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[1]; Pfaller et al.,

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

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1988). 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 Söllner et al. (1989). Western blotting was performed as described (Kyhse-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 quantitation of the data 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 (Pfaller et al., 1988). Transcription and translation reactions were performed according to Söllner et al. (1991) using [^{35}S]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 (Sebald et al., 1979). Hyphae (500 g wet wt) were mixed with 750 g quartz sand and 2.0 l SEMP buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH7.2, 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 (Pfanner 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 EMP 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 Instrs., Inc., Fullerton, CA) at 141,000 *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 flotation sucrose gradient consisting of 3 ml EMP buffer and 20 ml of 0.72 M sucrose in EMP 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 141,000 *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 was 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 $MgCl_2$, 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 μg/ml proteinase K for 15 min. After dilution of the samples with 1 ml SEMP buffer, mitochondria (12 min, 17,400 *g*), and vesicles (1 h, 226,000 *g*) were reisolated by centrifugation. Pelleted 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 and incubation for 5 min at 25°C. Vesicles and mitochondria were reisolated by centrifugation as detailed above. In the lysate control, MOM19 protein and derived fragments were immunoprecipitated with anti-MOM19 antibodies. The samples were subjected to SDS-PAGE and blotting onto nitrocellulose. The fragmentation of imported and endogenous MOM19 was analyzed by autoradiography and immunostaining, respectively, of the same blot.

Digitonin Fractionation, Sonication, and Alkaline 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. Protease digestion was stopped by addition of 2 mM PMSF or a 30-fold excess (wt/wt) of soybean trypsin inhibitor, respectively. For digitonin fractionations, digitonin was added from a 1% (wt/vol) stock solution in SEM buffer, and the samples were incubated for 2 min at 0°C, followed by 20-fold dilution 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 reisolated by centrifugation and analyzed by SDS-PAGE and fluorography. For sonication experiments, mitochondria or vesicles were reisolated and resuspended in 600 μl SEM buffer. 10 μg/ml proteinase K was added and the sample was sonicated for 1 min at 0°C (Branson Sonifier 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 Na_2CO_3 containing 1 mM PMSF and incubated for 30 min on ice (Fujiki et al., 1982). Insoluble material was spun down for 1 h at 166,000 *g* and subjected to SDS-PAGE and blotting onto nitrocellulose. Imported and endogenous protein was 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.

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 douncing the suspension in a glass-Teflon homogenizer. Purification of the outer membrane was achieved in two steps by sedimentation and flotation centrifugations in sucrose step gradients. The samples of the latter purification step were fractionated and analyzed by immunoblotting for various marker proteins of the outer and inner membranes (Fig. 1 *A*). As seen from the behavior of the ADP/ATP carrier (AAC), an inner membrane marker protein, the inner membrane contamination remaining from the first purification step stayed at the bottom of the second (flotation) gradient, whereas outer membranes were found to be highly enriched at the top of the gradient. The significant improvement in purity obtained by flotation centrifugation also becomes evident from the protein pattern of the two purification steps (Fig. 1 *B*). A number of protein bands which are still present after the sedimentation step are absent in the final preparation. The majority of the proteins present in the outer membrane fraction is represented by porin and the known components of the protein import apparatus (i.e., MOM7, MOM8, MOM19, MOM22, MOM38, and MOM72; Söllner et al., 1992) demonstrating that material transport is a prominent function of the mitochondrial outer membrane.

The purification of outer relative to inner membranes was quantified using MOM38 and AAC as marker proteins (Table I). In comparison to total mitochondrial protein, MOM38 was enriched fivefold after the first centrifugation step and 17-fold after the second step. For AAC a depletion by factors of 6 and 60, respectively, was obtained. Thus, the total enrichment of outer over inner membranes was 31-fold and 1,050-fold, respectively, after the two centrifugation steps. The overall yield of outer membranes was 4% taking into account that the outer membrane constitutes 6% of total mitochondrial protein (Table I). This rather low yield was probably due to the very gentle shearing of the swollen mitochondria during the homogenization procedure which only released a small fraction of the outer membrane.

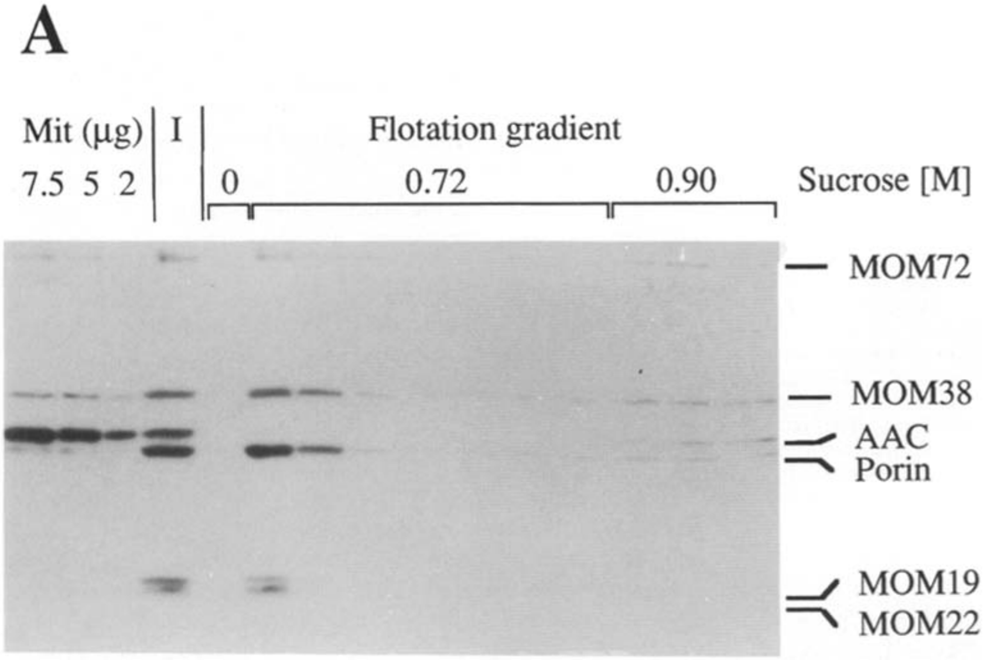
Biochemical Characterization of the Purified Outer Membrane Vesicles

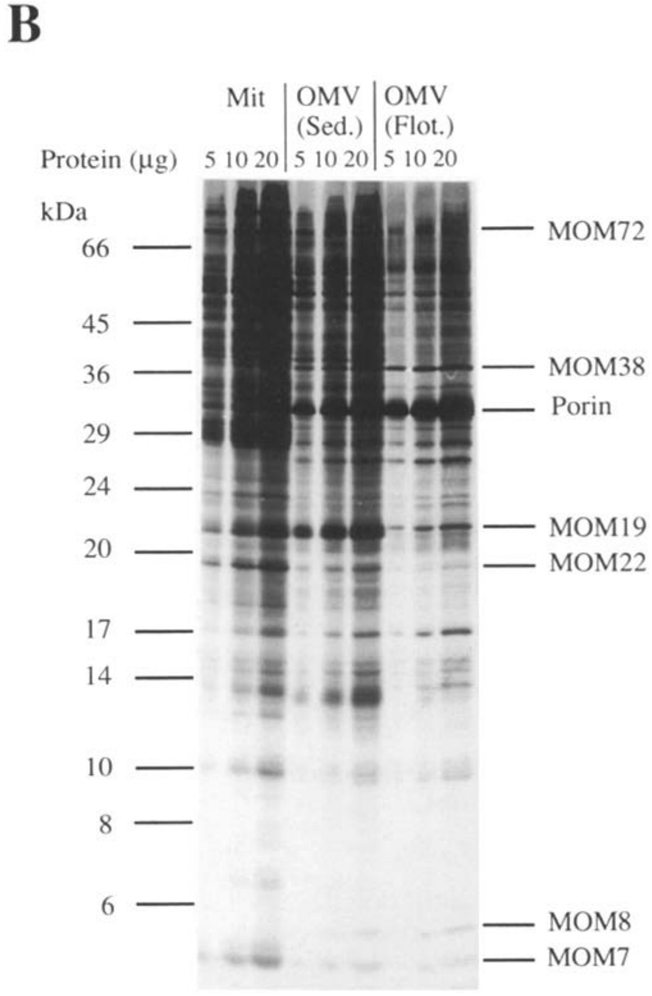
The functionality of the isolated outer membrane vesicles for protein import studies critically depends on the integrity of the protease-sensitive surface receptors MOM19 and MOM72 which are exceptionally prone to degradation during the isolation procedure. The relative abundance of these components 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 NH_2-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 mitochon-





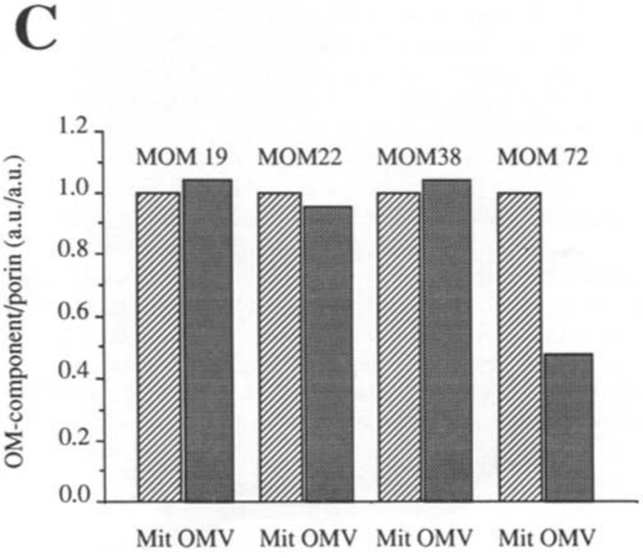


Figure 1. Purification of outer membrane vesicles. (*A*) Flotation sucrose gradient centrifugation. The outer membrane fraction obtained by sedimentation sucrose gradient centrifugation was adjusted to 0.9 M sucrose and loaded on the bottom of a sucrose gradient composed of 0 and 0.72 M sucrose steps. 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.

Table I. Purification of Outer Membranes from Isolated N. crassa Mitochondria

	MOM38/protein	AAC/protein	MOM38 enrichment	AAC depletion	MOM38/AAC	Protein	Yield
	(a.u./μg)					(mg)	(%)
Mitochondria	0.02	1.5	1	1	=1	200	=100
Pelleting centrifugation	0.10	0.24	5.0	6.3	31.5	2.0	16.7
Flotation centrifugation	0.35	<0.025	17.5	>60	>1050	0.5	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.

dria. After treatment with protease to digest non-imported precursors, the samples were analyzed by SDS-PAGE and fluorography (Fig. 3). In case of porin, a considerable fraction of the added precursor became protected against proteolytic attack (Fig. 3 *A*) indicating correct insertion and assembly to the protease-resistant conformation (Kleene et al., 1987). Import of porin was completely abolished by pretreating the vesicles with protease. Apparently, proteinaceous surface components are required for translocation in the same way as in intact mitochondria (Pfaller et al., 1988). Based on import experiments with comparable amounts of outer membranes, the efficiencies of porin insertion into vesicles and mitochondria were similar demonstrating that the translocation machinery was fully active in the isolated outer membrane (data not shown).

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., and P. Keil, 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, NH_2-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 MOM72 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

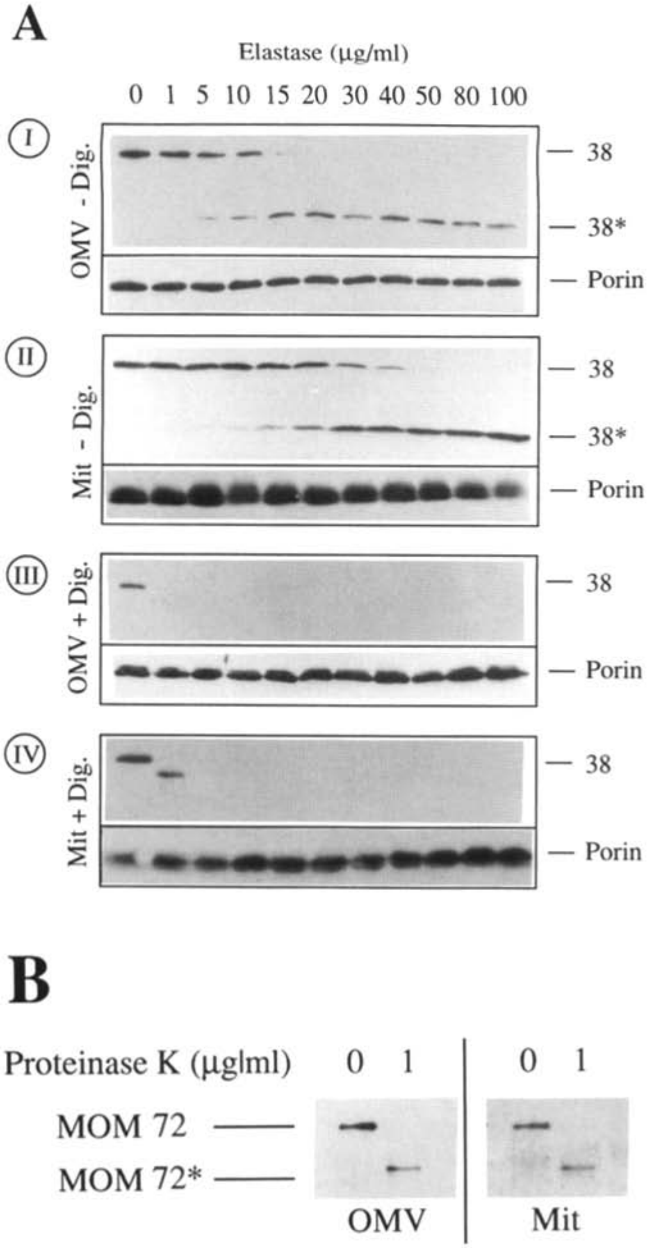


Figure 2. Outer membrane vesicles are sealed and in a right-side-out orientation. (*A*) Isolated outer membrane vesicles (*OMV*; 5 μg) or mitochondria (*Mit*; 50 μg) were suspended in SEM buffer. Where indicated, 0.12% (wt/vol) digitonin (*Dig.*) was added followed by incubation for 2 min on ice. After 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.

had no effect (Fig. 6, *A* and *B*). Import of MOM22 into mitochondria requires the function of both MOM19 and MOM72 (Keil, P., and M. Kiebler, unpublished results). Consistent with this observation, a substantial reduction of MOM22 import was observed, when the vesicles were pretreated with IgG against either MOM19 or MOM72 (Fig. 6 *C*). Thus, as in intact mitochondria, import of CCHL and porin depends on MOM19, whereas insertion and assembly of MOM22 requires the function of both MOM19 and MOM72.

A further characteristic of mitochondrial protein import is its dependence on cytosolic ATP, at least in part caused by the interaction of precursors with cytosolic Hsp70 (Deshaies et al., 1988). To investigate this for the vesicle system, the import mixtures were depleted of ATP by apyrase treatment before starting the import reaction. Insertion of porin into the vesicles was strongly diminished by reducing the ATP concentration, while the import of CCHL remained unaffected (Fig. 7). This parallels the situation found with intact mitochondria, where the depletion of ATP levels strongly affects the import of porin (Kleene et al., 1987), but not that of CCHL (Lill et al., 1992). Taken together, protein import into isolated outer membranes appears to employ the same components and follow the same mechanisms as in intact mitochondria.

Precursor Proteins Destined for the Matrix or the Inner Membrane Specifically Bind to Isolated Outer Membrane Vesicles, but Are Not Translocated

We asked whether the isolated outer membrane is able to translocate precursor proteins which normally undergo further potential-dependent transport into or across the inner membrane. To this end, the precursors of the β-subunit of matrix processing peptidase (PEP; Schneider et al., 1990), cytochrome c_1 (Hartl et al., 1987; Nicholson et al., 1989), and the AAC (Pfanner and Neupert, 1987) were incubated with the vesicles under conditions yielding efficient translocation into intact mitochondria. In neither case, however, specific import was detected as judged from the formation of protease-resistant material in a receptor dependent fashion (Fig. 8). The result was not influenced by the addition of ATP and reticulocyte lysate. Agents supporting the generation of a membrane potential across the inner membrane, like NADH, had no effect on the amount of protease-protected material confirming that inner membranes were absent in the vesicle preparation (data not shown). A number of other precursor proteins behaved in a comparable fashion, e.g., the β-subunit of F_1-ATPase ($F_1\beta$; Schleyer and Neupert, 1985), the α-subunit of the matrix processing peptidase (Schneider et al., 1990), Rieske iron sulfur protein (Fe/S; Hartl et al., 1986), and a fusion protein consisting of subunit 9 of the F_0-ATPase and dihydrofolate reductase (Su9-DHFR; Pfanner et al., 1987). Most likely, components of the inner membrane and/or the intermembrane space have been removed from the vesicle preparation which are essential for translocating these precursor proteins through the outer membrane. Interestingly, the precursors can still undergo specific interaction with the vesicles. Removal of the receptors by trypsin pretreatment significantly reduced the amount of precursor protein bound to the vesicles (Fig. 8, cf. lanes without proteinase K). Taken together, isolated outer membrane vesicles are functional in the initial specific recognition and binding of inner membrane and matrix precursor proteins, but they fail to subsequently transfer the polypeptide chain across the membrane.

Discussion

As reported here isolated outer membrane vesicles of *N.*

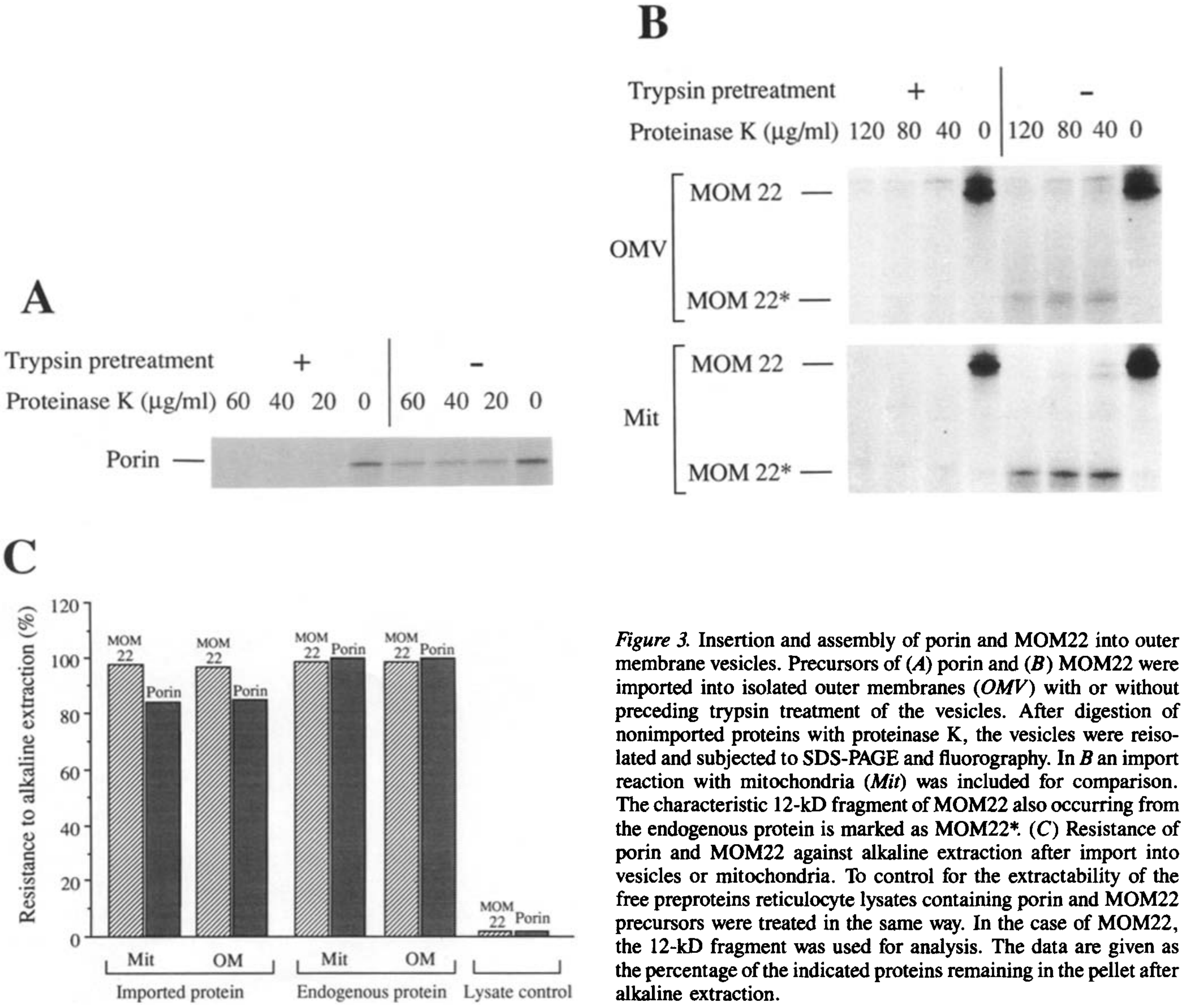


Figure 3. Insertion and assembly of porin and MOM22 into outer membrane vesicles. Precursors of (*A*) porin and (*B*) MOM22 were imported into isolated outer membranes (*OMV*) with or without preceding trypsin treatment of the vesicles. After digestion of nonimported proteins with proteinase K, the vesicles 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

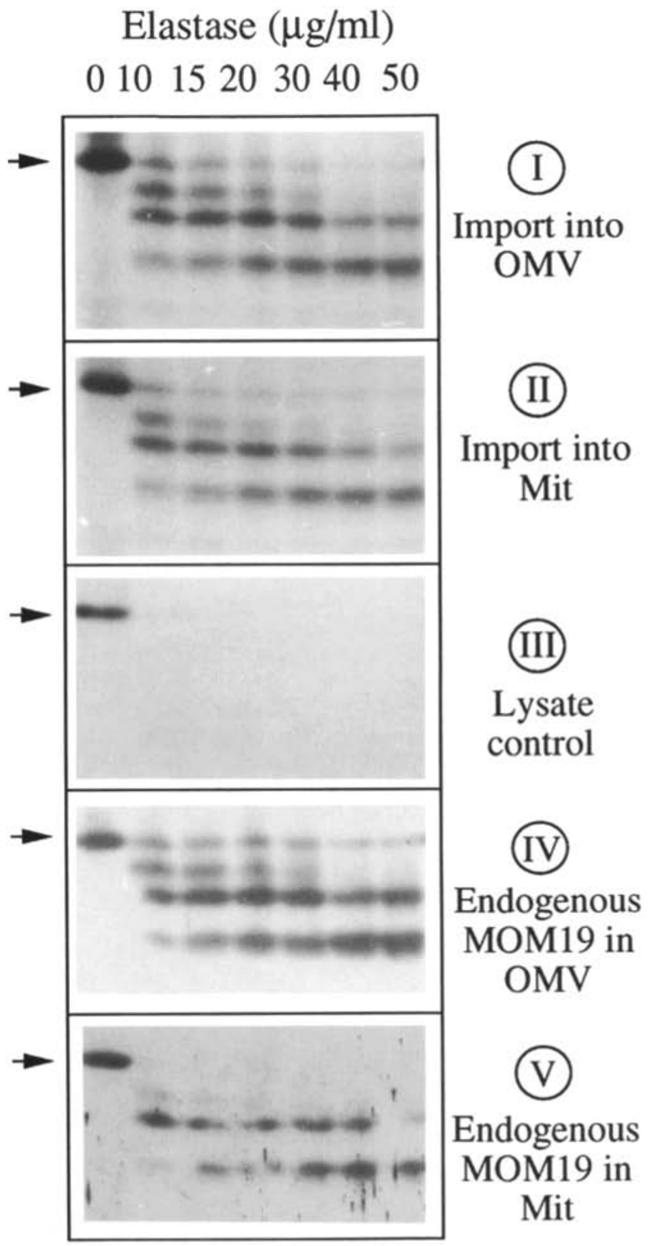


Figure 4. Insertion and assembly of MOM19 into outer membrane vesicles. MOM19 precursor was incubated with outer membrane vesicles (*OMV*), mitochondria (*Mit*), or import buffer only (*Lysate control*). After the import reaction the samples were treated with the indicated amounts of elastase. Vesicles 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.

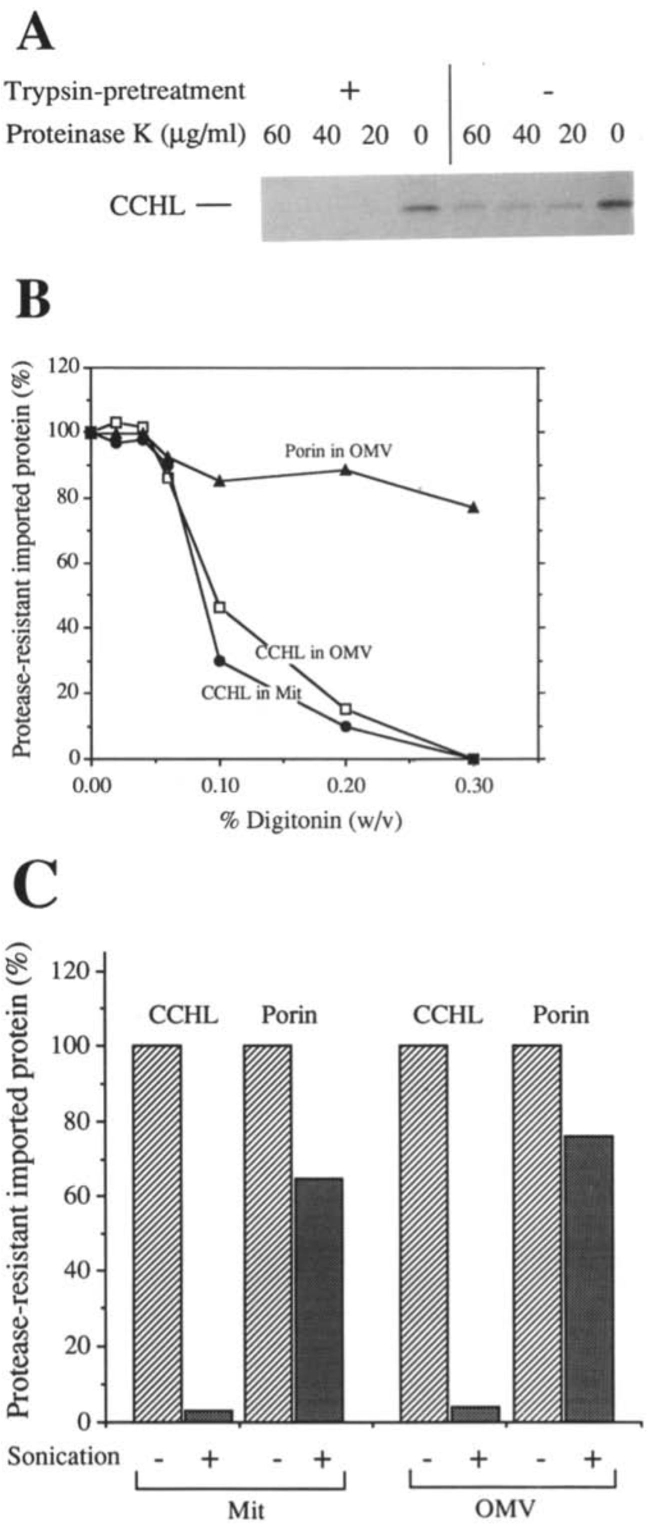


Figure 5. Translocation of CCHL into the lumen of outer membrane vesicles. (*A*) Import of radioactively labeled CCHL precursor into the vesicles and pretreatment with trypsin were performed as described for porin (see Fig. 3 *A*). (*B*) Digitonin fractionation of imported CCHL. Import into outer membrane 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, resuspended 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.

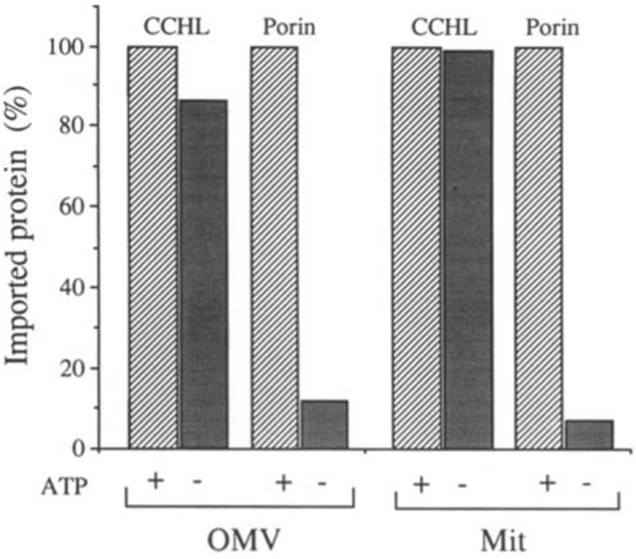


Figure 7. ATP requirement for import of porin and CCHL into outer membrane vesicles. Outer membrane vesicles (*OMV*), mitochondria (*Mit*), and reticulocyte lysate were pretreated with or without 15 U/ml apyrase (Sigma Chem., St. Louis, MO) for 15 min at 25°C. Import of porin and CCHL precursors was 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%.

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

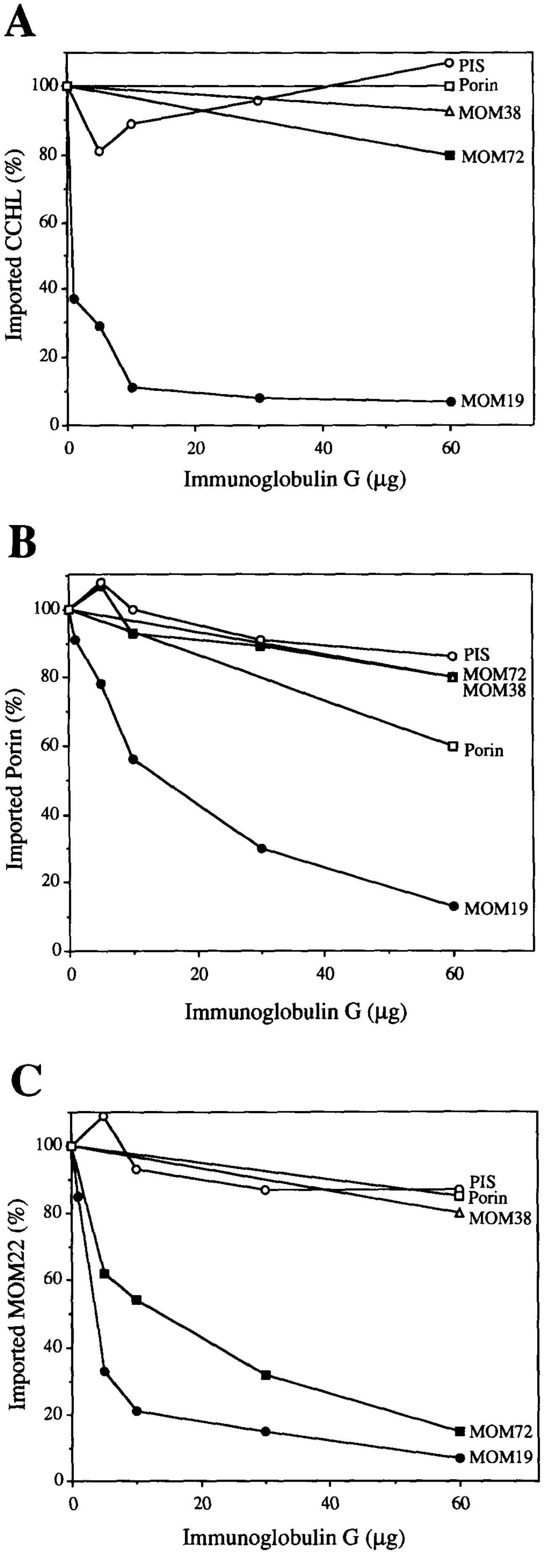


Figure 6. Import into isolated outer membrane vesicles requires the function of surface receptors. Outer membrane vesicles (1 µg per sample) were incubated for 30 min at 0°C with different amounts of IgGs in import buffer (total volume 110 µl). The IgGs were specific for MOM19, MOM72, MOM38, and porin, 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.

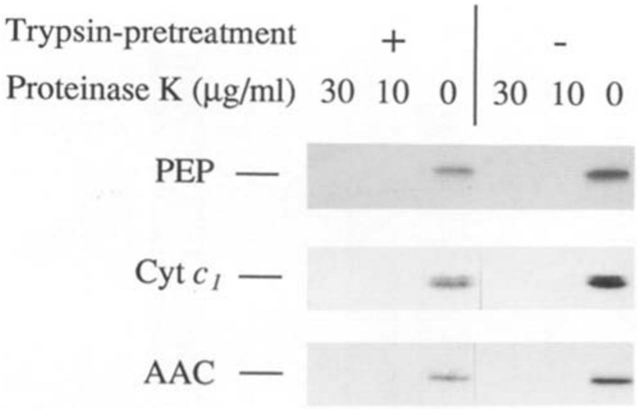


Figure 8. Proteins moving into or across the inner membrane of mitochondria specifically bind to isolated outer membrane vesicles, but are not translocated. Trypsin pretreatment of the vesicles and import reactions using the precursors of the indicated proteins were performed and samples analyzed as 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 c_1, cytochrome c_1; *AAC*, ADP/ATP carrier.

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 membrane. Mitochondrial Hsp70 engaged in "pulling" precursors into the matrix space (Neupert et al., 1990; Simon et al., 1992) needs ATP hydrolysis for its function and possibly acts in conjunction with other factors, such as DnaJ homologs. In the absence of matrix ATP, but in the presence of a membrane potential needed to insert the signal sequence into the inner membrane (Schleyer and Neupert, 1985), precursors will accumulate as two-membrane-spanning intermediates (Rassow et al., 1989). With isolated mitochondria under such conditions, a very slow movement across the outer membrane of the COOH-terminal segments of precursors has been observed (Hwang et al., 1991; Rassow and Pfanner, 1991). This may, however, represent an artifactual situation in vitro and one can only speculate about the energetic requirements for membrane passage. With inner membrane proteins such as the AAC, the situation is even more complex. It has been shown already some time ago that AAC in the absence of a membrane potential can accumulate at the GIP level ("stage 3 intermediate"; Pfanner and Neupert, 1987), where it is largely exposed to the intermembrane space but not to the mitochondrial surface. On the other hand, outer membrane vesicles are not capable of translocating AAC precursor. We speculate that AAC can overcome the outer membrane only by interacting with components of the intermembrane space and/or the inner membrane. Further movement and assembly into the inner membrane can occur by passing the membrane potential dependent step. Taken together, to achieve translocation across the outer membrane, different precursor proteins follow different strategies. For initiation of the process, the preproteins use the same receptor/GIP complex, but for completion of their translocation, various, so far, unknown components are used. The availability of a translocation system with mitochondrial outer membrane vesicles will now allow us to study the mechanisms, components, and energetics involved in the insertion and translocation of preproteins into and across the outer membrane.

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