Translocation and Insertion of Precursor Proteins into Isolated Outer Membranes of Mitochondria

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Abstract. Nuclear-encoded proteins destined for mitochondria must cross the outer or both outer and inner membranes to reach their final sub-mitochondrial locations. While the inner membrane can translocate preproteins by itself, it is not known whether the outer membrane also contains an endogenous protein translocation activity which can function independently of the inner membrane. To selectively study the protein transport into and across the outer membrane of Neurospora crassa mitochondria, outer membrane vesicles were isolated which were sealed, in a right-side-out orientation, and virtually free of inner membranes. The vesicles were functional in the insertion and assembly of various outer membrane proteins such as porin, MOM19, and MOM22. Like with intact mitochondria, import into isolated outer membranes was dependent on protease-sensitive surface receptors and led to correct folding and membrane integration. The vesicles were also capable of importing a peripheral component of the inner membrane, cytochrome c heme lyase (CCHL), in a receptor-dependent fashion. Thus, the protein translocation machinery of the outer mitochondrial membrane can function as an independent entity which recognizes, inserts, and translocates mitochondrial preproteins of the outer membrane and the intermembrane space. In contrast, proteins which have to be translocated into or across the inner membrane were only specifically bound to the vesicles, but not imported. This suggests that transport of such proteins involves the participation of components of the intermembrane space and/or the inner membrane, and that in these cases the outer membrane translocation machinery has to act in concert with that of the inner membrane.

Transport of proteins into mitochondria is a process of particular complexity, since many precursor proteins must be translocated across both the outer and inner mitochondrial membranes (Pfanner and Neupert, 1990; Glick and Schatz, 1991; Segui-Real et al., 1992). Protein components of the matrix and the inner membrane were found to pass the two membranes simultaneously at translocation contact sites (Schleyer and Neupert, 1985). Since polypeptide segments as short as 50 amino acid residues were found to be sufficient to span both membranes at these sites (Rassow et al., 1990), the translocation systems in the two membranes must be in close proximity. On the other hand, mitochondria in which the outer membrane was ruptured were able to import proteins directly across the inner membrane demonstrating the independence of the inner membrane translocation system (Hwang et al., 1989). Moreover, under certain conditions translocation intermediates en route from the outer to the inner membrane were observed to expose major segments in the intermembrane space (Hwang et al., 1991; Rassow and Pfanner, 1991; Jascur et al., 1992). This led to the suggestion that the two mitochondrial membranes contain separate translocation machineries which cooperate in a dynamic fashion during protein transfer into the matrix (Glick et al., 1991; Pfanner et al., 1992; Segui-Real et al., 1993).

The molecular nature of the two translocation machineries is only partially understood. That of the outer membrane has been studied in some detail. A multi-subunit complex from Neurospora crassa mitochondria has been characterized (Kiebler et al., 1990) containing two components exposed to the cytosol, MOM19 and MOM72, which function as receptors (Söllner et al., 1989, 1990). Several other membrane components including MOM38, MOM7, and MOM8 were found in contact with these receptors and also in contact with precursors arrested in transit (Söllner et al., 1992). These three components are believed to form at least part of the so-called general insertion pore (GIP); Pfaller et al.,

1. Abbreviations used in this paper: AAC, ADP/ATP carrier; CCHL, cytochrome c heme lyase; GIP, general insertion pore.
Materials and Methods

A complex of comparable protein composition has been identified in yeast mitochondria (Moczko et al., 1992; Baker et al., 1989; Steger et al., 1990; Hines et al., 1990). Relatively little is known about the translocation machinery of the inner membrane. Recently, two components have been described which may constitute part of it (Maarse et al., 1992; Scherer et al., 1992), but their function remains elusive.

The question arises as to whether the two machineries can act independently in the translocation and insertion of polypeptide chains. With the inner membrane this seems to be indeed the case, as import into mitoplasts was observed (Hwang et al., 1989). For the outer membrane it may be expected that it is active on its own, but direct evidence for that is missing, since the activity of this system has always been analyzed in intact mitochondria, i.e., in context with the inner membrane (Mihara et al., 1982; Freitag et al., 1982; Ono and Tuboi, 1987; Kleene et al., 1987; Schneider et al., 1991; Shore et al., 1992). So far it is not known whether the outer membrane machinery is only active when it functions in conjunction with the inner membrane machinery, e.g., at translocation contact sites, or whether the outer membrane is translocation-competent by itself just like the membranes of, e.g., peroxisomes or the ER. We therefore developed a procedure to purify outer membranes and analyzed the resulting vesicles for their ability to translocate precursor proteins. In particular it was important to know whether the isolated membranes would insert polypeptide chains into the membrane and whether they would be competent in translocating precursors whose final location is beyond the outer membrane.

We report here the preparation of mitochondrial outer membranes which are virtually free of inner membranes and which form tightly sealed vesicles in a right-side-out orientation. These vesicles are capable to insert and assemble various outer membrane proteins. In addition, they are competent in translocating cytochrome c heme lyase (CCHL; Lill et al., 1992), a peripheral protein of the inner membrane, across the outer membrane. They do, however, not translocate preproteins which are destined for the mitochondrial matrix and inner membrane, most likely because further components of the intermembrane space and the inner membrane are missing. These data demonstrate that the outer membrane contains an independent translocation activity, whose competence is limited to a subset of precursor proteins.

Materials and Methods

Biochemical Procedures

Antisera were raised and IgG prepared according to Stöllner et al. (1989). Western blotting was performed as described (Köhse-Anderson, 1984). Proteins blotted onto nitrocellulose were detected by chemiluminescence (ECL system, Amersham Corp., Arlington Heights, IL) according to the instructions of the supplier. For quantification of the data the time it was essential to evaluate signals in the linear range of detection. Therefore, each blot contained a titration of mitochondria and outer membranes, and a series of exposure times were evaluated. The resulting films were scanned on a laser densitometer (Pfüßer et al., 1988). Transcription and translation reactions were performed according to Stöllner et al. (1991) using [35S]methionine as a radioactive label. Immunoprecipitation by protein A-Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) was done as described by Zimmermann and Neupert (1980). Protein concentrations were determined by the Coomassie dye binding assay (Biorad Labs., Hercules, CA). SDS-PAGE and fluorography of the resulting gels were done as described by Nicholson et al. (1987).

Isolation of Mitochondria

N. crassa (wild-type strain 74A) was grown for 16 h under bright illumination, harvested by filtration, and washed as described (Sebah et al., 1979). Hyphae (500 g wet wt) were mixed with 750 g quartz sand and 2.01 SEM buffer (250 mM sucrose, 1 mM EDTA, 1 mM MOPS-KOH, pH 7.2, and 1 mM PMSF), and homogenized for 5 s in a Waring blender. Cells were gently broken by rapid passage through a grind mill. From the homogenate, mitochondria were isolated by differential centrifugation (Pfüßer and Neupert, 1985).

Purification of Outer Membrane Vesicles

The final mitochondrial pellet was resuspended at a protein concentration of 4-8 mg/ml in a hypotonic buffer (5 mM potassium phosphate, pH 7.2, 5 mM EDTA, and 1 mM PMSF) to allow swelling on ice for 10 min. Separation of the outer membrane from the remaining mitoplasts was achieved by 20 strokes in a glass-Teflon homogenizer. 15 ml homogenate was loaded onto a sucrose gradient consisting of 9 ml of 0.25 M sucrose (Roth, Karlsruhe) and 12 ml of 0.9-M sucrose steps in EM buffer (2.5 mM EDTA, 10 mM MOPS-KOH, pH 7.2, and 1 mM PMSF), after ultracentrifugation for 1 h in a SW28 rotor (Beckman Instruments, Inc., Fullerton, CA) at 140,000 g, the outer membrane fraction was harvested from the 0.25 and 0.9 M sucrose interface. The sample was brought to a concentration of 0.9 M sucrose by addition of 2 M sucrose. 14 ml of this solution was loaded on the bottom of a linear sucrose gradient consisting of 3 ml EM buffer and 20 ml of 0.72 M sucrose in EM buffer. The gradient was devised such that inner membranes due to their higher density do not float up the gradient. After isopycnic ultracentrifugation for 16 h in a SW28 rotor at 140,000 g the purified outer membranes were recovered from the interface of the 0 and 0.72 M sucrose layers. The sample was diluted fivefold in EM buffer (2.5 mM EDTA, 10 mM MOPS-KOH, pH 7.2) and concentrated by spinning for 3 h at 141,000 g in a SW28 rotor. The pellet, resuspended in EM buffer containing 15% sucrose at a protein concentration of 0.2-0.5 mg/ml, frozen in small aliquots in liquid nitrogen, and stored at -80°C until use.

Protein Import into Mitochondria and into Isolated Outer Membrane Vesicles

A typical protein import reaction consisted of either 25 µg freshly isolated mitochondria or 2 µg purified outer membrane vesicles and 5-10 µl rabbit reticulocyte lysate containing radioactively labeled precursor proteins in a total volume of 100 µl import buffer (10 mM MOPS, pH 7.2, 80 mM KCl, 5 mM MgCl2, 250 mM sucrose, and 3% fatty acid free BSA). Import was performed for 10 min at 25°C. Samples were chilled on ice and treated with 40 µl/ml protease K for 15 min. After dilution of the samples with 1 ml import buffer, mitochondria or 2 µg purified outer membrane vesicles were loaded onto a sucrose gradient consisting of 9 ml of 0.25 M sucrose (Roth, Karlsruhe) and 12 ml of 0.9-M sucrose steps in EM buffer (12.5 mM EDTA, 10 mM MOPS-KOH, pH 7.2, 0.72 M sucrose interface), harvested by filtration, and washed as described (Sebah et al., 1979).

Extraction of Vesicles and Mitochondria

After the import reactions, the samples were treated with protease (either 40 µg/ml proteinase K or 30 µg/ml trypsin) for 15 min at 0°C. Digestion was terminated by adding a 30-fold excess (wt/wt) of soybean trypsin inhibitor. Untreated vesicles went through the same procedure but received trypsin and its inhibitor simultaneously. To investigate the import of MOM19 the procedure was slightly modified. Parallel incubations of MOM19 precursor were performed together with either 50 µg mitochondria, 2 µg outer membrane vesicles, or without any addition (lysate control). After import (7 min, 25°C) the samples were treated with elastase at the indicated concentrations for 15 min at 25°C. Digestion was stopped by addition of 1 ml SEMP buffer, mitochondria (12.5 mM EDTA, 10 mM MOPS-KOH, pH 7.2, 0.72 M sucrose), 1 h, 226,000 g) were reisolated by centrifugation. Pellet material was subjected to SDS-PAGE, radioactive proteins were visualized by fluorography, and the resulting bands were quantified by densitometry. Pretreatment of the vesicles with 25 µg/ml trypsin was performed for 20 min at 0°C. Digestion was terminated by adding a 30-fold excess (wt/wt) of soybean trypsin inhibitor. Untreated vesicles went through the same procedure but received trypsin and its inhibitor simultaneously. To investigate the import of MOM19 the procedure was slightly modified. Parallel incubations of MOM19 precursor were performed together with either 50 µg mitochondria, 2 µg outer membrane vesicles, or without any addition (lysate control). After import (7 min, 25°C) the samples were treated with elastase at the indicated concentrations for 15 min at 25°C. Digestion was stopped by addition of 1 ml SEMP buffer, mitochondria, 1 h, 226,000 g).}

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Results

Purification of Outer Membrane Vesicles

To obtain outer membrane vesicles which are tightly sealed and in a right-side-out orientation, we optimized a previously published procedure (Söllner et al., 1989). Intact mitochondria freshly isolated from N. crassa hyphae were swollen in a hypotonic buffer. The outer membrane was separated from the remaining mitoplasts by budding the suspension in a glass-Teflon homogenizer. Purification of the outer membrane was achieved in two steps by sedimentation and flotation centrifugation in sucrose step gradients. The samples were resolated by centrifugation and analyzed by SDS-PAGE and fluorography. For sonication experiments, mitochondria or vesicles were resolated and resuspended in 600 μl SEM buffer. 10 μg/ml protease K was added and the sample was sonicated for 1 min at 0°C (Braunson, Sollifer 250 with a microtip, intensity 4, 30% duty cycle). Protease treatment was stopped after 14 min at 0°C by the addition of 1 mM PMSF. Finally, proteins were precipitated with TCA and analyzed by SDS-PAGE. For digitonin fractionation with SEM buffer containing 20 μg/ml proteinase K. After 15 min at 0°C, digestion was stopped by the addition of 1 mM PMSF. The samples were resolated by centrifugation and analyzed by SDS-PAGE and fluorography. For sonication experiments, mitochondria or vesicles were resolated and resuspended in 600 μl SEM buffer. 10 μg/ml protease K was added and the sample was sonicated for 1 min at 0°C (Braunson, Sollifer 250 with a microtip, intensity 4, 30% duty cycle). Protease treatment was stopped after 14 min at 0°C by the addition of 1 mM PMSF. Finally, proteins were precipitated with TCA and analyzed by SDS-PAGE, fluorography, and densitometry of the resulting x-ray films. For alkaline extraction, vesicles and mitochondria were pelleted and resuspended in SEM buffer. Samples were diluted 20-fold with 0.1 M Na2CO3 containing 1 mM PMSF and incubated for 30 min on ice (Fujiki et al., 1982). Insoluble material was spun down for 1 h at 16,000 g and subjected to SDS-PAGE and blotting onto nitrocellulose. Internal and endogenous proteins were visualized by autoradiography and immunostaining, respectively, of the same blot. Extractability of free precursor proteins was tested by diluting 1 μl of these proteins in reticulocyte lysate with 10 μl SEM buffer before alkaline treatment.

Biochemical Characterization of the Purified Outer Membrane Vesicles

The functionality of the isolated outer membrane vesicles for protein import studies critically depends on the integrity of the protease-sensitive surface receptors MOM19 and MOM72 which are exceptionally prone to degradation during the isolation procedure. The relative abundance of these components in mitochondria and in vesicles was assessed by comparing their amounts to those of porin which is highly insensitive to proteolytic attack. For MOM19 no change was observed (Fig. 1 C). The same result was obtained for two other components of the outer membrane import complex, MOM22 and MOM38. In contrast, the relative amount of MOM72 was about twofold lower in the outer membrane as compared to mitochondria. This decrease was not due to partial proteolysis of MOM72 during the isolation procedure. Rather, it is explained by the fact that MOM72 is enriched in contact sites (Söllner et al., 1990; Hines et al., 1990) and thus is partially depleted in pure outer membranes. Correspondingly, MOM72 was enriched over porin and MOM38 in vesicles of intermediate density (see Fig. 1 A, bottom fractions) which have been reported to contain contact sites (Pon et al., 1989). Taken together, these data demonstrate the physical integrity of the individual components of the outer membrane receptor complex.

Other important prerequisites for the suitability of the vesicles for protein import studies are their sealed nature and a right-side-out orientation. This was tested using the accessibility of various proteins to proteolytic attack. Upon treatment with increasing amounts of elastase MOM38 gave rise to a 26-kD fragment in both outer membrane vesicles and in intact mitochondria (marked as 38* in Fig. 2 A, panels I and II; Kiebler, M., unpublished observations). When protease digestion was preceded by treatment with limited amounts of digitonin to selectively open the outer membrane (Hartl et al., 1986), MOM38 became completely degraded even at low concentrations of protease (Fig. 2 A, panels III and IV). The results suggest that the majority of the vesicles was tightly sealed. This was also evident from electron micrographs of the isolated outer membranes which in addition demonstrated the vesicles to be homogeneous, unilamellar, and uniform in size (0.5 μm diameter; data not shown). The surface receptors MOM19 and MOM72 are highly sensitive to proteolytic attack (Söllner et al., 1989, 1990). Both in outer membrane vesicles and in intact mitochondria they were efficiently degraded to their characteristic fragments (Fig. 2 B and see Fig. 4, panels IV and V). Therefore, at least 95% of the vesicles expose the right side to the solvent.

The Protein Translocation Activity of Purified Outer Membrane Vesicles

Protein import into the vesicles was first tested for various outer membrane proteins. Precursors of such proteins do not contain NH2-terminal, cleavable signal sequences. After in vitro synthesis in reticulocyte lysate the precursor proteins were incubated with purified vesicles under conditions similar to those established for protein import into intact mitochondria.
Figure 1. Purification of outer membrane vesicles. (A) Flotation sucrose gradient centrifugation. The outer membrane fraction obtained by sedimentation sucrose gradient centrifugation was adjusted to 0.9 M sucrose and loaded on the bottom of a sucrose gradient composed of 0 and 0.72 M sucrose steps. After centrifugation at 141,000 g for 16 h, eleven fractions were collected and precipitated with TCA. Proteins were analyzed by SDS-PAGE, blotting onto nitrocellulose, and immunodecoration of the indicated outer membrane proteins and of AAC. For comparison the input fraction (I) and the indicated amounts of intact mitochondria (Mit) were treated in the same manner. (B) Protein pattern of isolated outer membrane vesicles. Mitochondria and outer membrane vesicles (OMV) from the sedimentation (Sed.) and the flotation (Flot.) centrifugation were analyzed by SDS-PAGE and silver-staining. Proteins of the receptor/GIP complex and porin were assigned according to Western blots run in parallel. The assignment of MOM7 and MOM8 was according to Moczko et al. (1992). Molecular mass markers are given on the left side of the figure. (C) Abundance of components of the outer membrane receptor/GIP complex in mitochondria and in vesicles. Mitochondria and vesicles were subjected to SDS-PAGE and proteins analyzed by ECL-Western-blotting. Quantitation was achieved by laser densitometry of the resulting films. The amounts of the indicated proteins in mitochondria and vesicles are given relative to the amount of porin. The ratio in intact mitochondria was set to 1.
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Table 1. Purification of Outer Membranes from Isolated *N. crassa* Mitochondria

| Method                  | Protein (mg) | Yield (%) |
|-------------------------|--------------|-----------|
| Pelleting centrifugation| 200          | 100       |
| Flotation centrifugation| 1050         | 4.2       |

The purification started from 200 mg of isolated mitochondria which were subjected to a swelling and douncing treatment. Outer membranes were purified from the homogenate by two successive sucrose step gradients (pelleting and floating). Details of the isolation procedure are outlined in Materials and Methods. From the purification steps, aliquots were withdrawn, TCA-precipitated, and analyzed by SDS-PAGE and Western blotting. The films emerging from the chemiluminescent detection of proteins (ECL, Amersham Corp.) were scanned on a laser densitometer. Each gel contained a titration of mitochondria and vesicles to guarantee that signals from the linear range of detection were chosen for quantitation. The calculation of the yield is based on the finding that the outer membrane comprises ~6% of total mitochondrial protein which was estimated from the enrichment of MOM38; the overall input of outer membrane (12 mg) was set to 100%. To assess the purification, MOM38 was chosen as an outer membrane marker protein, whereas AAC was used as an indicator for the inner membrane. Both MOM38 enrichment and AAC depletion are given relative to mitochondrial protein. The enrichment of MOM38 over AAC (MOM38/AAC) is obtained as the product of the enrichment and depletion, respectively, of the two markers. a.u., arbitrary units.

A similar result was obtained for another outer membrane protein, MOM22. Its import was assessed by the generation of characteristic proteolytic fragments found only after insertion and assembly of the precursor into the outer membrane (Kiebler, M., unpublished results). A fragment of 12 kD (marked as MOM22* in Fig. 3 B) was observed after incubation of the precursor with the vesicles, essentially in the same manner as with intact mitochondria (Fig. 3 B, lower panel). Pretreatment with trypsin prevented the formation of the fragment which is consistent with the notion that MOM22 requires the participation of surface receptors for its import into mitochondria (Kiebler, M., unpublished observations). Integration of imported MOM22 and porin into the lipid bilayer was verified by their resistance to alkaline extraction (Fujiki et al., 1982). Both imported MOM22 and porin were almost fully retained in the membrane after alkaline treatment (Fig. 3 C), whereas the precursor form was quantitatively extracted from reticulocyte lysate (cf. Sakaguchi et al., 1992). This behavior is identical to that of endogenous MOM22 and porin. Thus, the data indicate that both MOM22 and porin had become fully inserted into the outer membrane as expected for integral membrane proteins.

In contrast to most other precursor proteins, MOM19 is imported into mitochondria without the help of protease-sensitive receptors (Schneider et al., 1991). Specific import can be studied through the formation of characteristic proteolytic fragments which are not generated from unassembled MOM19. Outer membrane vesicles were competent for insertion and assembly of MOM19, since identical fragments were obtained as after import into intact mitochondria (Fig. 4, cf. panels I and II). These fragments did not arise from MOM19 precursor in reticulocyte lysate (Fig. 4, panel III). Fragmentation of endogenous MOM19 in both vesicles and intact mitochondria gave rise to an identical polypeptide pattern (Fig. 4, panels IV and V). Accordingly, MOM19 is inserted and folded correctly during in vitro import into the isolated outer membrane.

CCHL, a peripheral protein of the inner membrane facing the intermembrane space, is imported into mitochondria without the need of a membrane potential across the inner membrane (Lill et al., 1992). It does not contain a cleavable, NH₂-terminal signal sequence (Drygas et al., 1989). After incubation of in vitro translated CCHL with outer membrane vesicles, a fraction of the protein became protease-protected (Fig. 5 A) indicating that CCHL was transported across the outer membrane. The efficiency of CCHL translocation into vesicles was only slightly lower than that of import into mitochondria (data not shown). Import was fully dependent on the presence of proteinaceous surface components as shown by its sensitivity to pretreatment of the vesicles with trypsin.

To confirm that CCHL had been transported into the lumen of the vesicles, they were opened by treatment with digitonin or by brief sonication to make imported CCHL accessible to externally added protease. In both vesicles and intact mitochondria, imported CCHL became protease-sensitive at the same concentrations of digitonin (Fig. 5 B). In contrast, imported porin remained largely resistant to proteolytic attack independently of the digitonin concentration. Likewise, brief sonication in the presence of protease resulted in complete degradation of imported CCHL (Fig. 5 C), whereas the majority of imported porin was not digested. These data demonstrate that CCHL became transported across the membrane of the vesicles and rule out the possibility that components of the inner membrane participate in the translocation of CCHL.

Dependence on the function of the surface receptors MOM19 and MOM22 represents an important criterion to demonstrate that protein import into isolated outer membranes occurs along the authentic pathway. Preincubation of the vesicles with IgG against MOM19 strongly reduced the import of CCHL and porin, while IgG against porin, MOM38, MOM72, or IgG derived from preimmune serum
Figure 2. Outer membrane vesicles are sealed and in a right-side-out orientation. (A) Isolated outer membrane vesicles (OMV; 5 μg) or mitochondria (Mit; 50 μg) were suspended in SEM buffer. Where indicated, 0.12% (wt/vol) digitonin (Dig.) was added followed by incubation for 2 min on ice. After 20-fold dilution with SEM, the samples were incubated for 15 min at 25° C with the indicated concentrations of elastase. 1 mM PMSF was added and proteins were precipitated with TCA, and analyzed by SDS-PAGE and immunostaining of MOM38 (38) and porin. 38* indicates a 26-kD fragment of MOM38. (B) Vesicles (5 μg) or mitochondria (50 μg) suspended in SEM were treated with 1 μg/ml proteinase K for 15 min on ice and analyzed as in A by immunostaining of MOM 72. MOM72*: 60-kD fragment of MOM72.

Discussion

As reported here isolated outer membrane vesicles of N.
Figure 3. Insertion and assembly of porin and MOM22 into outer membrane vesicles. Precursors of (A) porin and (B) MOM22 were imported into isolated outer membranes (OMV) with or without preceding trypsin treatment of the vesicles. After digestion of nonimported proteins with proteinase K, the vesicles were reisolated and subjected to SDS-PAGE and fluorography. In B an import reaction with mitochondria (Mit) was included for comparison. The characteristic 12-kD fragment of MOM22 also occurring from the endogenous protein is marked as MOM22*. (C) Resistance of porin and MOM22 against alkaline extraction after import into vesicles or mitochondria. To control for the extractability of the free preproteins reticulocyte lysates containing porin and MOM22 precursors were treated in the same way. In the case of MOM22, the 12-kD fragment was used for analysis. The data are given as the percentage of the indicated proteins remaining in the pellet after alkaline extraction.

Crassa mitochondria are competent to translocate precursor proteins. Integral components of the outer membrane like MOM19, MOM22, and porin become inserted and assembled, and CCHL, a peripheral component of the inner membrane, is translocated into these vesicles. The import reactions fulfill criteria which are identical to those of import into intact mitochondria such as: (a) dependence on receptors, in particular on MOM19 and MOM72; (b) requirement for ATP in the case of some, but not all precursors; (c) integration into the membrane; and (d) folding of precursors into their native conformations as a result of translocation. These arguments demonstrate that in vitro protein import into isolated outer membranes occurs along authentic pathways and leads to products which are indistinguishable from the endogenous components. Thus, the outer mitochondrial membrane contains a translocation machinery which can function on its own, just like other protein translocation systems of eukaryotic cells found in, e.g., the ER, microbodies, and the inner mitochondrial membrane. An interesting, open question is whether components of the intermembrane space play a role in the transport reaction. Such components could reversibly associate with the translocation machinery at the inner face of the outer membrane, and, by interacting with preproteins in transit, they could participate in completion of translocation.

With most proteins targeted to mitochondria, in particular to the inner membrane and to the matrix space, precursors also have to use the translocation machinery of the inner membrane. It has been shown that this can happen by two sequential, independent steps (Segui-Real et al., 1993), but normally the two translocation machineries act in a highly concerted fashion in terms of time and space. We have previously suggested that the outer and inner membrane machineries cooperate in a dynamic manner and, during translocation of preproteins across the inner membrane, form the translocation contact sites (Pfanner et al., 1992). The data presented here support a model in which matrix proteins first become inserted into the receptor/GIP complex, and then reach the intermembrane space with their amino termini to make contact with components of the inner membrane machinery, and thereby initiate the second translocation event triggered by the membrane potential. Most interestingly, the
Figure 4. Insertion and assembly of MOM19 into outer membrane vesicles. MOM19 precursor was incubated with outer membrane vesicles (OMV), mitochondria (Mit), or import buffer only (Lysate control). After the import reaction the samples were treated with the indicated amounts of elastase. Vesicles and mitochondria were reisolated by centrifugation, whereas MOM19 from the lysate control was recovered by immunoprecipitation with anti-MOM19 antibodies. The proteins were subjected to SDS-PAGE and blotted onto nitrocellulose. Imported material was analyzed by autoradiography of the blot; endogenous MOM19 was visualized by immunodecoration of the same blot. The position of full length MOM19 is indicated by arrows.

Figure 5. Translocation of CCHL into the lumen of outer membrane vesicles. (A) Import of radioactively labeled CCHL precursor into the vesicles and pretreatment with trypsin were performed as described for porin (see Fig. 3 A). (B) Digitonin fractionation of imported CCHL. Import into outer membrane vesicles (OMV) or mitochondria (Mit) was performed as described in A. After trypsin treatment, digitonin at the indicated concentrations was added. Incubation for 2 min on ice was stopped by 20-fold dilution with SEM buffer containing proteinase K. After 15 min proteolysis was halted, the samples were reisolated, and analyzed by SDS-PAGE and fluorography. In case of the vesicles, porin was coimported to control for proper reisolation after the detergent treatment. (C) Protease accessibility of imported CCHL after sonication. After import of CCHL and porin into vesicles or mitochondria (see above), the samples were treated with proteinase K, reisolated, re-suspended in SEM, and divided into two aliquots. Proteinase K (10 μg/ml) was added to both aliquots. One was kept without further treatment while the other one was sonicated on ice for 1 min. After 15 min on ice, proteins were precipitated with TCA and analyzed by SDS-PAGE, fluorography, and densitometry. In B and C protease-resistant imported protein, without further treatment, was used as the 100% standard.
outer membrane vesicles studied in this report were incapable of translocating matrix and inner membrane proteins into their lumen. Apparently, the outer membrane machinery despite being competent to initiate translocation by specifically binding such proteins, cannot complete their transfer across the membrane. The same situation obviously holds true for import into intact mitochondria, since normally matrix precursors do not accumulate in the intermembrane space, even if translocation across the inner membrane is blocked by depletion of the membrane potential. Thus, in these cases transport across the outer membrane is apparently coupled to ongoing passage across the inner membrane.

Another important question raised by our results regards the driving force for protein insertion into and translocation across the outer membrane. In the case of integral proteins of the outer membrane, the insertion of hydrophobic segments into the lipid bilayer and assembly into oligomeric complexes may constitute a major driving force for leaving the putative translocation channel. For CCHL different

**Figure 6.** Import into isolated outer membrane vesicles requires the function of surface receptors. Outer membrane vesicles (1 µg per sample) were incubated for 30 min at 0°C with different amounts of IgGs in import buffer (total volume 110 µl). The IgGs were specific for MOM19, MOM72, MOM38, and porin, or were derived from preimmune serum (PIS). Import of radioactively labeled precursors of (A) CCHL, (B) porin, and (C) MOM22 was performed for 7 min at 25°C. The samples were chilled, treated with proteinase K (40 µg/ml), reisolated, and analyzed by SDS-PAGE, fluorography, and densitometry. The amount of imported material in the absence of antibodies was set to 100%. For MOM22, the characteristic 12-kD fragment (see Fig. 3 B) was used for the analysis.

**Figure 7.** ATP requirement for import of porin and CCHL into outer membrane vesicles. Outer membrane vesicles (OMV), mitochondria (Mit), and reticulocyte lysate were pretreated with or without 15 U/ml apyrase (Sigma Chem., St. Louis, MO) for 15 min at 25°C. Import of porin and CCHL precursors was performed and analyzed as in Figs. 3 and 5. Total lysate content was 1% (vol/vol) and temperature was kept at 5°C to make sure that the reaction was performed in the linear range of translocation. The amount of protein imported without apyrase pretreatment was set to 100%.
mechanisms must operate to support its transport. From the data presented here and from earlier observations (Lill et al., 1992), a membrane potential, cytosolic ATP, or binding to a putative “CCHL receptor” at the surface of the inner membrane, do not seem to be required for driving translocation. On the other hand, folding of CCHL in the intermembrane space could represent such an energetic source. Most likely, such a reaction involves the participation of folding mediators, as reported for the folding of proteins in the mitochondrial matrix (Ostermann et al., 1989; Kang et al., 1990; Manning-Krieg et al., 1991). Although protein folding has been discussed repeatedly as an energetic contribution towards completion of membrane passage, direct proof for the existence of such a pathway is still missing. The availability of an in vitro system to specifically study outer membrane translocation may now provide an experimental tool to directly address this question.

What is the driving force for moving matrix precursors through the outer membrane machinery? In line with the model discussed above, the major energetic contribution probably comes from the passage of the preproteins across the inner specifically bound to isolated outer membrane vesicles, but are not translocated. Trypsin pretreatment of the vesicles and import reactions using the precursors of the indicated proteins were performed and samples analyzed as in Fig. 3. The amount of reticulocyte lysate was adjusted to 5% (vol/vol), and the ATP concentration was 2 mM. PEP, β-subunit of matrix processing peptidase; Cyt c1, cytochrome c1; AAC, ADP/ATP carrier.

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