Biogenesis of a 35-Kilodalton Protein Associated with Outer Mitochondrial Membrane in Rat Liver

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Biochemical analyses following subcellular fractionation of rat liver indicated that the outer mitochondrial membrane contains a number of membrane-specific proteins of which a 35-kilodalton species (OMM-35) is a prominent component. These results were confirmed and extended by electron microscope immunocytochemical tests based on the protein A-gold technique. OMM-35 is tightly bound to the outer mitochondrial membrane, e.g. it was not released by sonication in the presence of 1.5 M KCl and 0.1% sodium deoxycholate. However, it did not react with the photoaffinity probe azidopyrene, which indicates that OMM-35 is located peripherally on the membrane rather than buried deep in the lipid bilayer as an intrinsic protein. Since low levels of detergent were required for OMM-35 in intact mitochondria to react with exogenous antibodies, OMM-35 is probably located on the side of the outer membrane which faces the interior of the organelle.

When rat liver mRNA was translated in a messenger-dependent cell-free system derived from rabbit reticulocytes, antisem against OMM-35 precipitated a single polypeptide product which migrated on sodium dodecyl sulfate-polyacrylamide gels with molecular weight characteristics of a protein slightly larger (by M, = 500) than OMM-35 obtained from isolated outer mitochondrial membrane. The mRNA coding for OMM-35 was recovered exclusively from membrane-free polysomes. Thus, the route followed for synthesis and subsequent insertion of OMM-35 into the outer membrane of mitochondria is the post-translational pathway which has been previously described for proteins destined for the interior compartments of this organelle.

The question of how content and membrane proteins of various organelles are channeled from their sites of synthesis in the cytoplasm to their final destination in the cell has come under considerable investigation in recent years. For organelles which make up the overall secretory pathway, for example, both their content proteins and many (but not all; see Ref. 16) of the intrinsic proteins buried deep in their delimiting membranes are initially assembled into the rough endoplasmic reticulum by a coupled translation-insertion mechanism prior to being transported to their final location (cf. Ref. 1). For proteins of a number of discrete organelles which do not obviously contribute to the exocytosis-endocytosis pathway, however, a very different mechanism appears to be operating. In the case of mitochondria (2-4), chloroplasts (5, 6), and peroxisomes (7), transmembrane uptake of newly synthesized proteins destined for the interior compartments of these organelles involves an exclusively post-translational pathway, i.e. following release of newly completed polypeptide chains from membrane-free polysomes. Very little is yet known, however, concerning the mechanism(s) whereby proteins are assembled into the delimiting membranes of these three organelles. Even though these membranes may share certain compositional and structural features in common with other cellular membranes (most notably the endoplasmic reticulum), they also contain a number of unique proteins which render them functionally distinctive.

In the present study, we have investigated the biogenesis of a 35-kilodalton protein which is located in the outer mitochondrial membrane in rat liver. OMM-35 does not penetrate significantly into the lipid bilayer of the membrane but, nevertheless, is very tightly bound to the membrane at its inner surface. Here, we show that synthesis of OMM-35 occurs on membrane-free polysomes in the cytoplasm. Thus, assembly of this protein into the outer mitochondrial membrane must follow a post-translational insertion mechanism.

MATERIALS AND METHODS

General—For most of the routine procedures used in this study, the methods followed have been outlined elsewhere (see Refs. 4, 8, and 9 and articles cited therein). These include protein measurements, assays of trichloroacetic acid-insoluble radioactive products by the filter disc method, extraction of mRNA by either the chloroform/phenol method or the guanidinium thiocyanate/CsCl method, protein synthesis in a messenger-dependent (10) cell-free protein-synthesizing system derived from rabbit reticulocytes, and SDS-polyacrylamide gel electrophoresis and radiautography of dried gels. Where individual protocols were employed only for a specific experiment, they are described in detail in the figures and tables. Young Sprague-Dawley rats (80-200 g) were used throughout this study and were deprived of food for 8-16 h prior to killing.

Purification of OMM-35—Outer mitochondrial membrane was isolated on three separate occasions from a total of 90 g of rat liver by the digitonin technique, exactly as described by Greenawalt (11). Electrophoresis of the combined outer mitochondrial membrane fractions was performed using a total of 16 SDS-polyacrylamide (10%) slab gels containing 400 μg of protein/slot and 10 slots/gel. The gels were stained with Comassie brilliant blue and destained, and the OMM-35 bands were excised. Bands were dried and embedded in 1% agarose plug containing 1 ml of normal hamster serum, and protein

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The abbreviations used are: OMM-35, a M, = 35,000 protein in outer mitochondrial protein; SDS, sodium dodecyl sulfate; pOMM-35, the primary translation product of OMM-35 mRNA.
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was electroeluted into a dialysis bag. The protein was dialyzed against a total of 8 liters of H2O at 4 °C for 2 days and then lyophilized.

**Antiserum against OMM-35**—Lyophilized OMM-35 and hamster serum proteins were suspended in 2 ml of phosphate-buffered saline and mixed with 2 ml of Freund's complete adjuvant. Subcutaneous injections of this mixture were given to a hamster at 2-week intervals in aliquots containing 6% of the total preparation for the first two injections and 10% of the total preparation for the last three injections. Seven days after the final injection, serum was collected from the animal by cardiac puncture.

**Immunocytochemical Localization of OMM-35 by the Protein A-gold Technique**—Small pieces of rat liver were fixed for 2 h at room temperature in 1% glutaraldehyde and 0.1 M sodium phosphate buffer, pH 7.4, and embedded in glycol/methyacrylate (12). Thin sections (600–800 Å) were obtained and mounted on 200-mesh nickel grids having a carbon-coated Parlodion film. OMM-35 was visualized by the protein A-gold technique (13, 14) as follows (14). Thin sections on grids were incubated for 5 min on a drop of phosphate-buffered saline containing 1% ovalbumin, followed by incubation for 1 h at room temperature on a drop of antiserum against OMM-35 (diluted 50:1 with phosphate-buffered saline). After a brief wash with phosphate-buffered saline, grids were placed on a drop of the protein A-gold complex (14) for 30 min. Finally, grids were washed thoroughly with phosphate-buffered saline, rinsed with distilled H2O, and stained with uranyl acetate and lead citrate. They were examined under a Siemens 101 electron microscope.

Control reactions involved: (a) preadsorbing antiserum with excess outer mitochondrial membrane protein to immunocytochemical tests, (b) performing immunocytochemical tests with nonimmune serum, and (c) performing tests in the absence of serum (protein A-gold alone). All three yielded negative results.

**Isolation of mRNA from Free and Membrane-bound Polysomes**—Free and membrane-bound polysomes were obtained from rat liver by the method of Ramsey and Steele (15) exactly as described (16). RNA was extracted from the resulting pellets by the guanidinium thiocyanate/CsCl procedure (17) and the poly(A)+ fraction was isolated by oligo(dT)-cellulose chromatography.

**Synthesis and Recovery of OMM-35 in Vitro—Synthesis in vitro** was performed by incubating liver RNA in 100 µl of a messenger-dependent (10) rabbit reticulocyte cell-free system containing 0.4–0.6 mCi/ml of [35S]methionine (8, 9). After 60 min at 29 °C, the reaction-mixture was diluted with 0.9 ml of ice-cold medium containing phosphate-buffered saline, 1% Triton X-100, 20 mM methionine, 0.02% NaN3, and centrifuged at 45,000 rpm for 45 min in the Beckman type 75 rotor. The supernatant was collected and 0.4 ml of 4 M NaCl and 15 µl of antiserum against OMM-35 were added. Following incubation overnight at 4 °C, protein A-coated Staphylococcus cells were added and the mixture was rotated for 1.5 h at room temperature (8). The mixture was centrifuged and the pellet was washed three times at room temperature with medium containing phosphate-buffered saline, 20 mM methionine, 0.02% NaN3, 0.9% Triton X-100, and 0.9% SDS, washed once with saline, and finally boiled in SDS-polyacrylamide electrophoresis buffer to elute immunoreactants which were then loaded directly onto an SDS-polyacrylamide slab gel.

**RESULTS**

**Association of OMM-35 with the Outer Mitochondrial Membrane**—The four submitochondrial compartments, i.e. matrix, inner membrane, the space between inner and outer membranes, and outer membrane, were obtained from purified rat liver mitochondria (Fig. 1). An estimate of the quantitative distribution of protein between the 4 fractions is shown in Table I and was found to agree with other reported values (11, 18). The outer membrane fraction constituted roughly 9% of total mitochondrial protein (Table I) and was obtained in good yields (77% of the outer membrane marker, monoamine oxidase was recovered here) and in a highly enriched form (as judged by the high specific activity of monoamine oxidase in this fraction). Cytochrome c oxidase measurements invariably showed that our outer mitochondrial membrane preparations were not significantly contaminated (<5%) with inner membrane.

In order to identify proteins which are located predominantly, or exclusively, in the outer membrane, samples of the 4 submitochondrial fractions were compared by SDS-polyacrylamide gel electrophoresis (Fig. 1). One of the most promi-

![Figure 1](left) Protein composition of submitochondrial fractions. Mitochondria were purified from 20 g of liver and treated with digitonin to separate outer mitochondrial membranes from mitoplasts (see "Materials and Methods"). Digitonin-released material was centrifuged at 135,000 × g for 1 h to pellet outer mitochondrial membranes; the supernatant contained soluble proteins which had been located between the inner and outer mitochondrial membranes. To separate inner membranes from matrix proteins, mitoplasts were suspended in 10 ml of 10 mM Tris-HCl, pH 8.5, and sonicated for eight 1-min periods (8). The inner membrane was sedimented by centrifuging at 135,000 × g for 1 h; the supernatant contained soluble matrix proteins. Aliquots of the 4 submitochondrial fractions (~100 µg of protein) were co-electrophoresed in an 8–12% polyacrylamide gel containing SDS. The gel was stained with Coomasie brilliant blue. Lane A, matrix fraction; lane B, inner mitochondrial membrane; lane C, intermembrane space; lane D, outer mitochondrial membrane.

Molecular weight distributions were calculated from the mobilities of standard protein markers: phosphorylase b, 94,000 (94K); bovine serum albumin, 67,000 (67K); ovalbumin, 43,000 (43K); carbonic anhydrase, 30,000 (30K); trypsin inhibitor, 20,100 (20.1K). The vertical arrow denotes bovine serum albumin which had been present during treatment of mitochondria with digitonin and, therefore, was recovered in the fraction containing proteins of the intermembrane space.

![Figure 2](right) OMM-35 is retained by outer mitochondrial membranes following sonication in 1.5 M KCl and 0.1% sodium deoxycholate (DOC). Outer mitochondrial membranes (1.3 mg of protein) were suspended in 3.0 ml of 10 mM Tris-acetate (pH 7.6). 5 mM disodium ethylenediaminetetraacetic acid, 1.5 M KCl, and 0.1% sodium deoxycholate (25, 26). The suspension was sonicated at 4 °C for six 10-s periods (MSE microsonicator operating with a peak to peak amplitude of 12 pm) and incubated at 25 °C for 30 min. Membranes were sedimented at 105,000 × g for 1 h, suspended in 2 ml of H2O, and centrifuged. A portion of the pellet (125 µg of protein) was electrophoresed in an 8–12% polyacrylamide-SDS gel along with a sample of the same preparation of outer mitochondrial membrane which had not been subjected to sonication in the presence of KCl and sodium deoxycholate. Closed arrow heads denote proteins associated with outer mitochondrial membrane which were no longer present following the sonication treatment (open arrow heads).

**Table I**

**Distribution of protein and monoamine oxidase activity between submitochondrial fractions**

Submitochondrial fractions were obtained as described in Fig. 1 and were assayed for their relative protein content (23) and for monoamine oxidase activity as described in Ref. 24. One unit of enzyme activity is defined as 1 pmol (2.2 × 106 cm) of indoleacetic acid formed from [3H]tryptamine/20 min.

| Fraction       | Protein | Monoamine oxidase activity |
|----------------|---------|----------------------------|
| Matrix         | 59      | 0.28                       |
| Inner membrane | 29      | 1.07                       |
| Intermembrane space | 2.7   | 2.04                       |
| Outer membrane | 9       | 19.4                       |

% units/µg protein % total activity
iment of these was a polypeptide demonstrating a size of 35,000 daltons (designated OMM-35). OMM-35 is not associated with the outer membrane merely in an adventitious manner but represents a tightly bound structural component. For example, it was not liberated by sonication in 0.1% sodium deoxycholate and 1.5 mM KCl (Fig. 2).

Antiserum Against OMM-35—Antibodies to OMM-35 were obtained by eluting the protein from SDS-polyacrylamide gels and injecting into a hamster. Subsequent screening of the antiserum was performed by reacting it with SDS-polyacrylamide gel profiles of total protein of both outer and inner mitochondrial membranes blotted onto nitrocellulose paper; IgG-antigen complexes were then visualized by reacting the paper with \(^{125}\text{I}\)-protein A (Fig. 3). Antiserum reacted only with OMM-35. Identity of OMM-35 was unequivocal since the autoradiogram of the nitrocellulose blot could be compared directly to the original SDS-polyacrylamide gel which had been subsequently stained with Coomassie brilliant blue (only about 30% of the OMM-35 band was transferred to the blot). The radioactive band on the blot (Fig. 3) demonstrated exactly the same position and shape as the stained OMM-35 band in the polyacrylamide gel. In this particular experiment, however, OMM-35 was detected in both outer and inner membrane fractions, perhaps because release of outer membrane from purified mitochondria by digitonin treatment had not been complete (but see below). That the antiserum was monospecific, i.e. reacted with a single antigen in the outer mitochondrial membrane, was demonstrated by two-dimensional rocket immunoelectrophoresis (Fig. 4).

Subcellular Distribution of OMM-35—Biochemical analyses indicated that OMM-35 is enriched in the outer mitochondrial membrane (Fig. 1); it was not present in detectable levels in either total microsomal preparations (Fig. 5) or in a post-microsomal cytosolic fraction (data not shown). These findings were confirmed and extended by immunocytochemical tests using the protein A-gold technique (Fig. 6). Of a total of 225 grains analyzed, the majority (82%) were located over mitochondria, of which 50% was present on the outer membrane and 30% was present on the inner membrane. The remainder (20%) could not be ascribed to a particular mitochondrial locus. Since there are three times more inner membrane protein compared to outer membrane protein (Table I), the immunocytochemical results indicate a clear enrichment of OMM-35 in the outer membrane. The significance of residual reactivity over the inner membrane, however, remains to be determined.

![Fig. 3. Specificity of antiserum against OMM-35. Samples of outer and inner mitochondrial membranes (200 µg of protein each) were subjected to electrophoresis in a 10% polyacrylamide gel containing SDS. A blot of the separated proteins was obtained (27) by electrophoretically transferring the proteins in the gel to a sheet of nitrocellulose paper. A strip of OMM-35 was transferred. Blots were rinsed in 100 µl of saline for 2-3 min and incubated for 1 h at 37 °C in 10 ml of a medium containing saline, 3% bovine serum albumin (Sigma type V, fatty acid free), and 0.02% NaN₃. Following a brief rinse with saline, the blot was incubated for 5 h at room temperature in a sealed plastic bag containing 100 µl of antiserum against OMM-35 and 10 ml of saline, 3% bovine serum albumin, 0.02% NaN₃. The blot was again rinsed with saline and agitated over night in 100 ml of saline, 3% bovine serum albumin, 0.02% NaN₃. It was then incubated in a sealed bag with 5 ml of saline, 3% bovine serum albumin, 0.02% NaN₃, and 2 pCi of \(^{125}\text{I}\)-protein A (20 mCi/mg, Amersham). The mixture was shaken for 1 h at room temperature. The blot was briefly rinsed with saline containing 3% bovine serum albumin and 0.02% NaN₃, and, finally, was washed extensively over a period of 16 h with six changes of saline containing 0.02% NaN₃. Excess liquid was removed from the blot and it was wrapped in Saran Wrap and exposed overnight at -70 °C to an x-ray film pressed onto a Cronex Lightning-plus (Dupont) intensifying screen. Lane A, outer mitochondrial membrane; lane B, inner mitochondrial membrane.](image)

![Fig. 4. Two dimensional rocket immunoelectrophoresis of OMM-35. Two samples of outer mitochondrial membrane (~200 µg of protein) were electrophoresed about 4 cm into a 10% SDS-polyacrylamide resolving gel. One lane was excised and stained; the other was used for rocket immunoelectrophoresis as follows. A narrow strip (1-2 mm wide and 2 cm long) was removed from the center of the track. Its long axis (2 cm) ran perpendicular to the protein bands and covered a portion of the gel containing OMM-35. The exact position of OMM-35 was determined by reference to OMM-35 in the stained companion lane. The strip was embedded in a section of the immunoelectrophoresis gel (3 cm wide) which contained 1% agarose (Bio-Rad), 0.1 M barbital III buffer (Bio-Rad), and 0.05% Triton-X-100, but no antiserum. The remaining portion of the gel contained 25 µl/ml of anti-OMM-35 antiserum, but no detergent. Electrophoresis was at 10 mA for 20 min and then at 4 mA for 1.5 h. Unprecipitated protein was removed from the gel by repeated drying and swelling in 0.1 M NaCl (28). The gel was then stained with Coomassie brilliant blue. The figure shows a section of stained SDS-polyacrylamide gel corresponding to the unstained strip which had actually been used in the experiment. The arrow denotes OMM-35.](image)
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**Fig. 5.** A comparison of levels of OMM-35 in purified outer mitochondrial membrane and in microsomal membranes. Mitochondria were purified as described under "Materials and Methods." Total microsomal membranes were recovered from the combined post-mitochondrial supernatants by centrifugation for 2 h at 105,000 × g. Outer mitochondrial membranes were isolated following digitonin treatment of the mitochondria. Samples of the two membrane preparations containing approximately 300 μg of protein were co-electrophoresed in a 10% polyacrylamide gel containing SDS. The gel was then stained and destained. A portion of the gel tracks in the region of OMM-35 is shown. Lane A, outer mitochondrial membranes; lane B, microsomes.

**Fig. 6.** Immunocytochemical localization of OMM-35 in rat hepatocytes by the protein A-gold technique. Thin sections from rat liver were incubated with antisera against OMM-35 and the resulting antibody-antigen complexes were then visualized following reaction with protein A-gold (see "Materials and Methods"). Magnification, × 75,000. Large arrows point to gold grains on the outer membrane and small arrows point to grains on the inner membrane. What appear to be cleared areas in thin sections are regions where glycogen particles had been leached from the tissue during the mild fixation techniques employed in this procedure.

**Topographical Orientation of OMM-35 in the Outer Mitochondrial Membrane**—Although OMM-35 was shown to be tightly bound to outer mitochondrial membrane, e.g. it was not released by sonication in the presence of 0.1% sodium deoxycholate and 1.5 M KCl (Fig. 2), such experimentation does not indicate if this tight association is mediated by peripheral interactions between OMM-35 and the surface of the membrane or results because the protein is buried, either wholly or in part, as an intrinsic component of the lipid bilayer. Fig. 7 provides strong evidence that OMM-35 is associated with outer mitochondrial membrane in a peripheral fashion; it does not enter the lipid bilayer to any significant degree. When isolated outer mitochondrial membrane was reacted with the membrane-penetrating photoaffinity reagent azidopyrene (19), OMM-35 was not among the proteins which became fluorescently labeled following subsequent photolysis. The major intrinsic proteins reacting with azidopyrene were located in the Mr = 30,000–33,000 range (Fig. 7).

The next step was to determine on which side of the outer membrane OMM-35 faces, i.e. toward the cytoplasm, toward the inside of the mitochondrion, or both. This was tested by incubating intact mitochondria with antisera against OMM-35 and measuring the accessibility of OMM-35 to the exogenous antisera in the presence and absence of low levels of detergent. The results showed (Table II) that detergent was required to facilitate antibody-antigen interaction, presumably because the detergent rendered the outer mitochondrial membrane porous to the antibody. Since OMM-35 was not noticeably available for interaction with antibody in the abs-

**Fig. 7.** Photoaffinity labeling of outer mitochondrial membrane proteins by the membrane-permeating agent azidopyrene. Azidopyrene (19) was dissolved in dimethyl sulfoxide in the dark to give a final concentration of 300 mM. An aliquot (5 μl) was added to outer mitochondrial membranes (~600 μg of protein) suspended in 50 μl of 4 mM Tris-HCl, pH 8.5, in a quartz vial. The suspension was irradiated with longwave UV light. An aliquot (10 μl) was removed and mixed with 10 μl of double-strength SDS-polyacrylamide electrophoresis sample buffer. Proteins were resolved in an 8–12% polyacrylamide gel containing SDS. Following a brief wash with H$_2$O, the gel was exposed to incident UV light and photographed through a blue filter and a clear plastic filter. The gel was then transferred to Coomassie brilliant blue staining solution. Lane A, fluorescence staining with azidopyrene; lane B, Coomassie brilliant blue stain of the fluorescent gel track shown in lane A.
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Table II

Accessibility of mitochondrial OMM-35 to exogenous antiserum

Purified mitochondria (350 μg protein) were suspended in 100 μl of medium A (phosphate-buffered saline, 0.3 M sucrose, 0.5% bovine serum albumin, and 0.02% Na3), either with or without 0.01% sodium deoxycholate. Then, 20 μl of either normal serum or antiserum against OMM-35 were added and the mixtures were briefly sonicated and incubated at 25 °C for 2.5 h. The reaction mixtures were diluted with 5 ml of medium A and centrifuged at 25 °C for 30 min, to remove the precipitate. The supernatants were diluted with 0.3 M phosphate-buffered saline, dissolved in NCS (Amersham), and assayed for radioactivity.

| Addition to mitochondria | 125I-Protein A bound | Difference (immune minus nonimmune) |
|--------------------------|-----------------------|-----------------------------------|
| Nonimmune serum          | 8.5                   | 10.9                              |
| Anti-OMM-35 serum         | 9.0                   | 0.5                               |
| Nonimmune serum + 0.01% sodium deoxycholate | 10.9               |                                    |
| Anti-OMM-35 serum + 0.01% sodium deoxycholate | 25.4               | 14.5                              |

Absence of detergent (Table II), it is concluded that the protein is located on the side of the outer membrane which faces the interior of the mitochondrion.

Biogenesis of OMM-35—When antiserum against OMM-35 was reacted against total polypeptide products synthesized in vitro in a messenger-dependent rabbit reticulocyte system programmed with rat liver mRNA, a single polypeptide was precipitated (Fig. 8). On SDS-polyacrylamide gels, this putative translation product of OMM-35 mRNA was found to demonstrate size characteristics just slightly larger (by about M, = 500) than the form of OMM-35 as it exists in isolated outer mitochondrial membrane (Fig. 8). Although this size difference is small, it is reproducible and has been observed recurrently using a number of different preparations of both outer mitochondrial membrane and messenger. In addition to the immunological evidence, however, it was impossible to demonstrate further equivalence between pOMM-35 synthesized in vitro and OMM-35 synthesized in vivo (e.g. by peptide map comparisons) because of very low levels of incorporation of radioprecursor into OMM-35 which occurred in vivo both in the intact animal and in liver explant cultures. Nevertheless, since only a single radioactive product was precipitated following immunoreaction of reticulocyte lysates with antiserum against OMM-35 (Fig. 8) and since the antiserum was shown to react only with OMM-35 (Figs. 3 and 4), it seems unlikely that the immunoprecipitated product of mRNA translation is anything other than the primary biosynthetic form of OMM-35.

In Fig. 9, translational assays were used to determine the subcellular location of poly(A') RNA coding for OMM-35. Free and membrane-bound polysomes were separated essentially according to the rapid procedure of Ramsey and Steele (15) and mRNA was then extracted from polysomal pellets by the guanidine thiocyanate/CsCl procedure (17). Following incubation of mRNA from free and bound polysomes in the reticulocyte protein-synthesizing system, pOMM-35 was precipitated only from lysates containing mRNA from free polysomes (Fig. 8); pOMM-35 was virtually undetectable in lysates programmed with membrane-bound mRNA. Conversely, when these same two messenger preparations were tested for their ability to direct synthesis of the secretory protein serum albumin, this polypeptide was detected only in reticulocyte lysates incubated with membrane-bound mRNA (data not shown).

Discussion

Two approaches were used in this study to ascertain the subcellular distribution of OMM-35: a biochemical analysis involving separation and isolation of various membrane fractions (Figs. 1 and 5) and an immunocytochemical analysis where antibody-antigen complexes were allowed to form on thin sections of liver tissue and then visualized following reaction with protein A-gold (Fig. 6). The immunological approach, of course, requires that the antiserum is monospecific and interacts only with OMM-35. That this was the case was demonstrated by incubating the antiserum with replicas of SDS-polyacrylamide gel patterns of outer and inner mitochondrial membrane proteins blotted onto nitrocellulose paper (Fig. 3). Only OMM-35 IgG complexes were detected following reaction with 125I-protein A; there was no evidence for the presence of even minor levels of contaminating antibodies. Moreover, two-dimensional rocket immunoelectrophoresis analyses showed that anti-OMM-35 reacted only with a single antigen in the outer membrane (Fig. 4), i.e. the OMM-35 band did not contain additional immunoreactive polypeptides which might have arisen by contamination from
the inner membrane. At least with respect to mitochondrial membranes, therefore, there is little doubt that the immunocytochemical technique is detecting anything but OMM-35 (Fig. 6).

When SDS-polyacrylamide gel patterns of outer and inner membranes were compared (Fig. 1), OMM-35 was found to be enriched in the outer membrane, even taking into account the fact that there is three times more inner than outer membrane protein (Table I). From experiment to experiment, however, there was a certain variability sometimes detected in rather significant levels in the inner membrane fraction as well (e.g. see Fig. 3). It was usually assumed that such variations arose because of variabilities encountered with the efficient release of outer membrane from intact mitochondria by the digitonin procedure. Immunocytochemical tests (Fig. 6), however, showed a positive reaction over both inner and outer membranes, albeit to a considerably lower extent (5-fold) in the inner membrane when calculated per unit membrane surface area. Whether the residual immunoreactivity over the inner membrane arose artifactually or is indicative of an additional location for OMM-35 in the inner membrane remains to be determined.

Evidence to elucidate the topographical orientation of OMM-35 in the outer mitochondrial membrane was obtained from a combination of approaches. First, the protein was shown to be tightly bound to the outer membrane (Fig. 2). However, it is not buried in the lipid bilayer as an intrinsic protein; OMM-35 did not react with azidopyrene when this membrane-permeating photoaffinity label was reacted with outer membrane (Fig. 7). Since treatment of intact mitochondrial membrane-permeating photoaffinity label was reacted with OMM-35, the receptor was low levels of detergent was required in order to render OMM-35 accessible to exogenous antibody (Table II), the protein is presumably located on the side of the outer membrane which faces toward the interior of the mitochondrion. Thus, in terms of functional compartmentalization, OMM-35 must be considered an intramitochondrial protein and, therefore, might be expected to conform to what is already known concerning assembly of proteins which are synthesized in the cytoplasm and transported to their final destination inside the mitochondrion. The main feature of this uptake process is that it occurs post-translationally (2-4); newly made polypeptides are transported across either one or both mitochondrial membranes only after release of completed polypeptide chains from cytoplasmic free polysomes has occurred. In the present study, we showed that OMM-35 is made on membrane-free polysomes (Fig. 9) and, therefore, its uptake into mitochondria must likewise follow the post-translational pathway for insertion into a membrane.

It is curious, however, that the primary translation product of OMM-35 mRNA demonstrates a slightly retarded mobility on SDS-polyacrylamide gels relative to the in vitro form of OMM-35, at least as it exists in isolated outer membrane (Fig. 8). The difference in size between the two is only on the order of about Mr = 500. Higher molecular weight biosynthetic precursors have been identified for a variety of mitochondrial proteins (3, 4, 20-22), but in all cases they exist with an extra Mr = 2,000-6,000 piece present in the precursor. A possibility which must be considered to explain the present results, therefore, is that OMM-35 is synthesized in vitro in its final form and the differential which is observed between OMM-35 synthesized in vitro and in vivo arises because of spurious trimming of OMM-35 which might occur during isolation of outer mitochondrial membrane. On the other hand, the extra Mr = 500 piece may indeed be important for directing the overall assembly process of this outer mitochondrial membrane protein. Resolution of this question will have to await reconstitution of synthesis, uptake, and assembly of OMM-35 in vitro since successful assembly should be accompanied by removal of the extra Mr = 500 piece if in fact this normally occurs in vivo.

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