major entry points of electrons into the mitochondrial electron-transport chain. It has been isolated as a water-soluble enzyme consisting of two subunits, one of 70 000 daltons containing the flavin moiety (Davis & Hatefi, 1971) and the active site (Kenney et al., 1976), and a smaller subunit of molecular weight 27 000. The isolated enzyme reacts with electron acceptors such as ferricyanide and phenazine methosulfate (PMS)[1] but not with its physiological electron acceptor ubiquinone. The simplest unit capable of succinate–ubiquinone reductase activity is complex II, a fragment of the mitochondrial inner membrane containing succinate dehydrogenase and in addition two polypeptides of approximate molecular weight 13 500 and 7000 (Capaldi et al., 1977). The 13 500-dalton component (CII-3) has been shown to be important for succinate–ubiquinone reductase activity (Ackrell et al., 1980). The role of CII-4 in this reaction remains obscure.

Our recent studies have been concerned with detailing the organization of complex II. Antibodies were made against succinate dehydrogenase and against the purified flavin-containing subunit, and these were shown to interact with submitochondrial particles and inhibit succinate-linked activities (Merli et al., 1979). The complex was also labeled with the water-soluble, lipid-insoluble, and protein-modifying reagent [$^{35}$S]diazobenzenesulfonate, first in the isolated complex and then with the complex in the mitochondrial inner membrane (Merli et al., 1979). Taken together, the antibody binding and chemical labeling experiments showed that complex II spans the mitochondrial inner membrane, with the flavoprotein subunit on the matrix side of the membrane, with the small subunit of the enzyme also on the matrix side of the membrane but partly shielded from water by interaction with the membrane and with CII-3 exposed at the cytoplasmic surface of the inner membrane. Both CII-3 and CII-4 were only poorly labeled by [$^{35}$S]DABS in the mitochondrial inner membrane, suggesting that they both are intercalated into the lipid bilayer (Merli et al., 1979). To test this possibility directly, we have now conducted experiments with (arylazido)phospholipids, phospholipid derivatives containing an azido group in one of the fatty acid tails (Bisson & Montecucco, 1980). Several recent studies have shown that these molecules are useful probes for the bilayer intercalated portions of intrinsic membrane proteins (Bisson et al., 1979a,b; Montecucco et al., 1979; Prochaska et al., 1980). Experiments were conducted both with isolated succinate dehydrogenase and with complex II. The results indicate that not only CII-3 and CII-4 but also the smaller subunit of succinate dehydrogenase penetrates into the interior of the lipid bilayer. This information is used to derive a more detailed model of complex II.

## Experimental Procedures

*Enzyme Preparations.* Succinate dehydrogenase prepared according to Ackrell et al. (1977) and complex II isolated by the procedure of Baginsky & Hatefi (1969) were the kind gifts of Dr. B. A. C. Ackrell, University of California, San Francisco. These preparations showed succinate–PMS and, in the case of complex II, succinate–DCIP activities similar to those reported previously (Ackrell et al., 1977; Singer, 1974; see Results).

*Lipid Preparations and Vesicle Formation.* The preparations of 1-myristoyl-2-[12-[(2-azido-4-nitrophenyl)amino]lauroyl]-*sn*-glycero-3-[$^{14}$C]phosphocholine (PLI) and 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[$^3$H]phosphocholine (PLII) have been described by Bisson & Montecucco (1980). The specific radioactivities of these (arylazido)-phospholipids were 177 Ci/mol and 3.9 Ci/mmol, respectively. Lipid vesicles incorporating the (arylazido)phospholipids were prepared according to Bisson et al. (1979a). Protein (succinate

† From the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received May 14, 1980.* This work was supported by a grant from the National Institutes of Health (HL 22050). R.A.C. is an Established Investigator of the American Heart Association. J.G. was supported by the Natural Science and Engineering Research Council of Canada.

‡ Present address: Instituto di Patologia Generale, Universita di Padova, 35100 Padova, Italy.

[1] Abbreviations used: PMS, phenazine methosulfate; DABS, diazobenzenesulfonate; DCIP, dichloroindophenol; PLI, 1-myristoyl-2-[12-[(2-azido-4-nitrophenyl)amino]lauroyl]-*sn*-glycero-3-[$^{14}$C]phosphocholine; PLII, 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[$^3$H]phosphocholine ; $NaDodSO_4$, sodium dodecyl sulfate; DMPC, dimyristoylphosphatidylcholine.

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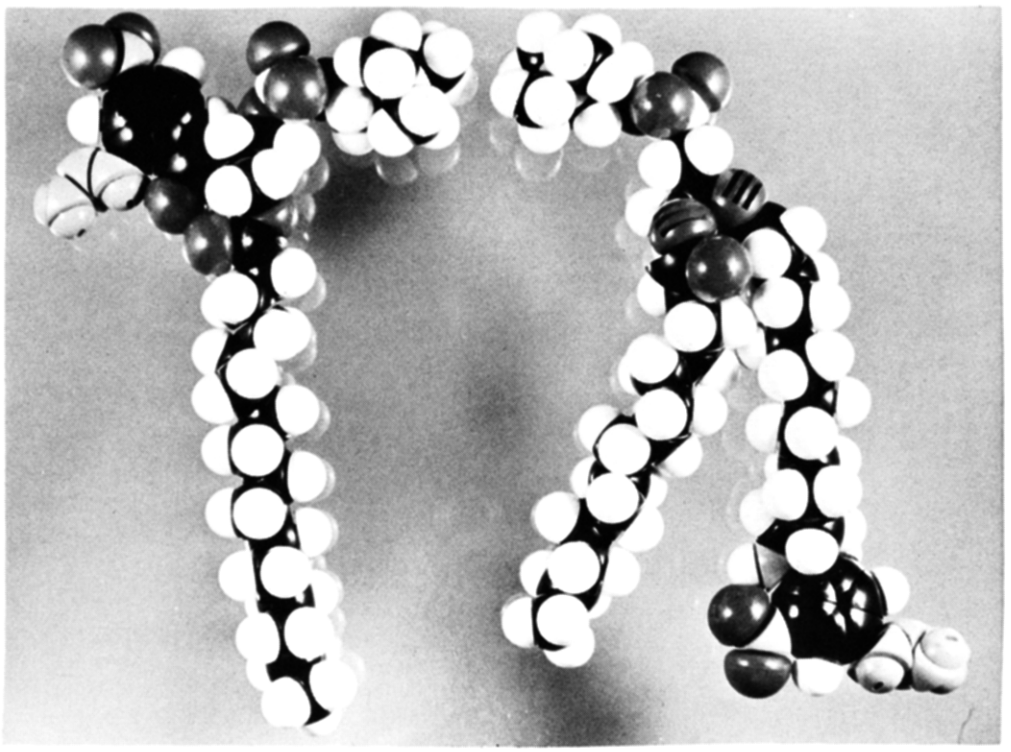

FIGURE 1: Space-filling models for (left) 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[$^3$H]phosphocholine (PLII) and (right) 1-myristoyl-2-[12-[(2-azido-4-nitrophenyl)amino]lauroyl]-*sn*-glycero-3-[$^{14}$C]phosphocholine.

dehydrogenase or complex II) was added to give a 2:1 (w/w) ratio of lipid to protein, and the mixture was either incubated on ice for 1 h (insertion procedure) or sonicated for 10 min in 1-min bursts on ice (sonication procedure), or dissolved in a small volume of 1% cholate and then dialyzed against 50 mM potassium phosphate, pH 7.0 (dialysis procedure), for vesicle formation.

*Cross-Linking of (Arylazido)phospholipids and Protein.* The cross-linking reaction was conducted in an atmosphere of $N_2$. In most experiments, covalent linking of the (arylazido)phospholipids with protein was effected by irradiating samples in glass vials for 1 h at 0 °C with a low-intensity ultraviolet lamp (Mineralight). Certain experiments were conducted at low temperature. In these, protein was incubated with vesicles of egg lecithin at 0 °C and this suspension drawn up into polyethylene tubing (Clay Adams; i.d. 0.58 mm, o.d. 0.965 mm) and immersed in liquid $N_2$ before irradiation for 15 min. Protein was isolated by centrifuging the irradiated samples through 7% sucrose with a Beckman SW50.1 rotor at 40 000 rpm for 3.5–4 h. The pellet was resuspended in a solution of 5% $NaDodSO_4$, 10 mM potassium phosphate, and 8 M urea (pH 6.8) containing 5% $\beta$-mercaptoethanol by heating to 100 °C for 2 min. Samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis on gels containing 15% acrylamide and 0.5% *N*,*N*′-methylenebis(acrylamide) according to Swank & Munkres (1971). Gels were stained with Coomassie brilliant blue and scanned at 560 nm by using a Gilford gel scanner. They were sliced in 1-mm-thick slices and counted as described previously (Merli et al., 1979).

## Results

Experiments were conducted with the two (arylazido)-phospholipids shown in Figure 1. PLII, containing the photoactive arylazido group attached directly to the glycerol backbone, was designed to label proteins from the head-group region of the bilayer. PLI, containing the arylazido group at the methyl terminus of a 12-carbon fatty acid, was designed to label proteins from within the bilayer.

These photoaffinity lipids were incorporated into vesicles made with egg lecithin in the ratio 1:1000 for PLII and 1:400 for PLI, and the reaction of protein (succinate dehydrogenase or complex II) with lipid vesicles was effected by incubating enzyme with the lipid for 1 h at 0 °C in an atmosphere of nitrogen (insertion procedure). Anaerobic conditions were used to prevent the air oxidation and concomitant denaturation of isolated succinate dehydrogenase. After incorporation of protein into vesicles, the membrane preparation was irradiated for 1 h with UV light filtered through a thin glass tube. The irradiation was done under nitrogen and at 0 °C (except where stated). This low intensity of UV irradiation [$(1–3) \times 10^3$ erg $cm^{-2}$ $s^{-1}$] was sufficient to photoactivate the arylazido group (as evidenced by covalent cross-linking of phospholipid to protein; see Figures 2 and 3) without significantly reducing enzymatic activity. The succinate–PMS reductase activity of isolated succinate dehydrogenase after incubation in the presence of phospholipids and after irradiation with UV light was 90–95% of that of an untreated control (different preparations ranged from 45 to 60 $\mu$mol of succinate oxidized $min^{-1}$ $mg^{-1}$ at 38 °C). The insertion procedure and irradiation with UV light had a similarly small effect on the activity of complex II. In a typical experiment, the $V_{max}$ for a control sample of complex II was measured as 24.7 $\mu$mol of succinate oxidized $min^{-1}$ $mg^{-1}$. After incubation with lipids followed by the irradiation step, the activity of this preparation was 21.7 $\mu$mol of succinate oxidized $min^{-1}$ $mg^{-1}$, i.e., an inhibition of less than 15%.

The covalent cross-linking of (arylazido)phospholipids with protein was monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 2 shows the labeling of purified succinate dehydrogenase which had been reacted with lipid vesicles containing PLII (solid line) and PLI (dotted line). It can be seen that both subunits of the enzyme were labeled equally by PLII while the small subunit was much more heavily labeled than the flavoprotein subunit by PLI. This difference

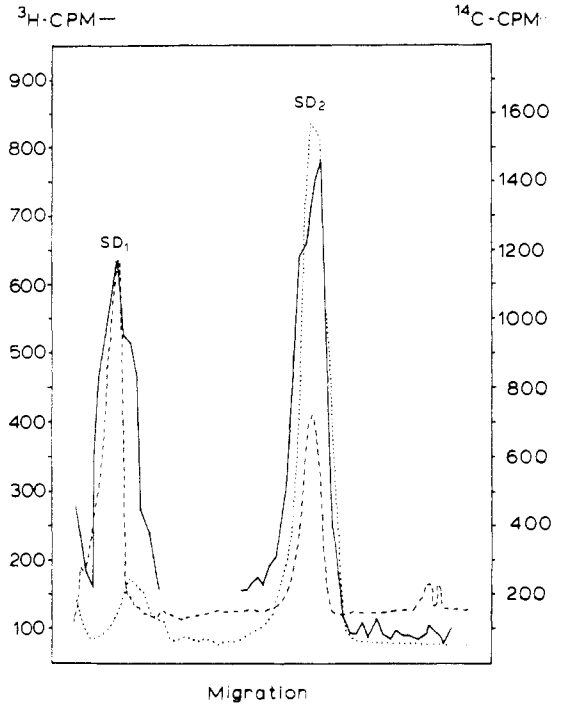


FIGURE 2: Labeling of succinate dehydrogenase with (arylazido)-phospholipids in egg lecithin vesicles at 0 °C. The dashed line shows the electrophoretic profile of protein. The solid and dotted lines show the distribution of radioactivity after irradiation with PLII and PLI, respectively.

in labeling is most reasonably explained by assuming that succinate dehydrogenase binds to the polar head groups of the phospholipids through both subunits and with the smaller subunit but not the flavoprotein subunit intercalated into the hydrophobic interior of the bilayer (for relatively heavy labeling by PLI). This interpretation, in turn, implies that the small amount of labeling of the flavoprotein subunit by PLI is due to folding back of the arylazido-containing fatty acid to the surface of the membrane.

Succinate dehydrogenase was also reacted with the two (arylazido)phospholipids in membranes held at below their phase transition temperature, rather than above it as in Figure 2. In one set of experiments, the enzyme was incubated with vesicles of DMPC at 0 °C (below the phase transition temperature of this lipid), and then the photolysis reaction was conducted at the same temperature. In a second set of experiments, the enzyme was incubated with vesicles of egg lecithin at 0 °C (above the phase transition temperature), the sample was then frozen to liquid nitrogen temperatures (below the phase transition temperature of the lipids), and the photolysis reaction begun. All experiments were conducted with the same ratio of PLI or PLII to protein and at the same ratios of total lipid to protein. A comparison of the extent of labeling of the two subunits by PLI in the different experiments (Table I) shows that the labeling of the flavoprotein subunit by this probe was similar under different conditions of the reaction while the labeling of the smaller subunit was 10 times or more higher in experiments done above the phase transition temperature than below it. Most factors which could affect the extent of labeling of subunits should affect both subunits to the same extent. These include changes in the collision frequency of probe and protein in a liquid crystalline vs. a gel-state membrane (because of changes in the viscosity of the bilayer) as well as an increase in looping back of the arylazido fatty acid in gel-state lipids than in a fluid membrane. [A similar effect is seen with nitroxide-containing fatty acids at below the phase transition temperature of lipids; see Cadenhead & Muller-Landau (1973).] The dramatic difference in labeling of the two subunits at above and below the phase transition temperature can only be explained by exclusion of the small subunit of succinate dehydrogenase from the interior of the bilayer in the gel-state lipids. For experiments done with DMPC, this would mean that succinate dehydrogenase does not intercalate into the bilayer at below the phase transition temperature. For experiments done with egg lecithin

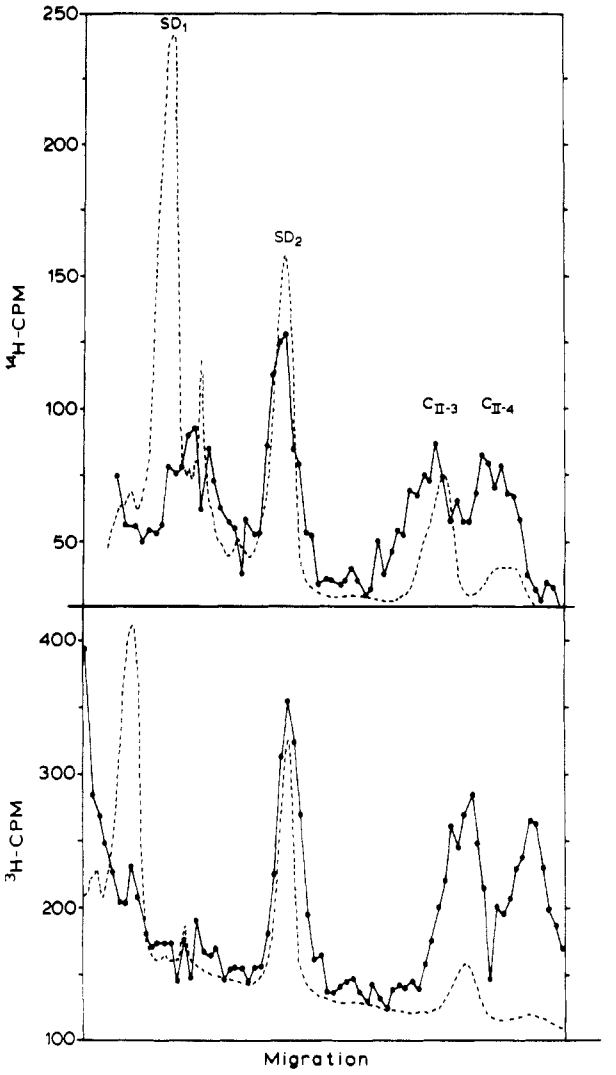


FIGURE 3: Labeling of complex II with (arylazido)phospholipids. The dashed line shows the electrophoretic profile of the protein. The solid line shows the profiles of radioactivity with PLI (top) and PLII (bottom).

Table I: Relative Labeling of Succinate Dehydrogenase Subunits by PLI under Different Experimental Conditions

| exptl conditions | incorporation of PLI as cpm in subunit: flavoprotein subunit | incorporation of PLI as cpm in subunit: 27 000-dalton subunit |
|---|---|---|
| egg lecithin vesicles, irradiation at 0 °C | 800 | 8600 |
| egg lecithin vesicles, irradiation at liquid $N_2$ temperature | 300 | 1300 |
| DMPC vesicles, incubated with enzyme at 0 °C, irradiated at same temperature | 500 | 600 |

at liquid nitrogen temperatures, when protein was inserted into the membrane above the phase transition temperature, it would mean that the enzyme was squeezed out of the bilayer upon freezing [see also Wunderlich et al. (1975), Borochov & Shinitsky (1976), and Armond & Staehelin (1979) for similar effects].

The labeling of complex II incorporated into vesicles of egg lecithin containing PLI and PLII is shown in Figure 3. The upper and lower traces show the Coomassie brilliant blue staining profile of two different complex II preparations. In addition to the two subunits of succinate dehydrogenase, complex II contains small molecular weight components labeled CII-3 and CII-4 in the figure. Under the gel conditions used here, CII-4 was always seen as a broad band and in some cases as a closely spaced doublet, indicating that there is probably more than one polypeptide in this fraction. Minor bands on the gels are impurities derived from complex III. The upper part of Figure 3 shows the labeling of complex II by PLI, the lower part of the figure the labeling by PLII. The large subunit of succinate dehydrogenase was not labeled by either probe in complex II preparations. The other components of complex II, namely, $SD_2$, CII-3, and CII-4, were all labeled, and the ratio of counts in these three polypeptides was nearly the same for both probes. The results shown in Figure 3 proved to be reproducible for different preparations of complex II. Moreover, the same labeling profile was seen whether protein was incorporated by insertion, sonication, or by the cholate dialysis procedure of membrane formation.

## Discussion

It is now generally accepted that the mitochondrial electron-transfer chain is made up of four multicomponent complexes, each of which is an intrinsic part of the mitochondrial inner membrane [for review, see Hatefi (1976)]. Complex II catalyzes the electron transfer from succinate to ubiquinone. More recent work has shown that this complex is constructed from the Krebs cycle enzyme succinate dehydrogenase tightly bound to two small molecular weight polypeptides labeled for convenience as CII-3 and CII-4 (Capaldi et al., 1977; Merli et al., 1979). The conditions of $NaDodSO_4$–polyacrylamide electrophoresis used in the present study indicate that CII-4 may in fact be a mixture of two (or possibly more) polypeptides, and this possibility warrants further study.

The major goal of the present work was to determine which of the components of complex II penetrates into the lipid bilayer for hydrophobic interaction with the lipid fatty acids. Experiments were conducted with purified succinate dehydrogenase and with complex II. These preparations were incorporated into lipid vesicles containing (arylazido)-phospholipids, and the covalent interaction of protein and lipid analogues was induced by UV irradiation of samples. Purified succinate dehydrogenase was labeled by both of the (arylazido)phospholipids used. The two subunits of the enzyme were labeled about equally by PLII containing the arylazido group in the head-group region of the molecule. The smaller subunit was also labeled heavily by PLI, which contains the arylazido group at the methyl terminus of one fatty acid. The flavoprotein subunit, in contrast, was labeled to less than 10% of the amount of the small subunit with this probe. These labeling data are taken to indicate that succinate dehydrogenase binds to phospholipid vesicles by ionic interactions involving either subunit of the enzyme and with hydrophobic interactions through insertion of the smaller subunit among the fatty acids of the membrane [see McPhail & Cunningham (1975) for a discussion of the role of lipid in succinate dehydrogenase activity].

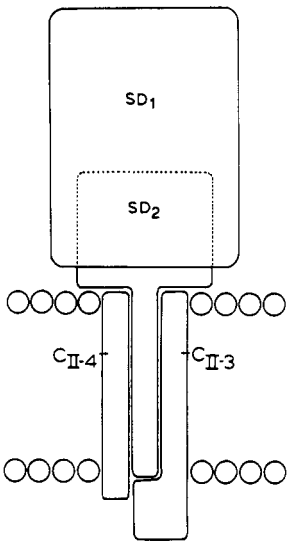


FIGURE 4: Schematic representation of the arrangement of subunits in complex II.

When succinate dehydrogenase was incorporated into lipid vesicles at below their phase transition temperature and when enzyme-containing membranes were cooled rapidly to below this temperature, the labeling of the small subunit was drastically reduced. This is consistent with the protein being excluded from the bilayer in gel-state lipids. Such an effect was not seen with intact complex II (results not shown), complex III, or cytochrome *c* oxidase, suggesting that with succinate dehydrogenase only a small portion of the molecule enters the bilayer.

The small amount of labeling of the flavoprotein subunit of isolated succinate dehydrogenase by PLI warrants comment. As described under Results, this labeling can best be explained by folding back of a fraction of the arylazido fatty acids to the surface of the membrane [as proposed by Bayley & Knowles (1978)]. The important point is that this rearrangement, if it occurs, is not a major event as judged by the relative labeling shown in Figure 2. Several recent studies have indicated the usefulness of (arylazido)phospholipids as probes of the interaction between proteins and lipids (Bisson et al., 1979a,b; Montecucco et al., 1979; Prochaska et al., 1980). The results with succinate dehydrogenase (and complex II) confirm this utility, but stress the importance of comparative studies with probes containing photoactive groups in the fatty acid and in the head-group region, in order to make meaningful assignments of which polypeptides in an enzyme or complex are extrinsic or intrinsic to the bilayer.

The labeling of succinate dehydrogenase in complex II by (arylazido)phospholipids was found to be different from that seen with the purified enzyme. In the complex, the flavoprotein subunit was not labeled by either PLI or PLII. The interaction of the enzyme with CII-3 and/or CII-4 must therefore shield (or raise) this polypeptide from the bilayer surface. The small subunit of succinate dehydrogenase, CII-3, and CII-4 were each labeled by both probes. Our previous experiments, in which [$^{35}$S]diazobenzenesulfonate was used to label the surface-exposed portions of complex II in intact mitochondria and submitochondrial particles (Merli et al., 1979), had provided suggestive evidence that CII-3 and CII-4 were intercalated into the lipid bilayer. The results reported here, provide direct evidence of this location and are consistent with a model for complex II shown in Figure 4. Sequencing of the bilayer-intercalated polypeptides of cytochrome *c* oxidase and the oligomycin-sensitive ATPase has revealed that these polypeptides each contain long stretches of amino acids (20 or more

residues in length) devoid of charged amino acids (Sebald, 1977; Steffens & Buse, 1978; Sacher et al., 1979; Macino & Tzagoloff, 1979, 1980). In the case of subunits II and IV of cytochrome *c* oxidase, definitive evidence has been obtained to show that these hydrophobic stretches contribute the bilayer-intercalated portion of the protein (Capaldi et al., 1980). It will be interesting to see whether the small subunit of succinate dehydrogenase, CII-3, and CII-4 each contain long stretches of uncharged amino acids in their sequences, and this work is now in progress.

Acknowledgments

The (arylazido)phospholipids were synthesized by R.B. and Dr. Cesare Montecucco, University of Padova, supported by the Consiglio Nazionale delle Ricerche Unit for the Study of Physiology of Mitochondria, Padova, Italy.

References

Ackrell, B. A. C., Kearney, E. B., & Coles, C. J. (1977) *J. Biol. Chem. 252*, 6963.

Ackrell, B. A. C., Ball, M. B., & Kearney, E. B. (1980) *J. Biol. Chem.* (in press).

Armond, P. A., & Staehelin, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1901.

Baginsky, M. L., & Hatefi, Y. (1969) *J. Biol. Chem. 244*, 5313.

Bayley, H., & Knowles, J. R. (1978) *Biochemistry 17*, 2414.

Bisson, R., & Montecucco, C. (1980) *Eur. J. Biochem.* (in press).

Bisson, R., Montecucco, C., Gutweniger, H., & Azzi, A. (1979a) *J. Biol. Chem. 254*, 9962.

Bisson, R., Montecucco, C., & Capaldi, R. A. (1979b) *FEBS Lett. 106*, 317.

Borochov, H., & Shinitsky, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4526.

Cadenhead, D. A., & Muller-Landau, F. (1973) *Biochim. Biophys. Acta 307*, 279.

Capaldi, R. A., Sweetland, J., & Merli, A. (1977) *Biochemistry 16*, 5707.

Capaldi, R. A., Bisson, R., & Prochaska, L. (1980) in *Interaction between Iron and Proteins in Oxygen and Electron Transport* (Ho, C., & Eaton, W. C., Eds.) Elsevier, Amsterdam (in press).

Davis, K. A., & Hatefi, Y. (1971) *Biochemistry 10*, 2509.

Hatefi, Y. (1976) *Enzymes Biol. Membr. 4*, 1–42.

Kenney, W. C., Mowery, P. C., Seng, R. L., & Singer, T. P. (1976) *J. Biol. Chem. 251*, 2369.

Macino, G., & Tzagoloff, A. (1979) *J. Biol. Chem. 254*, 4617.

Macino, G., & Tzagoloff, A. (1980) *Cell 20*, 507.

McPhail, L. C., & Cunningham, C. C. (1975) *Biochemistry 14*, 1122.

Merli, A., Capaldi, R. A., Ackrell, B. A. C., & Kearney, E. B. (1979) *Biochemistry 18*, 1393.

Montecucco, C., Bisson, R., Pitotti, A., Dabbeni-Sala, F., & Gutweniger, H. (1979) *Biochem. Soc. Trans. 7*, 954.

Prochaska, L., Bisson, R., & Capaldi, R. A. (1980) *Biochemistry* (in press).

Sacher, R., Steffens, G. J., & Buse, G. (1979) *Hoppe Seyler's Z. Physiol. Chem. 360*, 1385.

Sebald, W. (1977) *Biochim. Biophys. Acta 463*, 1.

Singer, T. P. (1974) *Methods Biochem. Anal. 22*, 123.

Steffens, G. J., & Buse, G. (1978) *Hoppe Seyler's Z. Physiol. Chem. 359*, 1005.

Swank, R. T., & Munkres, R. D. (1971) *Anal. Biochem. 39*, 462.

Wunderlich, F., Ronai, A., Speth, V., Seelig, J., & Blume, A. (1975) *Biochemistry 14*, 3730.