Protein Asymmetry in the Inner Membrane of Rat Liver Mitochondria*

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At least three groups of polypeptides of the inner membrane of rat liver mitochondria have been shown to be exposed to the exterior surface by several techniques: lactoperoxidase-catalyzed iodination of mitochondria and inner membrane/matrix vesicles (mitoplasts), reaction of mitochondria and mitoplasts with the membrane-impermeable diazonium salt of sulfanilic acid, and controlled proteolysis of mitoplasts. These classes of proteins, separated by dodecyl sulfate gel electrophoresis, have polypeptide molecular weights of 73,000, 31,000, and 26,000. In addition, four other groups have been shown to be exposed to the exterior surface by at least one of these techniques: these components have polypeptide molecular weights of 130,000, 87,000, 16,000, and 10,500.

A class of proteins, which makes up 50 to 60% of the total mitochondrial protein and which can be easily extracted from mitoplasts by freeze-thaw fractionation or other procedures designed to separate "matrix" protein from "membrane" protein, is shown not to be exposed to the outer surface of the inner membrane by these techniques. This class of proteins contains polypeptides of various molecular weights and includes the major 165,000 molecular weight polypeptide, identified with carbamyl phosphate synthetase.

The identification of membrane proteins that are exposed to the outer surfaces of cells and organelles and that may function in transmembrane phenomena is a central problem of membrane biochemistry. Experiments have generally been conducted by labeling membrane surfaces in intact and broken vesicles with membrane-impermeable reagents and identifying reacted proteins after the electrophoretic separation of the polypeptide chains. While the topography of membrane proteins in certain cells, especially human erythrocytes, has been relatively well established by these procedures (see Ref. 1 for a review), comparable results are lacking on the organization of proteins in the inner membrane of rat liver mitochondria, probably because several difficulties complicate these types of experiments. In the first place, liver homogenization and mitochondrial purification by differential centrifugation fragment some of the large tubular, branched structures that exist in vivo (2) to the small spherical organelles that are isolated and may irreversibly disrupt the structure of the two mitochondrial membranes. Secondly, the outer mitochondrial membrane limits the access of macromolecular reagents (lactoperoxidase, proteases) to the inner membrane and is a potential source of contaminating polypeptides. Finally, both mitochondria and inner membrane/matrix vesicles (mitoplasts) are fragile and one must be sure that these membranes are initially intact and do not become disrupted during the labeling procedure so that "interior" proteins are exposed to the reagents.

Two of the most widely used membrane-impermeable labeling reagents are the diazonium salt of sulfanilic acid (4) and the lactoperoxidase-iodide-hydrogen peroxide iodination system (5). The results obtained so far with these two procedures (6-10) give rather different and contradictory pictures for the orientation of the polypeptides of the rat liver inner mitochondrial membrane. The work reported here has been undertaken to clarify this situation using both of these techniques, and the additional technique of controlled digestion of membrane proteins by exogenous proteases. In this work, the central problem of membrane disruption during labeling has been followed by the extent of labeling of the major 165,000 molecular weight polypeptide, which has been identified with the "matrix" protein carbamyl phosphate synthetase (11), and apparently resides entirely within the inner mitochondrial membrane.

The evidence presented in this paper allows division of mitochondrial polypeptides into two classes. One of these consists of proteins which can be easily removed from the inner membrane/matrix by freeze-thaw procedures or mild detergent. These proteins account for 50 to 60% of the total mitochondrial protein and are not exposed to the exterior surface of the inner membrane. A second class consists of

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1 "Mitoplast" is used here to indicate the intact inner membrane + matrix complex of mitochondria after the outer membrane and intermembrane contents have been removed with digitonin (3).
proteins which are tightly bound to the inner membrane and which are exposed at the exterior surface of this membrane.

**EXPERIMENTAL PROCEDURES**

**Materials—**Butylated hydroxytoluene (used as a 1% solution in ethanol), 3-amino-1,2,4-triazole, and phenylmesasulfonful fluoride were obtained from Sigma. T-1-Tosylamido-2-phenylethyl chloromethyl ketone, trypsin, papain, and α-chymotrypsin (crystallized three times) were products of Worthington, while pronase (B grade) was from Calbiochem.

Bovine lactoperoxidase (A$_{280}$/A$_{280}$ ratio of 0.58 to 0.70) was obtained from Sigma. Sepharose 2B-bound lactoperoxidase (1 mg of protein/ml of packed resin) was prepared by coupling the enzyme to cyanogen bromide-activated resin (prepared according to March et al. (121)) in 0.1 M boric acid/NaOH, pH 8.5. Labeled lactoperoxidase was prepared by autocatalysis of 0.2 mg of enzyme in 30 µl of carrier-free iodide and 500 PM H$_2$O$_2$. Free iodide was separated from the iodinated protein (specific activity 2000 cpm/µg) by gel filtration on Sephadex G-25, and the purity of the labeled lactoperoxidase checked by polyacrylamide gel electrophoresis in dodecyl sulfate.

Sodium $^{35}$S iodide, carrier-free, reducer, was obtained from New England Nuclear in 0.1 N NaOH.

**Preparation of Mitochondria and Mitoplasts—**Rat liver mitochondria were prepared from young female albino rats (5 to 4 months old, Charles River CD strain) in a medium of 280 mM sucrose, 4 mM Tris, and 0.1 mM EGTA, pH 7.5. The outer membrane marker, monoamine oxidase, was assayed spectrophotometrically at 250 nm with a benzylamine substrate as described (11). Mitoplasts (inner membrane-matrix complexes) were prepared by the digitonin fractionation procedure of Schonbrunn and Trentham (130) at a final protein concentration of approximately 50 µg/ml in 0.5 M sucrose of serum albumin. Protein was quantitated in these experiments by the absorbance of dodecyl sulfate-solubilized aliquots at 280 and 310 nm as described (11).

For labeling experiments, serum albumin in the medium was replaced by two centrifugation-resuspension cycles.

**Enzymatic Assays—**Catalse was assayed by the decrease in A$_{422}$ of a 16 mM solution of hydrogen peroxide in 50 mM Tris H$_2$SO$_4$, pH 7.6. The outer membrane marker, monoamine oxidase, was assayed spectrophotometrically at 250 nm with a benzylamine substrate as described (11). A Cary 10 spectrophotometer, with a 0 to 0.1 A slide wire, was used at room temperature.

**Lactoperoxidase-catalyzed iodination—**Mitochondrial fractions were iodinated in a medium containing 280 mM sucrose, 10 mM Tris-acetic acid, and 0.1 mM EGTA, pH 7.5. Generally 0.5 ml of each fraction (1 to 2 mg of protein) in a medium containing 2 µg of butylated hydroxytoluene (14) and 30 µg of lactoperoxidase was mixed on ice for 0.5 ml of the medium made 30 µM in Na$^{35}$S (1.7 Ci/mmol) and 200 µM in hydrogen peroxide. The iodination was allowed to proceed for 20 min at 0°C and was terminated by the addition of 1 µl of β-mercaptoethanol.

Because of the difficulty with autolabeled lactoperoxidase (15, 16), iodinations were usually performed with a Sepharose 2B-bound lactoperoxidase catalyst. In these cases, the iodination mixture included 150 µl of packed resin containing 150 µg of bound enzyme in place of the soluble enzyme. After the iodination, the beads were removed from the mitochondria by centrifugation at 500 x g for 5 min.

Mitochondria and mitoplasts were generally separated from the reaction mixture by two or three centrifugation-resuspension cycles in the medium containing 0.1 mM NaI. Aliquots were then taken for dodecyl sulfate gel electrophoresis as described below.

In several experiments, especially those in which intact mitochondria were iodinated, two methods were given intraperitoneal injections of 3-amino-1,2,4-triazole (1 mg/g of body weight in aqueous solution) 1 hour before the animal was killed (17). Mitochondria from these animals had greatly reduced levels of catalase, which competes for hydrogen peroxide, the substrate of lactoperoxidase.

**Reaction of Mitochondria and Mitoplasts with Diazo Derivative of [35S]Sulfanilic Acid—**High specific activity [35S]sulfonic acid was obtained from Amersham/Seearle (1.7 Ci/mmol). The diazonium salt of hydrogen peroxide, the substrate of lactoperoxidase.

Peroxidase-protein (0.2 to 2 mg of protein) were incubated in a total volume of 1.0 ml of 0.1 M sucrose, 4 mM Na$_2$PO$_4$, and 0.1 mM EGTA, pH 7.5 (no serum albumin was used). The mitochondria (25 µg of protein/ml) were reacted with 70 µM diazobenzene sulfonate for 60 min at 0°C in a total volume of 4 ml. Labeling of smaller amounts of protein did not result in successful digitonin fractionation (see "Results").

**Purification of Cytochrome c from Labeled Mitochondria—**Cytochrome c was isolated from a 0.15 M KC1 mitochondrial extract after the procedure of Jacobs and Sanadi (22). [35S]Intact-labeled cytochrome c was isolated from a 0.15 M KC1 mitochondrial extract after the procedure of Jacobs and Sanadi (22). The isolated fractions were stored over NaOH pellets and did not correspond to any protein component.

**Gel Electrophoresis in Sodium Dodecyl Sulfate—**Tube gels (0.5 cm inner diameter x 8 cm) were prepared in the borate buffer system of Davies and Stark (19) and contained 7.5% acrylamide, 0.1% methylenebisacrylamide, and 0.1% dodecyl sulfate (11). Samples (50 to 250 µg of protein) were prepared for electrophoresis in 100 µl of dilute borate buffer containing 1% dodecyl sulfate and 30 mM β-mercaptoethanol. These samples were heated for 3 min at 100°C to minimize proteolysis (20). After electrophoresis, gels were stained with Coomassie blue R-250 (Sigma Chemical Co.) in 25% ethanol/7.5% acetic acid, and were destained by diffusion in the same solvent.

Stained gels were scanned for optical density to 490 to 560 nm as described (4) to determine the amount of protein in each band. Gels were then cut into 1-mm slices. Gel slices from iodinated samples were suspended in 0.5 ml of distilled water. The 0.5 ml of distilled water was then added to the gel slices with 35S-labeled proteins were first digested in 200 µl of 30% hydrogen peroxide for 2 hours at 70°C, then mixed with 2.5 ml of Aquasol (New England Nuclear) for counting on a Beckman model 250 liquid scintillation spectrophotometer.

Dodecyl sulfate gel slices (0.08 x 11 x 15 cm) containing a gradient of 5 to 10% acrylamide/0.06 to 0.13% methylenebisacrylamide were prepared in the borate buffer system (19) for use in the apparatus described by Studier (21). The gels were stained with Coomassie, destained, and dried onto filter paper as described (21). Radioautography was performed with Kodak X-Omat film (RP/R14 film).

**Purification of Cytochrome c from Labeled Mitochondria—**Cytochrome c was isolated from a 0.15 M KC1 mitochondrial extract after the procedure of Jacobs and Sanadi (22). [35S]or 35S-intact-labeled mitochondria (5 mg of protein/ml) were incubated at 0°C for 10 min in 0.15 M KC1, then collected by centrifugation at 6,000 x g for 10 min. The supernatant was resuspended in 0.15 M KC1 with 0.05 M Tris, and 0.05 M Tris, and 0.16 M KCl. The mitoplasts were spun down at 12,000 x g for 10 min, resuspended in the sucrose/KCl medium, spun again, and resuspended in the sucrose medium for dodecyl sulfate gel electrophoresis. The presence of 0.08 M KC1 in the washing medium was found to be effective in removing bound proteins from the mitochondrial membrane.
polypeptides of similar molecular weight. On the other hand, many of the cycle; carbamyl phosphate synthetase (11) and ornithine trans-appear to contain a single protein species. For example, compo-
methylenebisacrylamide. These fractions are designated by Roman numerals according to the calculated polypeptide molecular weight (Table I) and those numerals are used to iden-
tify these components in the photographs and optical density scans of gels in Figs. 1 to 7. Some of these polypeptide fractions appear to contain a single protein species. For example, compo-
ments I and Va have been identified with enzymes of the urea cycle; carbamyl phosphate synthetase (11) and ornithine trans-carbamylase, respectively. On the other hand, many of the other fractions are heterogeneous, containing several different polypeptides of similar molecular weight.

**RESULTS**

**Polypeptide Composition of Rat Liver Mitochondria and Inner Membrane/Matrix Vesicles (Mitoplasts)—Mitochondrial proteins were resolved into 17 fractions by electrophoresis in dodecyl sulfate tube gels containing 7.5% acrylamide/0.1% methylenebisacrylamide. These fractions are designated by Roman numerals according to the calculated polypeptide molecular weight (Table I) and those numerals are used to iden-
tify these components in the photographs and optical density scans of gels in Figs. 1 to 7. Some of these polypeptide fractions appear to contain a single protein species. For example, compo-
ments I and Va have been identified with enzymes of the urea cycle; carbamyl phosphate synthetase (11) and ornithine trans-carbamylase, respectively. On the other hand, many of the other fractions are heterogeneous, containing several different polypeptides of similar molecular weight.

**Determination of Proteins Exposed to Exterior Surface of Inner Membrane by Lactoperoxidase-catalyzed Iodination—** Most of the experiments reported here made use of a lactoperoxidase catalyst covalently bound to Sepharose 2B. The use of resin-bound enzyme was necessitated by the high efficiency of lactoperoxidase auto-labeling (15) and by the adhesion of these labeled molecules to mitochondria and mitoplasts (Table II). The use of soluble lactoperoxidase in labeling reactions resulted in proportional increases in Coomassie staining material and radioactivity in a band on dodecyl sulfate gels at the position of lactoperoxidase (Fraction IIc, apparent molecular weight 87,000).

Control experiments were performed to show an absolute requirement for lactoperoxidase in the iodination system (cf. Ref. 5). No radioactivity was incorporated into mitochondrial proteins after mitoplasts were reacted in the complete iodination mixture without this enzyme. Additionally, it was shown that no labeled materials were removed from the gel during the staining and destaining procedure. Duplicate samples gave identical labeling in gels sliced and counted immediately after electrophoresis or after the normal staining procedure. In the gel system used here, lipids ran well ahead of the tracker dye and did not contribute to the labeling patterns shown.

The results in Figs. 1 and 2 show that at least six components are exposed at the exterior surface of the inner membrane because they are iodinated by the membrane-impermeable labeling system in intact vesicles: II, III, VI, VII, VIII, and X. These polypeptides are associated with the inner membrane because they are labeled in mitoplasts where they are labeled in mitoplasts where the outer membrane has been removed (0.18 mg of digitonin/mg of protein). These polypeptides are also labeled in mitoplasts where the outer membrane has been only partially removed (0.11 mg of digitonin/mg of protein), as well as in intact mitochondria, where one might have expected that the outer membrane would prevent access of the lactoperoxidase catal-
yst. However, there is considerable evidence that preparations of intact mitochondria contain a significant population (about 10%) of mitochondria with disrupted or detached outer membrane proteins.

<table>
<thead>
<tr>
<th>Band designation</th>
<th>Average mobility</th>
<th>Polypeptide M&lt;sub&gt;0&lt;/sub&gt;</th>
<th>Per cent of total protein&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.259</td>
<td>165,000</td>
<td>16.9</td>
</tr>
<tr>
<td>Ia</td>
<td>0.286</td>
<td>140,000</td>
<td>2.5</td>
</tr>
<tr>
<td>II</td>
<td>0.320</td>
<td>130,000</td>
<td>2.3</td>
</tr>
<tr>
<td>IIa</td>
<td>0.340</td>
<td>120,000</td>
<td>2.8</td>
</tr>
<tr>
<td>IIb</td>
<td>0.365</td>
<td>107,000</td>
<td>3.2</td>
</tr>
<tr>
<td>IIc</td>
<td>0.412</td>
<td>87,000</td>
<td>5.7</td>
</tr>
<tr>
<td>III</td>
<td>0.448</td>
<td>73,000</td>
<td>4.5</td>
</tr>
<tr>
<td>IIIa</td>
<td>0.478</td>
<td>64,000</td>
<td>6.2</td>
</tr>
<tr>
<td>IV</td>
<td>0.543</td>
<td>51,000</td>
<td>12.9</td>
</tr>
<tr>
<td>V</td>
<td>0.599</td>
<td>41,000</td>
<td>8.2</td>
</tr>
<tr>
<td>Va</td>
<td>0.614</td>
<td>36,000</td>
<td>3.0</td>
</tr>
<tr>
<td>VI</td>
<td>0.696</td>
<td>31,000</td>
<td>8.8</td>
</tr>
<tr>
<td>VII</td>
<td>0.743</td>
<td>26,000</td>
<td>8.6</td>
</tr>
<tr>
<td>VIIa</td>
<td>0.812</td>
<td>20,000</td>
<td>3.5</td>
</tr>
<tr>
<td>VIII</td>
<td>0.880</td>
<td>16,000</td>
<td>2.8</td>
</tr>
<tr>
<td>IX</td>
<td>0.945</td>
<td>13,000</td>
<td>5.3</td>
</tr>
<tr>
<td>X</td>
<td>1.022</td>
<td>10,500</td>
<td>3.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mobilities calculated relative to the tracker dye, bromphenol blue (3',3''5',5''-tetrabromophenolsphtalein), in 7.5% acrylamide/0.1% methylenebisacrylamide gels.
<sup>b</sup> Calculated from the following standard proteins (polypeptide molecular weight in parentheses): myosin (220,000), β-galactosidase (135,000), serum albumin (68,000), catalase (60,000), fumarase (48,500), ovalbumin (45,500), malate dehydrogenase (32,000), carbonic anhydrase (30,000), and cytochrome c (11,500).
<sup>c</sup> Determined from integrated stain intensity of two gels.

**TABLE I**

**Identification of Coomassie staining bands from dodecyl sulfate gel electrophoresis of rat liver mitoplasts**

The qualitative pattern of Coomassie staining for whole mitochondria is not different from that of mitoplasts. However, there is a greater amount of material in component IIIa and IX.

**TABLE II**

**Adhesion of 131I-labeled lactoperoxidase to mitochondria and mitoplasts**

The indicated amount of intact mitochondria and digitonin pre-
pared mitoplasts were incubated with 15 μg of autolabeled 131I-labeled lactoperoxidase (2000 cpm/μg) in 1.0 ml of sucrose medium for 20 min at 0°C. The mixture was then centrifuged for 15 min at 4000 x g and the mitochondrial pellet was resuspended in 1 ml of sucrose medium, recentrifuged, and the pellet resuspended in 0.4 ml of medium. An aliquot of this final suspension (0.1 ml) was subjected to electrophoresis on dodecyl sulfate gels and the amount of bound lactoperoxidase was determined by the radioactivity in the gel at the migration position of this enzyme. The amount of outer membrane remaining was calculated from the specific activity of monamine oxidase.

<table>
<thead>
<tr>
<th>Material</th>
<th>Outer membrane remaining</th>
<th>Amount of protein</th>
<th>131I-labeled lactoperoxidase bound mg bound/ mg mitochondrial protein</th>
<th>% of total bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>100</td>
<td>1.7</td>
<td>1.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Mitoplasts (0.11 mg of digitonin/mg protein)</td>
<td>74</td>
<td>1.6</td>
<td>2.9</td>
<td>31.5</td>
</tr>
<tr>
<td>Mitoplasts (0.18 mg of digitonin/mg protein)</td>
<td>2.8</td>
<td>1.4</td>
<td>2.8</td>
<td>25.4</td>
</tr>
</tbody>
</table>

<sup>b</sup> The serum albumin used in the mitochondrial isolation did not adhere to the membrane and was washed away before the labeling reaction. In this gel system, albumin migrated between components III and IIIa.
Fig. 1. Distribution of protein and radioactivity after dodecyl sulfate tube gel electrophoresis of \(^{125}\)I-iodinated mitochondria and digitonin-prepared inner membrane/matrix vesicles (mitoplasts). The lower three traces indicate the radioactivity present in the polypeptides of mitochondria and mitoplasts prepared with 0.11 and 0.18 mg of digitonin/mg of protein and labeled with Sepharose BB-bound lactoperoxidase as indicated under “Experimental Procedures.” The uppermost trace is a scan of the optical density of the Coomassie-stained gel containing labeled polypeptides of the 0.18 mg/mg digitonin sample, taken before the gel was sliced and counted. The spectrophotometric traces for the lower two gels (not shown) were identical except that more staining material was present in bands IIIa and IX in the mitochondrial sample. The designation of staining bands by Roman numerals in order of decreasing molecular weight is described in Table I. Approximately 300 \(\mu\)g of protein were applied to each gel. The specific activity of monoamine oxidase, a marker for the outer membrane, was 3.2, 2.4, and 0.1 nanomole/min/mg of protein for the mitochondria, 0.11 and 0.18 mg/mg digitonin pellets, respectively.

These results also suggest that the major labeled component of band IX is in the outer membrane because the amount of label corresponds to the amount of outer membrane present (see figure legend). Similarly, the labeled component migrating between bands III and IV in labeled mitochondria is probably a nonmitochondrial protein because it is not labeled in mitoplasts prepared with low amounts of digitonin (0.11 mg/mg of protein) that separate mitochondria from proteins of the endoplasmic reticulum, lysosomes, and peroxisomes (6, 7, 26).

The data in Fig. 1 also show that there is some label present in bands I, IV, and V. However, since the amount of label in these components is small and increases with increasing amounts of digitonin, the possibility exists that these proteins are not exposed to the exterior surface of the inner membrane and that their labeling results from the exposure of these proteins in a small population of disrupted vesicles.

It has been possible to show that this is the case for component I, which contains only one polypeptide species and has been identified with carbamyl phosphate synthetase (11). Fig. 3 shows the result of an experiment in which mitoplasts (95% outer membrane removed) were labeled in the presence and absence of Triton X-100 to determine what fraction of the total tyrosine residues exposed by the detergent treatment were being labeled in the intact mitoplasts. As discussed previously (6, 7, 11), this comparison cannot be made directly because the detergent releases factors which inhibit the labeling reaction. The extent of this inhibition can be estimated by the inclusion of an internal standard in the labeling mixture. In this case, a 100-fold excess of exogenous cytochrome c was added to the mitoplasts in the presence and absence of

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**Fig. 2.** Comparison of labeling patterns obtained from intact mitochondria and mitoplasts iodinated with either soluble lactoperoxidase or Sepharose 2B-bound lactoperoxidase. Mitochondrial fractions were labeled exactly as in Fig. 1 except that additional samples were iodinated with the soluble enzyme. Aliquots of the samples (30 \(\mu\)g of protein) were subjected to electrophoresis on dodecyl sulfate 5 to 10% acrylamide slab gels, which were then stained with Coomassie, dried onto filter paper, and radioautographed for 44 days against x-ray film. A photograph of the stained gel is shown on the left; the arrows mark the top of the gel and the position of the tracker dye. A photograph of the autoradiograph is shown on the right. Mitochondrial samples are: 1 and 4, intact mitochondria; 2 and 3, 0.11 mg/mg digitonin pellet; 5 and 6, 0.18 mg/mg digitonin pellet (see Fig. 1). Samples 1 to 3 were labeled with soluble lactoperoxidase; samples 4 to 6 were labeled with Sepharose 2B-bound enzyme. Protein bands are labeled as in Fig. 1. The data in Fig. 1 also show that there is some label present in bands I, IV, and V. However, since the amount of label in these components is small and increases with increasing amounts of digitonin, the possibility exists that these proteins are not exposed to the exterior surface of the inner membrane and that their labeling results from the exposure of these proteins in a small population of disrupted vesicles.
that about 95% of the outer membrane had been removed. The activity of the outer membrane marker, monoamine oxidase, was 0.25 nmol/min/mg of protein in this preparation of mitoplasts, indicating that iodination was performed with soluble lactoperoxidase, an additional intact labeled mitoplasts. The amount of protein applied to these gels was 0.05% Triton X-199. The upper trace is a scan of the optical density of protein. Iodinations were performed in the presence and absence of 0.20 mg of digitonin/mg of protein sample (where only 6% of the outer membrane remained) contained four major radioactive bands. Nevertheless, because the outer membrane was apparently labeled at a much higher specific activity than the inner membrane, not all of the radioactivity in these components is of inner membrane proteins. For example, the radioactivity in the region between IX and X is probably associated with a protein of the outer membrane because its distribution parallels that of the outer membrane marker enzyme monoamine oxidase (Fig. 6). This component does not appear to be labeled lipid because the dodecyl sulfate lipid mixed micelles migrated ahead of the tracker dye in these experiments and because the labeled material is not eluted during extensive washing of the gel in 40% methanol/7.5% acetic acid. When the radioactivity in component VII was analyzed as for band IX/X in Fig. 6, it was also apparent that most of the labeled material in this fraction had an outer membrane origin. However, the specific activity of band III was not consistent with such a location, and this labeled material is likely to be derived from the inner membrane alone.

Proteolysis of Membrane Components in Intact and Disrupted Mitoplasts—Table III is a summary of the results obtained when intact and Triton-disrupted mitoplasts were incubated with various proteases. Almost all of the major protein components of mitoplasts were digested when exposed to detergent by chymotrypsin. However, in intact mitoplasts, components I, Ia, and Ila, and perhaps IIIa, IV, V, and Va are not cleaved, presumably because these components are located inside the inner membrane and no chymotrypsin-sensitive bond is exposed to the protease. On the other hand, four components are cleaved in intact mitoplasts, and these components are likely to be exposed to the exterior surface of the membrane.

\*The digestions were terminated by the addition of the appropriate protease inhibitor. To ensure that no proteolysis occurred after this time, especially when all of the mitochondrial polypeptides were exposed by dodecyl sulfate, controls were performed where the inhibitor was added prior to the protease (see "Experimental Procedures"). In all of these cases, the polypeptide distribution of gels was identical with a control gel not treated with proteases except for the additional band(s) due to the polypeptides of the protease itself.
Fig. 4. Labeling of intact and Triton X-100-disrupted mitoplasts (0.20 mg of digitonin/mg of protein) with \([\text{[9]}\text{S}'\text{diazobenzenesulfonate}\]) labeled mitoplasts (150 \(\mu\)g of protein) before the gel was sliced and counted for radioactivity (lower trace). The center trace is the radioactivity of labeled Triton X-100-disrupted mitoplasts (110 \(\mu\)g of protein). Monoamine oxidase activity of these mitoplasts was 0.06 mmol/min/mg of protein, indicating that 98% of the outer membrane had been removed.

inner membrane: II, IIC, III, and VII. Fig. 7 (upper left) shows the difference in the optical density scan of stained gels from intact mitoplasts treated with active or inactivated chymotrypsin (see Footnote 6). The effect of trypsin, papain, and pronase digestion of intact mitoplasts is also shown in Table III. These effects are in general similar to those demonstrated with chymotrypsin.

Experiments were also performed where lactoperoxidase- or diazobenzenesulfonate-labeled mitoplasts were treated with these proteases. Fig. 7 shows the effect of chymotrypsin, trypsin, and papain on the distribution of radioactivity in proteins of Sepharose 2B-lactoperoxidase intact labeled mitoplasts. The usual zero time controls were performed to eliminate the possibility that proteolysis continued during the solubilization by dodecyl sulfate. These results are summarized in Table IV. Similar results were obtained when \([\text{[9]}\text{S}'\text{diazobenzenesulfonate}\]) labeled mitoplasts were treated with chymotrypsin.

Labeling of Cytochrome c in Intact Mitochondria by Lactoperoxidase Iodination or Reaction with \([\text{[1]}\text{S}'\text{diazobenzenesulfonate}\]—The polypeptide components shown here to be exposed to the outer surface of the inner membrane are in general functionally unidentified. It was desired to correlate the amount of radioactivity in these components with at least one protein whose localization has been determined by other techniques. Cytochrome c, whose position on the exterior surface of the inner membrane has been well established by both chemical and functional criteria (see Ref. 27 for a review) has been used here for this purpose. This protein migrates on dodecyl sulfate gel electrophoresis between components IX and X. It does not appear as a separate band, however, because it makes up only 0.2% of the total mitochondrial protein (28) and is obscured by the protein in components IX and X which contain 5.3% and 3.4% of total Coomassie-staining material, respectively (Table I). However, it is possible to use the approach of Schneider et al. (23) and purify cytochrome c from labeled mitochondria and determine its specific activity (see "Experimental Procedures"). Table V shows the results of such experiments. Cytochrome c is labeled at a comparable specific activity of components identified with exterior inner membrane locations (such as component III) in both \([\text{[9]}\text{S}'\text{diazobenzenesulfonate}\] and \([\text{[1]}\text{S}'\text{iodide}\]) labeled mitochondria. The iodination result is of particular interest because it demonstrates that a functional inner membrane protein (cytochrome c) can be labeled with the lactoperoxidase system in intact mitochondria where one might have expected the outer membrane to exclude the catalyst. This labeling is presumably due either to the presence of a small fraction of isolated "intact" mitochondria that have a broken outer membrane or to the participation in the reaction of free activated iodide (see "Discussion").
to note that although the bulk of component VI is found in the membrane after freeze-thaw treatment. This result shows to be the case for component I. It is clear that the bulk of the membrane and are not tightly bound to it. It is also of interest that the outer surface of the inner membrane are in fact tightly bound to 80 to 90% of components III, VII, IX, and X are found tightly associated with the inner membrane. Material from bands II, III, VII, and VIII is distributed in both fractions.

Table VI shows that most of the loosely bound proteins are present in large amounts in a polypeptide band on gels and are not exposed to the outer surface of the inner membrane. For other components, one can fractionate intact labeled mitoplasts and determine the attachment of these labeled species to the inner membrane. Mitochondria were labeled with this reagent and were then fractionated with various amounts of digitonin as described in Fig. 5. The specific radioactivity (counts per min/mg of mitochondrial protein) of the 12,000 molecular weight fraction in the pellet is shown relative to the specific radioactivity of this material in the pellet fraction of mitochondria carried through the fractionation without digitonin. Similarly, the relative specific activity of the outer membrane marker, monoamine oxidase (MAO), is shown for the same samples.

Fractionation of Mitochondrial Polypeptides by Freeze-Thaw or Mild Detergent Treatment—It is possible to separate the proteins of mitoplasts into a fraction tightly associated with the inner membrane and a fraction containing matrix proteins and more loosely attached inner membrane proteins. This separation can be achieved by freeze-thawing mitoplasts in buffers of high or moderate ionic strength and then centrifuging a “soluble” fraction (not sedimenting at 100,000 x g for 60 min in a tube of 4-cm path length) and a “pellet” fraction containing all of the phospholipid. Alternatively, mitoplasts can be treated with low amounts of a nonionic detergent and can then be separated into two fractions by centrifugation as above (13). Under these conditions, 60 to 90% of components I, Ia, IIa, IIIa, IV, V, Va, and VI are found in the loosely bound fraction, while 80 to 90% of components III, VII, IX, and X are found tightly associated with the inner membrane. Material from bands II, IIc, VIIa, and VIII is distributed in both fractions.

For components containing a single polypeptide species, one can directly correlate the membrane position and the degree of membrane attachment. For example, component I is loosely bound to the inner membrane and is not exposed to the outer surface of the inner membrane. For other components, one can fractionate intact labeled mitoplasts and determine the attachment of these labeled species to the inner membrane.

Table VI shows that most of the protein fractions labeled from the outside of the inner membrane are in fact tightly bound to the membrane. The labeled species in components IV and V are not tightly bound, but the possibility exists that these proteins were labeled in disrupted mitoplasts as has been shown to be the case for component I. It is clear that the bulk of fractions IV and V are, however, located inside the inner membrane and are not tightly bound to it. It is also of interest to note that although the bulk of component VI is found in the loosely bound fraction, the labeled component fractionates with the membrane after freeze-thaw treatment. This result indicates that only a small fraction of the molecules in this component are exposed to the outside. This conclusion is substantiated by the fact that proteolysis removed the label without removing a measurable amount of Coomassie-staining protein from this band.

No components were found in this study that were tightly bound to the inner membrane but were not exposed to its outer surface. The failure to detect such components may result from the limits of resolution of the gel electrophoretic separation or of the fractionation of polypeptides by freeze-thaw disruption. Proteins tightly bound to the interior surface of the inner membrane would not be observed in this study unless they were present in large amounts in a polypeptide band on gels that did not contain other polypeptide species.

Table VI also shows that most of the loosely bound proteins are not exposed to the outer surface of the inner membrane. If all of these components are not true “matrix” proteins (see Refs. 7 and 11), these results suggest that the inner surface of the mitochondrial inner membrane is capable of weak interactions with a number of proteins, while the outer surface is not.

DISCUSSION

Two requirements must be met to determine the localization of specific mitochondrial inner membrane proteins by the
FIG. 7. Proteolysis of intact labeled mitoplasts. Mitoplasts were iodinated as described under “Experimental Procedures,” and were then exposed to various proteases under the conditions described in Table III. Upper left, scans of the optical density of Coomassie-stained dodecyl sulfate gels of control mitoplasts (solid line) and chymotrypsin-treated mitoplasts (dashed line). Polypeptide bands affected by proteolysis are shown by arrows. Each gel contained 100 μg of mitochondrial protein. Lower left, the radioactivity in both control and chymotrypsin gels from above. The hatched area indicates the counts from the chymotrypsin-treated sample. Right, radioactivity patterns of trypsin- and papaain-treated samples. Here the radioactivity was adjusted for the amount of protein on the gel so that these results could be compared to those on the left. The pattern of radioactivity in the pronase sample was identical with the chymotrypsin sample. Mitoplasts were prepared with 0.16 mg of digitonin/mg of protein, and 98% of the outer membrane was removed.

**TABLE IV**

<p>| Effect of proteases on 125I-intact-labeled polypeptides of mitoplasts |</p>
<table>
<thead>
<tr>
<th>Mitoplasts were iodinated intact as under “Experimental Procedures” with Sepharose 2B lactoperoxidase. These mitoplasts were then incubated with the following proteases at the concentrations and under the conditions given in Table III. The approximate degree of removal of the radioactive band is given by: +, 10 to 40%; ++, 40 to 70%; and ++++, 70 to 100%. Blank spaces indicate inconclusive results. In these cases, either there was no change in the radioactivity but the possibility remained that removal of the component was masked by the presence of radioactive fragments of higher molecular weight polypeptides, or there was an actual increase in the radioactivity from these fragments. No data are given for components I and IV, where the large part of the labeling is not thought to be on outer surface polypeptides.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>125I-Labeled component</strong></td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>VI</td>
</tr>
<tr>
<td>VII</td>
</tr>
<tr>
<td>VIII</td>
</tr>
<tr>
<td>IX</td>
</tr>
<tr>
<td>X</td>
</tr>
</tbody>
</table>

It is necessary to show both that the labeled or proteolyzed polypeptides are of inner membrane origin and that the results are obtained with populations of well sealed, right-side-out vesicles. In this work, I have shown that the labeled proteins are from the inner membrane by the digitonin fractionation of labeled mitochondria and by labeling or digesting purified inner membrane-matrix complexes free of outer membrane. The digitonin fractionation used here removes the outer membrane, intermembrane proteins, endoplasmic reticulum, and other nonmitochondrial membranes.

**TABLE V**

Determination of label incorporated into cytochrome c and other protein components of intact mitochondria reacted with [125I]iodide/lactoperoxidase system or [35S]diazobenzenesulfonate

<table>
<thead>
<tr>
<th>Material</th>
<th>Specific radioactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>53.5</td>
</tr>
<tr>
<td>Carbamyl phosphate synthetase (component I)</td>
<td>2.0</td>
</tr>
<tr>
<td>Component III</td>
<td>62.1</td>
</tr>
<tr>
<td>Component IX</td>
<td>75.4</td>
</tr>
<tr>
<td>Component X</td>
<td>72.8</td>
</tr>
<tr>
<td>12,000 M₉ outer membrane component</td>
<td>137</td>
</tr>
</tbody>
</table>

* Determined from the ratio of radioactivity to Coomassie staining intensity in the appropriate band on gels containing samples of purified cytochrome c or mitochondria which underwent electrophoresis in dodecyl sulfate (see “Experimental Procedures”).

from the mitoplast fraction (6, 7, 13, 26). The amount of disruption of the preparation has been monitored by measuring the label incorporated into a protein that appears to be totally contained within the inner membrane. This protein (component I) has been identified as carbamyl phosphate synthetase, makes up 15 to 20% of the total mitochondrial protein, and migrates in a unique position on dodecyl sulfate gel electrophoresis.

The extent of outer membrane removal after digitonin treatment has been monitored here by the marker enzyme monoamine oxidase (28). Although at least two other outer membrane components fractionate in the same way (rotenone-insensitive NADH cytochrome c reductase, kynurenine hydroxylase (30)), the possibility remains that some outer membrane proteins are redistributed during this procedure to a location in the inner membrane/matrix fraction.
sulfonate; 3, reaction with exogenous proteases. Exposed to the outer surface of the inner membrane. 1, lactoperoxidase-catalyzed iodination; 2, reaction with diazobenzene-sulfonate; 3, reaction with exogenous proteases. Not exposed to the outer surface of the inner membrane.

<table>
<thead>
<tr>
<th>Exposed to the outer surface of the inner membrane</th>
<th>Fractions tightly bound to inner membrane</th>
<th>Fractions loosely bound to inner membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>II (1, 3)*</td>
<td>IV (1, 2)</td>
<td></td>
</tr>
<tr>
<td>III (1, 2, 3)</td>
<td>V (1, 2)</td>
<td></td>
</tr>
<tr>
<td>VI (1, 2, 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII (1, 2, 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII' (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X (1, 3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The numbers in parentheses indicate the techniques used to assign the component to the exterior surface of the inner membrane. 1, lactoperoxidase-catalyzed iodination; 2, reaction with diazobenzene-sulfonate; 3, reaction with exogenous proteases.

& The component is not tightly bound.

* At least one nonlabeled component in this fraction is not tightly bound.

Low concentrations (35 to 70 μM) of [35S]diazobenzene-sulfonate were used in these experiments to avoid the disruption seen in human erythrocytes (18) at the concentrations of this reagent (2 to 5 mM) used in previous studies (4, 10). At these lower concentrations, Berg and Hirsh (18) showed that a variety of exterior cell surfaces could be specifically labeled.

The results obtained in this study by the proteolytic digestion of mitoplasts show that the polypeptides labeled with lactoperoxidase or diazobenzene-sulfonate are not proteins present only in trace amounts. Because these labels were used at high specific radioactivities (1,700 cpm/picomole for both 32P and 35S), the labeling observed in this study could have resulted from a very small number of reacting molecules. For example, labeling of the magnitude seen here would result if 10 tyrosine residues were quantitatively iodinated on a 50,000 molecular weight protein that made up only 0.01% of the total mitochondrial protein. However, because the proteases could remove a substantial amount of protein as well as label, probably only a small fraction of the available sites are iodinated under the conditions described here. For example, a significant fraction of components IIc, III, and VII is removed by protease treatment (Fig. 7). If this amount of material was quantitatively labeled, approximately 100 times more radioactivity should have been incorporated, assuming that these proteins have the average amino acid composition of rat liver mitochondrial proteins (7). It is not clear why the labeling with these reagents does not proceed to a greater degree and thus does not indicate directly the number of sites exposed to the outside. In the case of lactoperoxidase labeling, it is known that mitochondrial fractions contain substances which inhibit the iodination reaction. These substances may compete for the hydrogen peroxide substrate (glutathione, glutathione peroxidase, catalase (7, 11I)), sequester free iodide, or directly inhibit the enzyme. 8

Although there have been several attempts to show the localization of mitochondrial polypeptides as separated by dodecyl sulfate gel electrophoresis, various results have been obtained. For example, Farber (6) and Clarke and Farber (7) iodinated mitochondria and mitoplasts and found that the majority of the polypeptides were exposed to the outer surface of the inner membrane. It was recognized at that time that this result might have been obtained with preparations of mitoplasts that were not completely sealed. That this was the case is indicated by the present work which shows that the 165,000 molecular weight component is located within the inner membrane. The amount of labeling of this component (1) seen previously (6, 7) indicated that approximately 10% of the mitoplasts used were disrupted. A further complication of these previous studies was that the large peak of autolabeled lactoperoxidase did not allow the resolution of the radioactivity in component III.

Huber and Morrison (8) have also used lactoperoxidase to study the location of mitochondrial polypeptides. However, they did not consider the possibility that one of the labeled bands corresponded to autolabeled lactoperoxidase, and made no attempt to correlate the radioactive bands with specific polypeptide bands on the dodecyl sulfate gels. Furthermore, although small amounts of the intermembrane marker enzyme adenylyl kinase were released in their preparation of mitochondria, they did not consider that the breakage of the outer membrane in a small population of mitochondria would result in the labeling of inner membrane proteins. In fact, when they did fractionate labeled mitochondria with digitonin, most of

8 The pretreatment of rats with aminonitazol to inhibit the bulk of the catalase activity was important in reducing the competition; little or no protein iodination took place with samples of intact mitochondria prepared from untreated rats.

Location of Mitochondrial Proteins by Labeling Reagents

<table>
<thead>
<tr>
<th>Fractions tightly bound to inner membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
</tr>
<tr>
<td>Ia</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>IIa</td>
</tr>
<tr>
<td>IIb</td>
</tr>
<tr>
<td>IIc</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>Va</td>
</tr>
<tr>
<td>VI</td>
</tr>
</tbody>
</table>

Fraction I primarily consisted of a protein that is well known to be a component of the inner membrane (12, 18). Fraction Ia likely contained one or more proteins that were loosely bound to the inner membrane. Fraction II (1, 2) contained a protein of molecular weight 50,000. In a previous study (6), fraction II was shown to correspond to a protein that was present in a protein complex of molecular weight 80,000. Fraction IIa represented a protein of molecular weight 50,000. Fraction IIb contained a component of molecular weight 30,000. Fraction IIc was a protein of molecular weight 25,000. Fraction III (1, 2, 3) contained two closely related proteins of molecular weight 58,000 and 49,000. Fraction IV (1, 2, 3) contained a protein of molecular weight 30,000. Fraction Va (1, 2, 3) contained a protein of molecular weight 25,000. Fraction VI (1, 2, 3) contained a protein of molecular weight 20,000.

Localization of polypeptides in inner membrane/matrix of rat liver mitochondria

* Some of this component is not tightly bound.

* At least one nonlabeled component in this fraction is not tightly bound.

The results obtained in this study by the proteolytic digestion of mitoplasts show that the polypeptides labeled with lactoperoxidase or diazobenzene-sulfonate are not proteins present only in trace amounts. Because these labels were used at high specific radioactivities (1,700 cpm/picomole for both 32P and 35S), the labeling observed in this study could have resulted from a very small number of reacting molecules. For example, labeling of the magnitude seen here would result if 10 tyrosine residues were quantitatively iodinated on a 50,000 molecular weight protein that made up only 0.01% of the total mitochondrial protein. However, because the proteases could remove a substantial amount of protein as well as label, probably only a small fraction of the available sites are iodinated under the conditions described here. For example, a significant fraction of components IIc, III, and VII is removed by protease treatment (Fig. 7). If this amount of material was quantitatively labeled, approximately 100 times more radioactivity should have been incorporated, assuming that these proteins have the average amino acid composition of rat liver mitochondrial proteins (7). It is not clear why the labeling with these reagents does not proceed to a greater degree and thus does not indicate directly the number of sites exposed to the outside. In the case of lactoperoxidase labeling, it is known that mitochondrial fractions contain substances which inhibit the iodination reaction. These substances may compete for the hydrogen peroxide substrate (glutathione, glutathione peroxidase, catalase (7, 11I)), sequester free iodide, or directly inhibit the enzyme. 8

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the labeled material above 15,000 molecular weight did fractionate with the inner membrane. Because they assumed that all of these labeled polypeptides were from the outer membrane alone, they concluded that the outer membrane was heterogeneous; a simpler interpretation may be that the labeled proteins fractionating with the inner membrane were in fact inner membrane proteins.

The results of Tinberg et al. (10) in which mitochondria and inner membranes were labeled with diazobenzenesulfonate at much higher concentrations than used here (2 mM versus 35 to 70 μM) are similar to the results shown here in Fig. 4. Their results indicate exterior components at mobilities of 0.36 (component III), 0.60 (VII and VIII), 0.85 (outer membrane band?), and 0.92 (?).

Although the work reported here is concerned with the overall pattern of mitochondrial protein organization with respect to the membrane, and although the location of the polypeptide components has been determined without regard for function, it is possible to use these general labeling techniques to demonstrate the position in the membrane of specific functional components if these components can be subsequently purified (23). This has been done in this study for cytochrome c and carbamyl phosphate synthetase to demonstrate the usefulness and applicability of these techniques. The components identified here as being exposed to the outer surface of the inner membrane (Table VI) are candidates for the polypeptide chains of a number of mitochondrial proteins which would be expected to transverse the membrane bilayer. A large group of these proteins is made up of the molecules involved in substrate transport across the inner mitochondrial membrane. Although there is no present evidence to link any one of these polypeptides to a transport function, some progress has been made by Coty and Pedersen (35) who have identified five polypeptides which are possible components of the phosphate transport system in rat liver mitochondria. Two of these polypeptides correspond in molecular weight to components identified here as being exposed to the outer surface: III (73,000) and VI (31,000). Thus, the results presented here can serve as a starting point for the process of matching transmembrane functions with specific mitochondrial polypeptides. Furthermore, it is clear that several components of the electron transport system are exposed to the outer surface of the inner membrane (see Ref. 27 for a review) and it may be possible to correlate these proteins with the polypeptides identified in this study.

Acknowledgments—Thanks are due to Guido Guidotti for his kind support while these studies were carried out in his laboratory and to Joshua Farber for his contributions to this work. I am indebted to Mark Bretscher for helpful discussions on the mechanism of lactoperoxidase-catalyzed iodination and to Robert Jackson, Lloyd Waxman, and Kurt Drickamer for their critical reading of this manuscript.

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S Clarke


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