Biochimica et Biophysica Acta, 345 (1974) 118–128

BBA 76600

STUDIES ON MITOCHONDRIAL PROTEINS

II. LOCALIZATION OF COMPONENTS IN THE INNER MEMBRANE: LABELING WITH DIAZOBENZENESULFONATE, A NON-PENETRATING PROBE

HAROLD M. TINBERG, RONALD L. MELNICK*, JOHN MAGUIRE and LESTER PACKER

Physiology Research Laboratory, Veterans Administration Hospital, Martinez, Calif. 94553, and Department of Physiology-Anatomy, University of California, Berkeley, Calif. 94720 (U.S.A.)

SUMMARY

The topography of the inner membrane of rat liver mitochondria was studied using a probe, diazobenzenesulfonate, which interacts preferentially with surface components. Inner membranes were examined both in a native orientation as found in the intact mitochondrion or in an inverted state as found in isolated inner membranes prepared by sonication.

Enzyme inactivation as a consequence of diazobenzenesulfonate labeling was employed to determine the localization of a number of inner membrane activities. In inner membranes labeled on the outer surface, NADH and succinate oxidation were strongly inhibited while ATPase and ascorbate-*N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD) oxidase activities were unaffected. In inner membranes labeled on the inner surface, ATPase and succinate oxidation were inactivated while NADH oxidation and ascorbate-TMPD oxidase were unaffected. Succinate dehydrogenase was inhibited only by labeling the inner surface while NADH dehydrogenase was inhibited to a similar extent by treatment of either surface.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed that diazobenzenesulfonate labeled two polypeptides (66 000 and 26 000) on the outer surface of the inner membrane and five polypeptides (80 000, 66 000, 51 000–48 000, and 26 000) on the inner surface. These results indicate a highly asymmetric localization of inner membrane components.

---

* Present address: Department of Life Science, Polytechnic Institute of Brooklyn, Brooklyn, New York 11201.

Abbreviations: TMPD, *N*,*N*,*N'*,*N'*,-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol; OSP preparation, inner membranes obtained from labeled mitochondria; ISP preparation, inner membranes labeled directly; IOSP preparation, inner membranes obtained by labeling the OSP preparation.

INTRODUCTION

At the molecular level, membrane function may depend upon a precise spatial arrangement of components. Components may reside on one or both surfaces or in the core of the membrane. Numerous approaches have been employed to localize proteins in membranes. These include immunological methods [1], cytochemical methods [2], enzymatic degradation [3], substrate accessibility [4], and interaction with impermeable probes [5–8].

Recently, we have reported on the polypeptide composition of the inner membrane of rat liver mitochondria [9]. The present study was undertaken to investigate the disposition of these proteins with respect to the plane of the membrane. The inner membrane is particularly well suited for such studies because it can be obtained in either a normal orientation as found in mitochondria or in an inverted orientation as in sonically prepared submitochondrial preparations. The fact that different membrane surfaces face the external medium in the two preparations allows one to probe each surface of the inner membrane separately. These surfaces were probed using a membrane-impermeable alkylating agent, diazobenzenesulfonate.

MATERIALS AND METHODS

Isolation of membrane preparations

Mitochondria were isolated from rat liver as described previously [10]. Mitoplasts (inner membranes plus matrix) were prepared by the digitonin method of Schnaitman and Greenawalt [11]. Inner membranes were obtained by sonicating mitoplasts (in 0.03 M sodium phosphate buffer, pH 7.0) and sedimenting the suspension at 140 000 $\times g$ (45 min) after removing unbroken mitoplasts by centrifuging for 15 min at 25 000 $\times g$. All experiments were performed on fresh preparations.

Preparation of diazobenzenesulfonate

Sulfanilic acid (0.25 mmole) was dissolved in 1 ml 2.5% sodium carbonate by warming. The solution was placed on ice, and sodium nitrite (0.53 mmole) was added. When the nitrite had dissolved, 0.15 ml concentrated HCl was added and the solution returned to the ice bath. Diazobenzenesulfonate precipitated within 5–10 min. The material was isolated by centrifugation (2000 rev./min, 5 min, 4 °C, Sorvall RC2B centrifuge) washed with 1 ml ice-cold water, and dissolved in 2.5 ml of 0.10 M sodium phosphate buffer (pH 8.0) containing 0.25 M sucrose. The diazobenzenesulfonate was stored on ice and used within a few hours. Diazobenzene-[^{35}S]sulfonate was prepared as described above by including [^{35}S]sulfanilic acid in the reaction mixture. The diazobenzene-[^{35}S]sulfonate was prepared at a specific activity of 1–5 Ci/mole.

Diazobenzenesulfonate labeling

Mitochondria (10 mg protein/ml) and inner membranes (4 mg protein/ml) were treated with diazobenzenesulfonate (2 μmole/ml) at 4 °C in 0.05 M sodium phosphate buffer (pH 8.0) containing 0.25 M sucrose (Fig. 1). The reaction was terminated after 15 min by dilution with 0.25 M sucrose containing 0.05 M Tris–HCl (pH 8.0), which complexed unreacted diazobenzenesulfonate. The treated mitochondria were washed in 0.25 M sucrose and resuspended in sucrose for the preparation of mitoplasts.

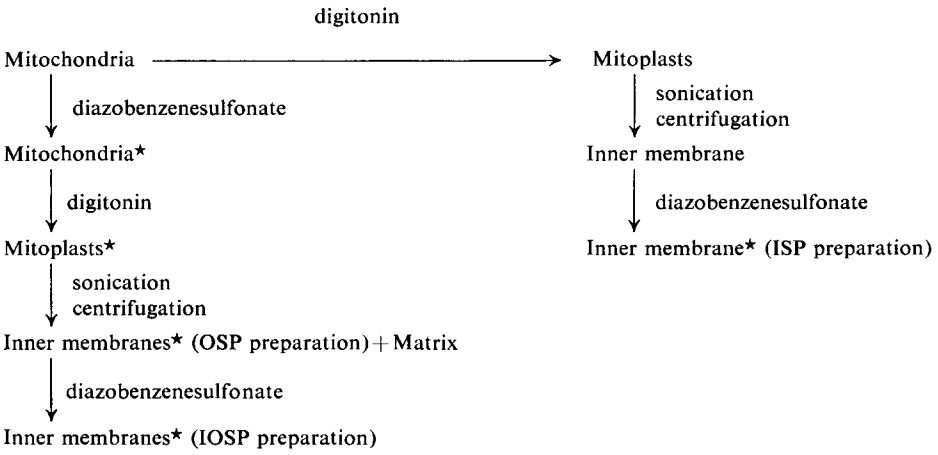


Fig. 1. Labeling of inner membrane preparations with diazobenzenesulfonate. Labeled preparations are designated by an asterisk (*).

Inner membranes were isolated from these mitoplasts as described above. Inner membranes treated directly with diazobenzenesulfonate were diluted with the Tris–sucrose buffer and isolated by centrifugation at 140 000 $\times g$ for 45 min. Both preparations were suspended in water at a concentration of 2 mg protein/ml and prepared for electrophoresis by solubilization with 1% sodium dodecylsulfate and 1% mercaptoethanol [9].

To determine the amount of diazobenzene-[^{35}S]sulfonate bound, the solubilized samples (20–50 μl) were added to scintillation vials containing Beckman Biosolve: scintillation fluid (1:2, v/v). The vials were shaken until the solution clarified. Scintillation fluid (15 ml) was added and the samples counted in a Packard Tricarb Scintillation Counter.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in gels containing 6% acrylamide and 0.1% sodium dodecylsulfate as described previously [9]. Gels were stained for protein with Coomassie brilliant blue as described by Fairbanks et al. [12]. Radioactivity was localized in unstained gels by fractionation on a Maizel-type apparatus (Savant Instruments). After the fractions had been dissolved in 1 ml hydrogen peroxide (overnight, at 50 °C), 15 ml of Aquasol scintillation cocktail was added and the samples were counted in a liquid scintillation counter.

Enzyme assays

ATPase activity was determined as described by Caterall and Pedersen [13]. All other activities were measured in a reaction mixture containing 0.05 M Tris–HCl buffer (pH 8.0) and 0.25 M sucrose. The following activities were determined essentially as described by published methods: NADH–cytochrome *c* reductase [14], succinate dehydrogenase [15], and succinate–cytochrome *c* reductase [16]. NADH oxidase was determined using 0.3 mM NADH and monitoring the decrease in absor-

bance at 340 nm on the Cary 14 spectrophotometer. NADH dehydrogenase was determined by measuring the decrease in absorbance at 340 nm of a reaction mixture containing 0.3 mM NADH, 40 μM dichlorophenolindophenol (DCIP), and antimycin A ($\approx$1 μg/mg protein). Succinate oxidase and ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) oxidase activities were determined polarographically using 5 mM succinate and 4 mM ascorbate plus 0.3 mM TMPD respectively. All enzymes were assayed at 30 °C.

Miscellaneous analyses

Free amino groups were determined by the trinitrobenzenesulfonate method [17]. Protein was determined by the method of Lowry et al. [18], using bovine serum albumin as standard. Lipids were extracted from membrane samples using the method of Folch et al. [19].

Materials

Sulfanilic acid was obtained from J. T. Baker Chemical Company (Phillipsburg, N. Y.). [^{35}S]Sulfanilic acid and Aquasol Scintillation Cocktail was purchased from New England Nuclear (Boston, Mass.). Trinitrobenzenesulfonate was purchased from Sigma Chemical Company (St. Louis, Mo.). Biosolve was obtained from Beckman Instruments (Fullerton, Calif.).

RESULTS

Labeling of inner membranes with diazobenzenesulfonate

Mitochondria and isolated inner membranes were treated with diazobenzenesulfonate as shown in Fig. 1. This scheme was devised to specifically label either or both of the surfaces of the inner membrane. The following preparations resulted from this protocol: (a) Outer surface labeled preparations (OSP preparation): as mitochondria were used as starting material to prepare the OSP preparation these particles should be labeled on the outer surface of the inner membrane, i.e., on the surface facing the outer membrane. (b) Inner surface labeled preparations (ISP preparation): since inner membranes invert upon sonication, these particles should be labeled on the inner surface of the inner membrane, i.e., on the membrane surface originally facing the matrix. (c) Doubly-labeled particles (IOSP preparation): these particles were obtained by labeling OSP preparations and should be labeled on both membrane surfaces. Inner membranes constitute approximately 30–40% of the total mitochondrial protein [20]. To ensure similar membrane concentrations during diazobenzenesulfonate treatment of the three systems, reaction of mitochondria was done at a higher protein concentration (10 mg/ml) than that used to label isolated inner membranes (4 mg/ml).

Diazonium salts react with histidyl, tyrosyl and lysyl residues in proteins [21]. However, no decrease in the content of free amino groups was detected in the three inner membrane preparations relative to controls. Nevertheless, we suggest that diazobenzenesulfonate is a relatively non-specific labeling agent and that incorporation of this label into proteins reflects the extent to which these proteins are exposed to the bulk aqueous phase. Diazobenzene-[^{35}S]sulfonate was used to determine the extent of labeling in the various inner membrane preparations (Table I). Only 2–6%

TABLE I

INCORPORATION OF DIAZOBENZENE-[^{35}S]SULFONATE INTO MITOCHONDRIAL INNER MEMBRANE PREPARATIONS

Intact mitochondria (10 mg protein/ml) and isolated inner membranes (4 mg protein/ml) were labeled with diazobenzene-[^{35}S]sulfonate (2 μmoles/ml, $5 \cdot 10^6$ cpm/μmole) as described in Materials and Methods and in Fig. 1. Aliquots of the membrane fractions were dissolved and the extent of radioactivity determined in a liquid scintillation counter as outlined in Materials and Methods.

Preparation	Diazobenzene-[^{35}S]sulfonate labeling (cpm/mg protein)
OSP	106 000
ISP	357 000
IOSP	447 000
Matrix	20 000

of the radioactivity present in the inner membrane could be extracted with chloroform: methanol (2:1, v/v) indicating that essentially all the label present in the samples resides in the protein component. Under the conditions of labeling, the ISP preparation possessed a specific activity over three times that of the OSP preparation, while the preparation labeled on both surfaces approximated the sum of the other two preparations. Leakage of diazobenzenesulfonate through the inner membrane was investigated by determining the level of radioactivity present in the matrix derived from treated mitochondria which had been washed to remove unreacted reagent. The specific activity of the OSP preparation was slightly more than five times that of the matrix. A considerable percentage of the radioactivity present in the matrix is due to contamination from fragmented inner membranes which failed to sediment under the centrifugal fields employed to isolate these membranes [9]. Thus, we suggest that extensive penetration of the diazobenzene-[^{35}S]sulfonate through the inner membrane does not occur.

In initial experiments, the OSP preparation was also prepared by labeling mitoplasts (inner membranes plus matrix). Inner membranes obtained in this way possessed a specific activity only twice that of the matrix, indicating some leakage of the reagent through the membrane. Leakage was also indicated by the fact that the specific activity of the ISP preparation was only twice that of the OSP preparation. Therefore, we prepared the OSP preparation from mitochondria rather than mitoplasts in subsequent experiments.

Enzyme activities of diazobenzenesulfonate-labeled inner membranes

Since diazonium salts react with a number of amino acid residues, coupling may result in the inactivation of the modified protein molecule. Therefore, a comparison of enzyme activities in membrane preparations labeled at different surfaces may provide information concerning the localization of these activities. A comparison of enzyme activities in the labeled inner membrane preparations was carried out and the results are summarized in Table II. Extensive inhibition of NADH oxidation occurred in the OSP preparation while more than 80% of this activity was retained in the ISP preparation. Succinate oxidation was strongly inhibited in both preparations. To obtain further information concerning the site of inhibition, various seg-

TABLE II

THE EFFECT OF DIAZOBENZENESULFONATE LABELING ON MITOCHONDRIAL INNER MEMBRANE ENZYME ACTIVITIES

Preparation of membranes, labeling with diazobenzenesulfonate, and enzyme assays were carried out as described in Materials and Methods and in Fig. 1. Activities have been normalized to unlabeled controls and are given either as the means of two experiments or as the means ± standard deviations.

Enzyme system	Activity (% of control)	
	OSP preparation	ISP preparation
ATPase	114	20
NADH oxidase	19 ± 6	80 ± 24
NADH dehydrogenase	45	46
NADH–cytochrome *c* reductase	53	99
Succinate oxidase	14	5
Succinate dehydrogenase	84 ± 12	7 ± 1
Succinate–cytochrome *c* reductase	28	13
Ascorbate–TMPD oxidase	110	156

ments of the respiratory chain were measured. Cytochrome oxidase (measured as ascorbate–TMPD oxidase) was unaffected by diazobenzenesulfonate treatment at either membrane surface. Although NADH oxidase and NADH–cytochrome *c* reductase were inhibited significantly only in the OSP preparation, NADH dehydrogenase (measured as NADH–DCIP reductase) was inhibited to the same extent (≈ 60 %) at either membrane surface. Succinate dehydrogenase was strongly inhibited only in the ISP preparation while succinate–cytochrome *c* reductase activity was reduced in both preparations. ATPase was sensitive to diazobenzenesulfonate labeling only in the ISP preparation. In doubly-labeled membranes (the IOSP preparation), ATPase was inhibited to a similar degree (76%) as in the singly treated ISP preparation.

During the course of these experiments, some variability was noted in the extent to which various enzyme activities were inactivated by diazobenzenesulfonate. Variable leakage of the label in different preparations could account for these results.

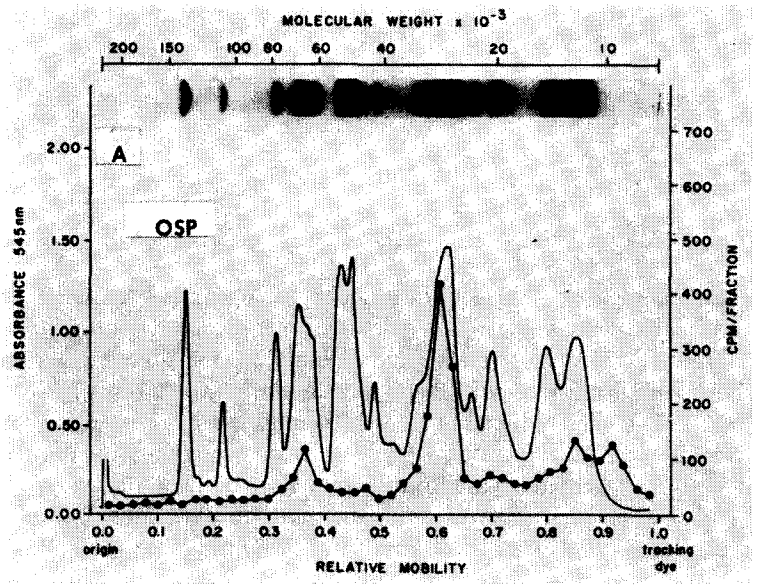
Sodium dodecylsulfate–polyacrylamide gel electrophoresis of diazobenzene-[^{35}S]sulfonate treated membranes

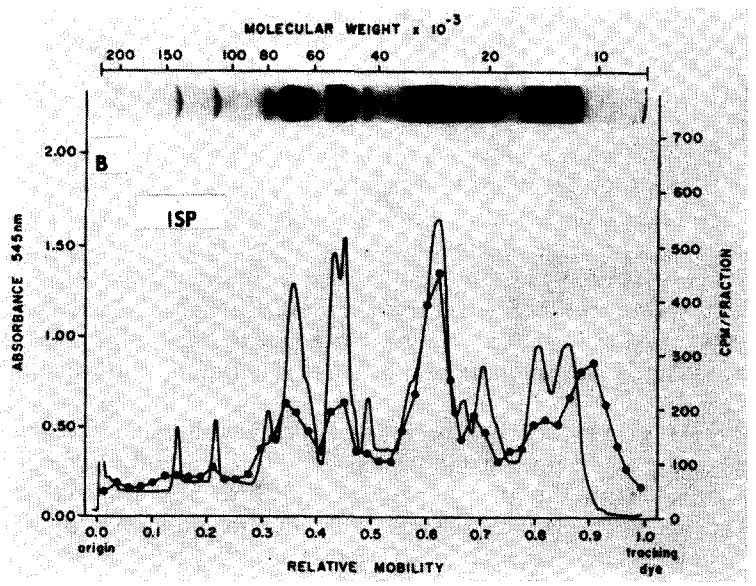
To ascertain which membrane polypeptides were labeled with diazobenzene-[^{35}S]sulfonate, the membrane preparations were analyzed by sodium dodecylsulfate gel electrophoresis. The treated preparations gave electrophorograms similar to untreated controls, indicating that under the conditions employed diazo coupling did not radically alter the mobility of any membrane polypeptide. Furthermore, although the three inner membrane samples contained different levels of label, the electrophorograms of these samples were similar to each other and to those of untreated membranes, suggesting that derivitization did not interfere significantly with staining of the polypeptides.

The electrophoretic pattern of inner membranes contained about 15 components ranging in molecular weight from 10 000 to 140 000. However, only 2 components appeared to contain label in the OSP preparation (Fig. 2A). The radioactivity coincided with stained bands possessing apparent molecular weights of 66 000 and 26 000. Approx. 50% of the counts present on the OSP preparation electrophorogram

resided in the 26 000 molecular weight component. A small but reproducible quantity of radioactivity was localized in a region unstained by coomassie blue (relative mobility, 0.9).

The ISP preparation treated with diazobenzene-[^{35}S]sulfonate contained 5 labeled bands (Fig. 2B). As with the OSP preparation, one additional region was not stained (relative mobility, 0.9). This unstained region contained more counts in ISP preparation than in OSP preparation gels. The labeled bands possessed apparent





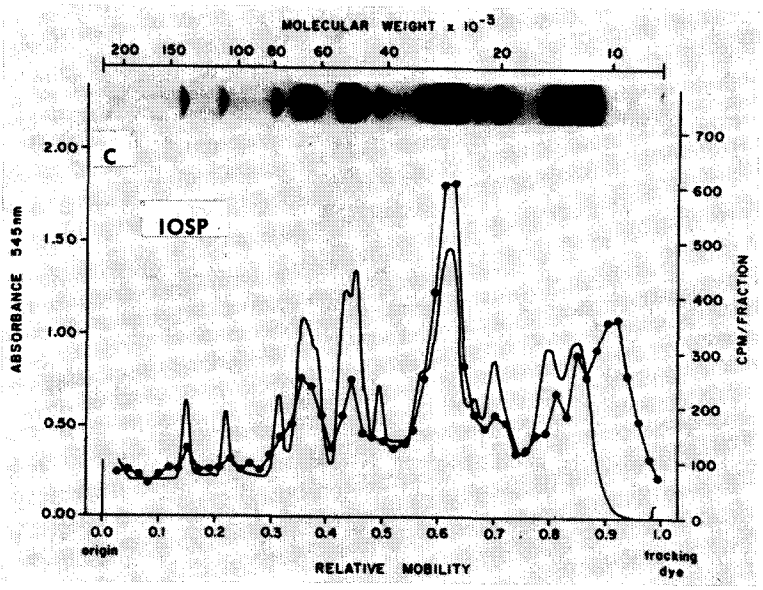


Fig. 2. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of diazobenzenesulfonate-labeled inner membranes. Labeling with diazobenzene-[^{35}S]sulfonate was performed as described in Materials and Methods and in Fig. 1. Inner membranes (2 mg protein/ml) were solubilized in 1 % sodium dodecylsulfate and 1% mercaptoethanol and subjected to electrophoresis, in duplicate, in 6% polyacrylamide gels containing 0.1 % sodium dodecylsulfate as previously described [9]. Each gel contained 45 μg protein. One gel was stained with coomassie blue [12] while the other was fractionated prior to radioactivity determination as described in Materials and Methods. Coomassie blue stain (absorbance 545 nm) (—): cpm [^{35}S] per fraction (●–●).

molecular weights of 80 000, 66 000, 51 000–48 000 (unresolved doublet) and 26 000. The IOSP preparation yielded a radioactivity distribution approximating a composite of that of the OSP preparation and the ISP preparation (Fig. 2C).

DISCUSSION

The topography of inner membrane enzymes

This study has demonstrated that diazobenzenesulfonate can inactivate inner membrane enzymes differentially at each surface and that this property of the probe is useful for localizing surface proteins. It may be argued that the asymmetric inactivation of inner membrane enzymes by diazobenzenesulfonate was due to a failure of labeled mitoplasts to invert during sonication. A failure to invert would result in anomalous enzyme activities due to differences in substrate accessibility in inner membranes of opposite orientation. It appears, however, that inversion of labeled mitoplasts was obtained. Doubly labeled inner membranes (IOSP preparation) displayed a similar level of ATPase inhibition as the single labeled ISP preparation. Furthermore, similar polypeptides are labeled in both isolated inner membranes and OSP preparation (to prepare IOSP) again indicating inversion. It can be concluded, therefore, that differences in sensitivity to diazobenzenesulfonate result from an asymmetric distribution of enzyme components.

Results obtained in this study suggest that ATPase and succinate dehydrogenase are both located on the inner surface of the inner membrane (i.e., the surface facing the matrix in intact mitochondria), a conclusion reached by other investigators using different experimental approaches [1, 4]. Our results agree with those of Schneider et al. [22] who isolated oligomycin-insensitive ATPase from mitochondria and inner membranes both labeled with diazobenzene-[^{35}S]sulfonate and found that the complex isolated from inner membranes possessed a specific activity eight times that of the mitochondrial preparation.

Interesting results were obtained concerning the effect of diazobenzenesulfonate labeling on NADH oxidation. Although both NADH oxidase and NADH–cytochrome *c* reductase activities were relatively unaffected by treatment of the inner surface of the inner membrane, NADH dehydrogenase was inactivated at this surface. Moreover previous studies, involving "substrate accessibility" [4], suggest that NADH dehydrogenase is localized on the inner surface of the inner membrane. These apparent contradictions may actually reflect a difference in the way the dehydrogenase interacts with either its natural or with artificial electron acceptors (e.g., DCIP). The results may be explained if diazobenzenesulfonate reacts with the segment of the dehydrogenase involved in binding or interacting with DCIP, but not with the part of the enzyme involved in the passage of electrons to the respiratory chain. The inhibition of NADH dehydrogenase by labeling at the outer surface of the membrane suggests that some portion of the molecule may be localized at this surface.

Cytochrome oxidase activity (measured as ascorbate–TMPD oxidase) was insensitive to diazobenzenesulfonate labeling at either membrane surface. However, previous studies have indicated that cytochrome oxidase may be located at both membrane surfaces [1] and in fact, Schneider et al. [22] have shown that diazobenzenesulfonate labels this complex to a limited extent at both surfaces in heart mitochondria. Furthermore, these workers have demonstrated that treatment of bovine heart mitochondria with diazobenzenesulfonate resulted in the labeling of cytochrome *c*. However, in our study, ascorbate–TMPD oxidase, which involves cytochrome *c* as an intermediate electron carrier, was unaffected by labeling of mitochondria. Assuming that cytochrome oxidase and cytochrome *c* are labeled, we must therefore conclude that this labeling takes place at residues which are not requisite for the proper functioning of these components.

The present study also provided some information concerning the localization of the cytochrome b–c_1 region of the electron transfer chain about which little is known. Diazobenzenesulfonate labeling of the outer surface of the inner membrane resulted in the inactivation of succinate oxidation, but left succinate dehydrogenase and cytochrome oxidase largely unaffected. The locus of attack, therefore, lies in the region of cytochrome b–c_1 and suggests that this segment of the respiratory chain exists on the outer surface of the membrane.

These results discussed above must be interpreted with some degree of caution for a number of reasons. First, labeling of a component will cause inactivation only if those groups alkylated are required for activity. In some instances, this deficiency may be remedied by employing nonpenetrating probes which react with other amino acid residues (e.g., sulfhydryl reagents). Second, inactivation may result from binding of the label at a site other than the enzyme proper, thereby making determination of binding sites equivocal.

Identification of labeled proteins

The asymmetry of the inner membrane, suggested by diazobenzenesulfonate-induced enzyme inactivation, was also evident from an analysis of the nature of polypeptides alkylated at each membrane surface. The inner surface of the membrane was found to contain a greater number of exposed proteins than the outer surface. Furthermore, the outer surface of the inner membrane appeared to contain one major exposed polypeptide (mol. wt 26 000).

Two polypeptides (26 000 and 66 000 mol. wt) appear to be labeled on both membrane surfaces. Several explanations are possible to account for this observation. First, these polypeptides may represent single molecules which traverse the membrane, thereby protruding from either surface. Secondly, these polypeptides may possess a dual localization, being on both surfaces concurrently. Thirdly, as our electrophoretic system separates components only on the basis of molecular weight, the results can be explained if one labels different polypeptides of similar size on each membrane surface. At present, we cannot distinguish between these possibilities, although a transmembrane localization of the 26 000 mol. wt component would be unlikely on the basis of the size of this component if present in a globular configuration.

Although molecular weights of a number of inner membrane proteins have been reported [13, 23–26], this information is of limited value in identifying labeled polypeptides on our electrophorograms. These components may not possess these apparent molecular weights in our gel system, thereby affording equivocal conclusions. Furthermore, the presence of unknown polypeptides of mol. wt similar to those of known membrane components would make identification difficult.

A comparison of stained and fractionated gels revealed an unstained region (relative mobility, 0.9) which contained considerable radioactivity but was shown not to be phospholipid (e.g., phosphatidylethanolamine). We are currently investigating the possibility that this material consists of low molecular weight labeled polypeptides, which are eluted from gel during fixation and staining.

ACKNOWLEDGMENTS

The authors would like to thank Calvin Lee for expert technical assistance. This work was supported by a United States Public Health Service grant (No. AM06438-10) and the Veterans Administration.

REFERENCES

1 Racker, E., Burstein, C., Loyter, A. and Christiansen, R. O. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., eds), p. 35, Adriatica Editrice, Bari
2 Seligman, A. M., Karnovsky, M. J., Wasserkrug, H. L. and Hanker, J. S. (1968) J. Cell Biol. 38, 1–14
3 Steck, T. L., Fairbanks, G. and Wallach, D. F. H. (1971) Biochemistry, 10, 2617–2624
4 Klingenberg, M. and Von Jagow, G. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C. eds), p. 281, Adriatica Editrice, Bari
5 Berg, H. C. (1969) Biochim. Biophys. Acta 183, 65–78
6 Bretscher, M. S. (1971) J. Mol. Biol. 58, 775–781
7 Pardee, A. B. and Watanabe, K. (1968) J. Bacteriol. 96, 1049–1054
8 Vansteveninck, J., Weed, R. I. and Rothstein, A. (1965) J. Gen. Physiol. 48, 617–632

9 Melnick, R. L., Tinberg, H. M., Maguire, J. and Packer, L. (1973) Biochim. Biophys. Acta 311, 230–241
10 Stancliff, R. C., Williams, M. A., Utsumi, K. and Packer, L. (1969) Arch. Biochem. Biophys. 131, 629–642
11 Schnaitman, C. A. and Greenawalt, J. W. (1968) J. Cell Biol. 38, 158–175
12 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
13 Catterall, W. A. and Pedersen, P. L. (1971) J. Biol. Chem. 246, 4987–4994
14 King, T. E. and Howard, R. L. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman M. E., eds), Vol. 10, pp. 275–294, Academic Press, New York
15 King, T. E. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 323–331, Academic Press, New York
16 King, T. E. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 216–225, Academic Press, New York
17 Habeeb, A. F. S. A. (1966) Anal. Biochem. 14, 328–336
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 235–275
19 Folch, J., Lees, M. and Stanley, G. H. S. (1957) J. Biol. Chem. 226, 497–509
20 Allmann, D. W., Bachmann, E., Orme-Johnson, N., Tan, W. C. and Green, D. E. (1968) 125, 981–1012
21 Riordan, J. F. and Vallee, B. L., in Methods in Enzymology (Hirs, C. H. W. and Timasheff, S. N., eds), Vol. XXV, pp. 521–531, Academic Press, New York
22 Schneider, D. L., Kagawa, Y. and Racker, E. (1972) J. Biol. Chem. 247, 4074–4079
23 Goldberger, R., Smith. S. L., Tisdale, H. and Bomstein, R. (1961) J. Biol. Chem. 236, 2788–2793
24 Singer, T. P., Kearney, E. B. and Bernath, P. (1956) J. Biol. Chem. 223, 599–613
25 Criddle, R. S. and Bock, R. M. (1959) Biochem. Biophys. Res. Commun. 1, 138–142
26 Criddle, R. S., Bock, R. M., Green, D. E. and Tisdale, H. (1962) Biochemistry 1, 827–842