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In vitro synthesis and assembly of a 68 kDa outer mitochondrial membrane protein from rat liver

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Outer mitochondrial membrane was purified from rat liver. Its constituent proteins were analyzed by SDS-polyacrylamide gel electrophoresis and by electrophoretic immunoblotting employing antibodies raised against total outer mitochondrial membrane. Anti-outer mitochondrial membrane antiserum reacted with only one polypeptide (15 kDa) in rough microsomes, whereas no immunological cross-reactivity was observed with other mitochondrial compartments (intermembrane space, inner membrane, or matrix) or with lysosomes or total cytosol. The antiserum was employed to characterize precursors of outer mitochondrial membrane proteins synthesized in vitro in a rabbit reticulocyte cell-free system. One product (a 68 kDa polypeptide designated OMM-68) bound efficiently to mitochondria in vitro but did not interact with either dog pancreas or rat liver microsomes, either co-translationally or post-translationally. OMM-68 was synthesized exclusively by the membrane-free class of polyribosomes. Attachment of precursor OMM-68 to mitochondria was not accompanied by processing of the polypeptide to a different size.

Introduction

Of the four subcompartments of the mitochondrion (outer membrane, intermembrane space, inner membrane and matrix), the outer membrane is perhaps the most poorly understood, both functionally and structurally. Its relevance to mitochondrial physiology, however, is essential: it contains a number of important enzymatic functions, it contains proteinaceous pores for transporting small metabolites, it contains structural components which interact with cytoplasmic elements, including the cytoskeleton, and it has exposed at its surface receptors which mediate transport of nuclear-coded precursor proteins into the interior of the organelle (see Refs. 1 and 2 for reviews). How these various protein components are themselves assembled into the outer membrane, however, has only recently been studied. To date, seven proteins of the outer mitochondrial membrane have been investigated: OMM-35 [3] and monoamine oxidase [4] in rat liver, porin [5–7] in yeast and *Neurospora,* and 70, 45 and 14 kDa polypeptides in yeast [7]. Assembly of these proteins into the outer mitochondrial membrane occurs by a post-translational mechanism and does not seem to involve proteolytic removal of a signal sequence from the precursor protein (although a 500 dalton difference was observed between pre-

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Abbreviations: OMM, outer mitochondrial membrane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

OMM-35 and the mature form of the protein as it exists in purified outer membrane; Ref. 3). Of the various proteins, only those from yeast and *Neurospora* have been demonstrated to insert into mitochondrial outer membrane in vitro.

In order to further investigate the composition and assembly of outer mitochondrial membrane proteins in rat liver, we have raised highly specific antisera against the purified membrane. The antibodies show little or no cross-reactivity against other mitochondrial compartments, including inter-membrane space, inner membrane, and matrix, or against endoplasmic reticulum, cytosol, or lysosomes. The antibodies have been employed to analyze in vitro synthesis of outer mitochondrial membrane proteins. One such protein product, OMM-68, was found to be synthesized exclusively by membrane-free polyribosomes. It bound efficiently to purified mitochondria, but not to dog pancreas or rat liver microsomes, in vitro; binding to mitochondria was not accompanied by proteolytic processing.

Experimental procedures

General. Livers from starved male Sprague-Dawley rats (150–250 g) were used throughout this study. For most of the routine procedures which were employed, the methods followed have been outlined elsewhere [8,9]. These include protein and RNA measurements, isolation of cytoplasmic mRNA, protein synthesis in a messenger-dependent [10] cell-free system derived from rabbit reticulocytes, and SDS-polyacrylamide gel electrophoresis and fluorography of dried gels. Further details are provided in the figure legends.

Purification of rat liver outer mitochondrial membrane. Purification was achieved by a modification of the French press procedure described by Greenawalt [11]. Mitochondria were first purified from about 30 g of liver as follows. The tissue was homogenized in isolation medium (70 mM sucrose, 220 mM mannitol, 2 mM Hepes (pH 7.6) and 0.5 mg/ml bovine serum albumin; Ref. 12) and centrifuged in an IEC-DPR6000 swing-out rotor (No. 243) at 2100 rpm ($1000 \times g$) for 10 min at 4°C. The supernatant was centrifuged for 10 min in the Sorval SS34 rotor at 5300 rpm and the pellet was resuspended and recentrifuged. Following another resuspension, a final mitochondrial pellet was obtained by centrifuging for 10 min at 7000 rpm. The ratio of tissue wet weight to volume of isolation medium employed at each stage was the same as in Ref. 11. Also, care was taken to remove any unpacked, fluffy material (broken mitochondria and lysosomes) from the top of mitochondrial pellets. The purified mitochondria were uniformly suspended in twice-concentrated isolation medium, to a protein concentration of about 45 mg/ml. Approx. 10 ml were passed through the French press at 1500 p.s.i. at a rate of 1–2 ml/min. The eluate was centrifuged at 10000 rpm in the Sorval SS34 rotor for 10 min, the supernatant was recentrifuged as before, and then finally centrifuged for 10 min at 18000 rpm; the majority of mitoplasts (inner membrane plus matrix) were thus removed. The supernatant was centrifuged at 45000 rpm in the Beckman Ti75 rotor for 2.5 h to recover outer mitochondrial membrane vesicles; they were suspended in 5% sucrose and 20 mM Tris-HCl (pH 7.6), layered (0.5 ml) on a 20–50% (w/v) sucrose density gradient containing 20 mM Tris-HCl (pH 7.6) and centrifuged in the Beckman SW40 rotor at 20000 rpm (2°C) for 2 h. The turbid band near the top of the gradient contained purified outer mitochondrial membrane. It was recovered and diluted with 4 vol. of 20 mM Tris-HCl (pH 7.6), centrifuged at 45000 rpm as above, suspended in 20 mM Tris-HCl (pH 7.6) and 50% (w/w) glycerol, and stored at −20°C until use. The final yield was 3–5 mg protein, representing about 10% of the starting amount of outer mitochondrial membrane.

Anti-serum against outer mitochondrial membrane proteins. Two intra-muscular injections of total outer mitochondrial membrane (3 mg protein) in Freunds complete adjuvant were given to a rabbit on days 1 and 7, followed by multiple subcutaneous injections of 3 mg outer mitochondrial membrane protein in incomplete adjuvant on day 21 and again on day 42; the animal was bled 6 days later.

In vitro import of outer mitochondrial membrane precursor proteins. Liver mRNA was incubated in a rabbit reticulocyte cell-free protein synthesizing system supplemented with 1.0 mCi/ml [^{35}S]methionine (approx. 1000 Ci/mmol, Amersham) for 60 min at 30°C, at which time

sucrose was added to a final concentration of 0.25 M. An aliquot (50 μl) was then mixed with 17 μl of freshly-purified mitochondria [9] and suspended to 2.9 mg protein/ml in 10 mM Hepes (pH 7.4), 0.25 M sucrose, 1.0 mM dithiothreitol, 10 mM sodium succinate, 0.15 mM ADP, 2.5 mM K_2HPO_4 and 0.1 mg/ml cycloheximide [9,13]. After incubating for 60 min at 30°C, the sample was centrifuged in a Beckman microfuge for 5 min at 4°C. The supernatants and pellets were then dissolved in 1.0 ml of ice-cold medium containing phosphate-buffered saline, 1.0% Triton X-100, 20 mM methionine, 10 mM EDTA, 0.02% (w/v) NaN_3 and 150 μg/ml phenylmethylsulfonylfluoride. Following centrifugation at $130000 \times g$ for 45 min to remove particulate material, 15 μl of anti-outer mitochondrial membrane antiserum and 0.4 ml of 4.0 M NaCl were added. Immunoprecipitates were obtained and processed as described in Ref. 8.

Results

The post-translational route for insertion of polypeptides into outer mitochondrial membrane was first described for OMM-35 [3]. In that study, we employed a monospecific antibody against OMM-35 to show that the precursor protein was synthesized exclusively by membrane-free polyribosomes. To date, however, we have not been able to achieve insertion of precursor OMM-35 into the outer membrane of isolated intact mitochondria from either liver or heart, despite the fact that these same mitochondrial preparations efficiently import and process the precursor to the liver mitochondrial matrix enzyme, ornithine carbamyl transferase [9,13]. In order to further investigate biogenesis and assembly of proteins located in the outer mitochondrial membrane, therefore, we have purified outer membrane from rat liver and have raised antibodies against its constituent protein population.

Purification and characterization of rat liver outer mitochondrial membrane

Purification was achieved by a slight modification of Greenawalt's French press cavitation procedure: additional fractionation by sucrose density gradient sedimentation was essential for removing contaminating fragments of inner mitochondrial membrane (see Experimental procedures). The yield of outer mitochondrial membrane by this procedure is relatively low (5–10%), but we have found that its purity is superior to outer mitochondrial membrane prepared by the more popular digitonin procedure [12]. Our outer mitochondrial membrane preparations routinely give monoamine oxidase activities (an outer mitochondrial membrane marker, Ref. 14) which are on average 13-times higher than purified whole mitochondria, indicating that outer mitochondrial membrane contains about 8% of total mitochondrial protein.

Fig. 1 presents an SDS-polyacrylamide gel electrophoresis profile of purified rat liver outer mitochondrial membrane; the overall pattern of polypeptides is completely different than that obtained from purified inner membrane or from ribosome-stripped rough microsomes from rat liver. The most abundant protein in outer mitochondrial membrane is porin, a pore-forming protein made up of three identical subunits of 32 kDa in size (Ref. 15 and Colombini, M., personal communication). Other major species include OMM-35 and polypeptides located in the 60–70 kDa range (Fig. 1). At the 15 kDa position, there is a polypeptide which is common to outer mitochondrial membrane and ribosome-stripped rough microsomes (Fig. 2). This is presumably the same 15 kDa protein which Fujiki et al. [16] found in endoplasmic reticulum and total mitochondrial membranes; Fig. 2, however, demonstrates that its mitochondrial location is restricted to the outer membrane.

The relative purity of outer mitochondrial membrane preparations was assessed immunologically (Fig. 2). Antibodies were raised against total outer mitochondrial membrane and subsequently analyzed by immunoblotting against outer mitochondrial membrane and rough microsomes. The reactivity against inner membrane was low and demonstrated the same pattern as for outer mitochondrial membrane, indicating that the inner membrane preparation was slightly contaminated with outer mitochondrial membrane. With the exception of the 15 kDa polypeptide, rough microsomes similarly showed very low cross-reactivity (the two minor staining bands in the 60–70 kDa

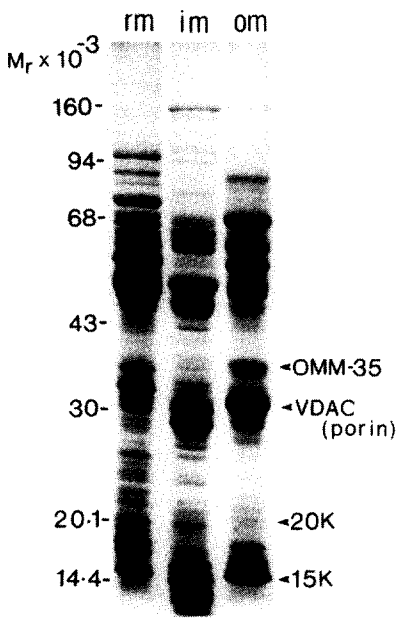

Fig. 1. Resolution of proteins from outer mitochondrial membrane, inner mitochondrial membrane, and ribosome-stripped rough microsomes by SDS-polyacrylamide gel electrophoresis. Outer mitochondrial membrane (om) was purified as described in Experimental procedures, inner mitochondrial membrane (im) was prepared as described in Ref. 3, and ribosome-stripped rough microsomes (rm) from rat liver were obtained according to Ref. 27, and were kindly supplied by Dr. Jacques Paiement (University of Montreal). Samples of each preparation (100 μg protein) were resolved in a 8–14% SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue, destained and photographed. Relative mobilities of various marker proteins are shown on the left: they are phosphorylase *b* (94 kDa); albumin (68 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). OMM-35 denotes a 35 kDa polypeptide previously shown [3] to be located in rat liver outer mitochondrial membrane; VDAC (voltage-dependent anion channel, Ref. 15), also called porin, is a major intrinsic protein component of outer mitochondrial membrane.

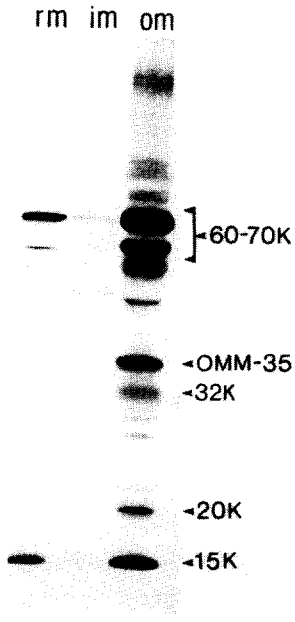

Fig. 2. Immunoblot of outer (om) and inner (im) mitochondrial membranes and of ribosome-stripped rough microsomes (rm) employing anti-outer mitochondrial membrane antiserum. Proteins from the three sources (100 μg) were resolved by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose [23], and incubated with a 1000:1 dilution of anti-outer mitochondrial membrane antiserum [3] followed by goat anti-rabbit IgG coupled to horseradish peroxidase. The immuno reaction was visualized by staining with a solution containing 0.1% 3,3′-diaminobenzidine tetrahydrochloride, 0.1% imidazole, 0.03% hydrogen peroxide, 150 mM NaCl and 10 mM Tris-HCl (pH 7.4). The relative masses (kilodaltons) of various immunoreactive polypeptides are indicated. 32 kDa is at the position of the voltage-dependent anion channel.

range probably arose from contamination of microsomes with outer mitochondrial membrane). Mitochondrial inter-membrane space and matrix, lysosomes, and cytosol did not contain polypeptides reacting with the antiserum (data not shown).

In vitro synthesis of outer mitochondrial membrane polypeptide precursors

Anti-outer mitochondrial membrane antiserum was employed to analyze synthesis of outer mitochondrial membrane primary translation products encoded by mRNA isolated from either free or membrane-bound polyribosomes. Separation of free and bound polyribosomes was achieved by slight modifications [9] of the Ramsey and Steele protocol [17], followed by extraction of RNA by the guanidinium/CsCl procedure [18]. The advantage of the Ramsey and Steele technique is that recovery of the two mRNA populations is quantitative; in particular, all membranes which bear ribosomes in vivo, including microsomes, rapidly-sedimenting endoplasmic reticulum [19] and nuclei, are used as the source of membrane-bound polyribosomes. To assess the purification of free and

bound polyribosomes by this method, aliquots of their respective mRNAs were translated in vitro in a messenger-dependent rabbit-reticulocyte cell-free system and the radioactive products were analyzed for the presence of prepro-albumin (made only by membrane-bound polyribosomes) and pre-ornithine carbamyl transferase (made only by free polyribosomes). The results are shown in Fig. 3. We calculated that cross-contamination between the two mRNA populations was less than 10%.

Fig. 4 shows the pattern of polypeptides synthesized under the direction of mRNA from free and

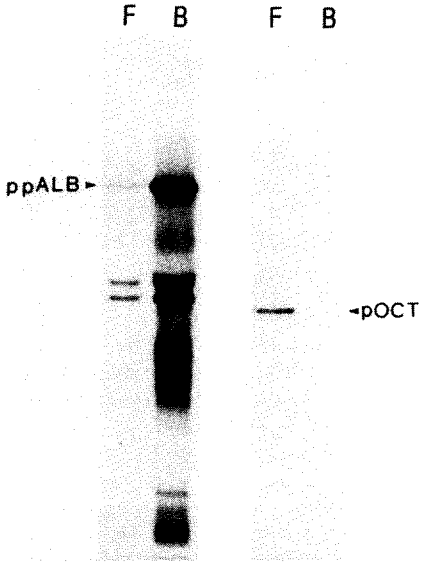

Fig. 3. In vitro synthesis of prepro-albumin (ppALB) and pre-ornithine carbamyl transferase (pOCT) employing mRNA from membrane-bound and membrane-free polyribosomes. Free (F) and bound (B) polyribosomes were isolated from rat liver exactly as described [9], and further extracted to recover mRNA [18]. The two mRNA populations were incubated for 60 min in a messenger-dependent rabbit reticulocyte cell-free protein synthesizing system in the presence of [^{32}S]methionine (1 mCi/ml, approx. 1000 Ci/mmol). The translational efficiency of the free and bound mRNA was similar. Aliquots of radioactive translation products (equivalent to $4.0 \cdot 10^6$ cpm) were subjected to immunoprecipitation by IgG antibodies monospecific for either rat serum albumin [24] or rat mitochondrial ornithine carbamyl transferase [13]. Precipitates were resolved by 10% SDS-polyacrylamide gel electrophoresis and the gel was fluorographed. The two radioactive bands appearing below prepro-albumin presumably represent premature translational termination products since they did not appear when pre-immune IgG was employed for immunoprecipitation (not shown).

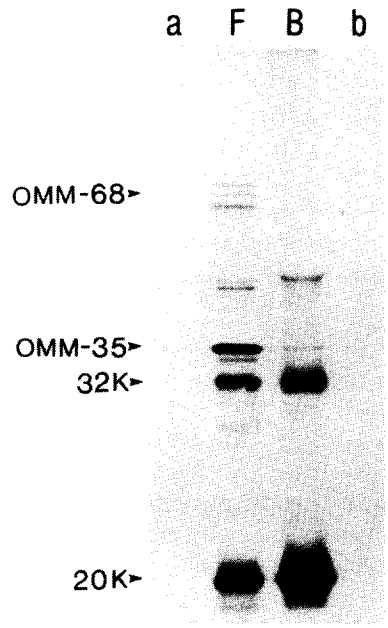

Fig. 4. Translation products of mRNA from free and membrane-bound polyribosomes which are precipitated by anti-outer mitochondrial membrane antiserum. mRNA from free (F) and bound (B) polyribosomes were translated in vitro as described in Fig. 3. Products ($6.9 \cdot 10^6$ cpm for free polyribosomes and $3.8 \cdot 10^6$ cpm for bound polyribosomes) were precipitated with anti-outer mitochondrial membrane antiserum, resolved by 12% SDS-polyacrylamide gel electrophoresis, and fluorographed. Lanes a and b, pre-immune serum was employed against products synthesized under the direction of mRNA from free and bound polyribosomes, respectively. The relative masses (kilodaltons) of certain translation products are indicated.

bound polyribosomes and precipitated by anti-outer mitochondrial membrane antiserum. The identity of OMM-35 in all our translations has been confirmed by parallel immunoprecipitations employing a mono-specific anti-OMM-35 antibody. As described in Ref. 3, OMM-35 is made exclusively by free polyribosomes. Fig. 4 extends this observation to include polypeptides in the 60–70 kDa range; they too are made by free polyribosomes. However, considering the relative purity of the two messenger populations (Fig. 3) and the comparative specificity of the antiserum (Fig. 2), it was surprising to find that synthesis of two polypeptides, at 20 and 32 kDa, was clearly expressed by mRNA from both free and bound polyribosomes. Whether this occurs because the respective mRNAs from free and bound poly-

ribosomes encode different proteins of similar molecular weight, or because their polyribosome characteristics cause them to non-specifically partition into free and bound polysomes following tissue homogenization, has not been determined. Interestingly, however, 20 and 32 kDa polypeptides recognized by the anti-outer mitochondrial membrane antiserum are not found in rough microsomes (Fig. 2).

In vitro import of OMM-68 by isolated mitochondria from rat liver

As outlined earlier, we have been unsuccessful to date in achieving import of in vitro synthesized precursor to OMM-35, despite the fact that the same mitochondrial preparations efficiently import and process the precursor to the matrix enzyme, ornithine carbamyl transferase. Since import of pre-ornithine carbamyl transferase is extremely sensitive to structural disruptions of the organelle [13], it is unlikely that failure to import precursor to OMM-35 resides at this level. It may be, however, the OMM-35 precursor synthesized in reticulocyte lysates assumes a tertiary conformation which is different from its native form, rendering the molecule incapable of binding to the mitochondrial surface.

Of the various primary translation products precipitated by anti-outer mitochondrial membrane anti-serum, only one bound to mitochondria in vitro (Fig. 5). It demonstrated a molecular mass of 68 kDa following SDS-polyacrylamide gel electrophoresis and, therefore, was designated OMM-68. As shown in Fig. 5, when translation products were combined with mitochondria and then immediately centrifuged for 5 min, OMM-68 did not sediment and was recovered in the supernatant. Following incubation with mitochondria for 60 min, however, about 60% of OMM-68 sedimented following centrifugation, indicating that it was bound to the organelle. The remainder of OMM-68 (about 40%) was recovered in the supernatant (Fig. 5). Other polypeptide products in the 60–70 kDa range did not bind to mitochondria, nor did their net amounts change during the import incubation (Fig. 5). The data in Fig. 5 indicate, therefore, that binding of OMM-68 to mitochondria occurred without concomitant proteolytic processing.

Finally, binding of in vitro synthesized OMM-68

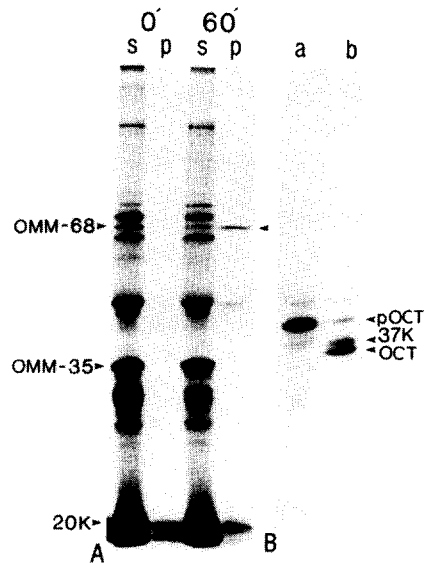

Fig. 5. In vitro import of OMM-68 and precursor to ornithine carbamyl transferase by rat liver mitochondria. Panel A: In vitro translation products ($10.4 \cdot 10^6$ cpm) from total liver mRNA were incubated with purified liver mitochondria for 0 or 60 min (see Experimental procedures), after which mitochondria were pelleted by centrifugation for 5 min at $12000 \times g$. Supernatants (s) and pellets (p) were subjected to immunoprecipitation with anti-outer mitochondrial membrane antiserum, resolved in a 10% SDS-polyacrylamide gel, and fluorographed. The relative masses (kDa) of certain products are indicated. Panel B: The same mitochondrial preparation which was employed in panel A was also used to measure import and processing of the precursor to ornithine carbamyl transferase [13]; electrophoresis, however, was performed in a different gel. Lane a, 0 min incubation; lane b, 60 min incubation. OCT, ornithine carbamyl transferase; pOCT, precursor to OCT; 37K, a 37 kDa fragment of pOCT which is an artifact of the in vitro system [13].

to mitochondria was membrane-specific. When dog pancreas microsomes were incubated either co-translationally or post-translationally with reticulocyte lysate programmed with liver mRNA, OMM-68 did not subsequently sediment with the membranes (Fig. 6); the concentration of dog pancreas microsomes in these incubations was higher than the content of outer mitochondrial membrane present when intact mitochondria were employed for import (Fig. 5). Identical results were obtained using rat liver microsomes (data not shown). Interestingly, the 20 kDa polypeptide, representing a major product precipitated by anti-outer

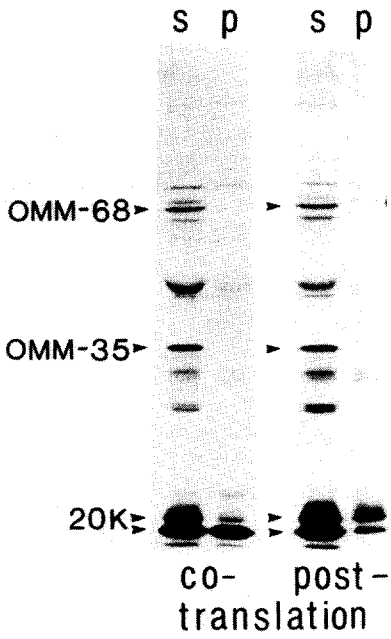

Fig. 6. OMM-68 does not bind to dog pancreas microsomes either co-translationally or post-translationally. In vitro translation in the reticulocyte cell-free system was performed for 60 min at 30°C in the presence or absence of dog pancreas microsomes, exactly as described in Ref. 26. The reaction mixture (50 μl) containing polypeptide products ($3.0 \cdot 10^6$ cpm) made in the presence of microsomes (320 μg protein/ml) was then maintained on ice. Products made in the absence of membranes (25 μl, $8 \cdot 10^6$ cpm) were post-translationally incubated with microsomes (320 μg protein/ml) for 60 min at 30°C. Microsomes from both reaction mixtures were then recovered by centrifugation in the Beckman airfuge operating for 10 min at 30 lb/in^2. Products in the supernatant (s) and pellet (p) were precipitated with anti-outer mitochondrial membrane antiserum, resolved on a 12% SDS-polyacrylamide gel, and fluorographed. The positions of OMM-68, OMM-35 and 20 kDa polypeptides are indicated. OMM-68 was identified in this experiment by co-electrophoresis with product imported by mitochondria (not shown). The position of 20 kDa polypeptide was identified following immunoprecipitation of translation products which had not been incubated with dog microsomes.

mitochondrial membrane antiserum, was co-translationally (but not post-translationally) inserted into dog microsomes. During incubation, a portion of the 20 kDa product was converted to 19 kDa by the microsomes, but this occurred post-translationally as well so that it presumably represented an artifact of the in vitro system. We are currently attempting to purify the 20 kDa polypeptide in order to unequivocally determine (by electron microscope immunocytochemistry, see Ref. 3) whether this product is indeed a bona-fide protein of the mitochondrial outer membrane, or a contaminant.

Discussion

To date, in vitro insertion of precursor proteins into the outer membrane of mitochondria has been demonstrated for a number of proteins in yeast and *Neurospora,* the best studied of which is the pore-forming protein, porin [5–7]. In the present communication, we extend such observations to include a 68 kDa polypeptide (OMM-68) from rat liver and show that, like porin, it is made exclusively by membrane-free polyribosomes; it can be imported post-translationally by mitochondria in vitro and is not further processed to a smaller (or larger) size.

A major question arising from the present work, however, is why binding of precursor OMM-68 to mitochondria can be achieved in vitro whereas binding of another polypeptide, OMM-35, cannot (Fig. 5 and Ref. 3). Preliminary evidence indicates that, although newly-bound OMM-68 is very tightly associated with mitochondria in vitro (less than 50% is removed following sonication in 0.1 M Na_2CO_3 (pH 11.5)), a large part of the molecule is exposed at the surface of the organelle, rendering it accessible to digestion by trypsin and chymotrypsin (unpublished data); in this respect, it shows characteristics similar to a 70 kDa outer mitochondrial membrane polypeptide in yeast [27]. OMM-35, on the other hand, is largely located at the inner surface of the outer membrane [3] and, therefore, the majority of its polypeptide chain must be transported across the lipid bilayer of the outer membrane. Still, failure to import OMM-35 by rat liver mitochondria is curious when these same mitochondria are capable of importing and processing the precursor to the matrix enzyme, ornithine carbamyl transferase (Fig. 5); successful import of the latter requires transport across the lipid bilayers of both outer and inner mitochondrial membranes. It is unlikely that essential cytosolic components are lacking for successful import of OMM-35 to take place, because the reticulocyte lysate, present during such incubations, provides

sufficient levels of import factors required by a number of other precursors [13,20,21]. One explanation may be that in vitro synthesized OMM-35, unlike OMM-68, assumes a tertiary conformation different from its native form in vivo. Alternatively, it may be that some damage is incurred to the mitochondrial surface during isolation of the organelle, e.g., breaking of cytoskeleton-mitochondrial interactions [22], thereby inactivating essential features of the outer membrane which are required for insertion of OMM-35. If so, however, such features are not required for import of OMM-68 nor of pre-ornithine carbamyl transferase.

Acknowledgments

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