Purified and Protein-Loaded Mitochondrial Outer Membrane Vesicles for Functional Analysis of Preprotein Transport

Mayer, Andreas; Driessen, Arnold; Neupert, Walter; Lill, Roland

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tion. Both of these types of experiments will help delineate the stage of import at which the particular component is required.

The analysis of translocation intermediates will also help determine the sequential series of interactions that occurs between precursor proteins and import components during protein import into the matrix. Determination of these reactions will provide an estimate of the affinity of different import components for the presequence and mature regions of precursor proteins. This information will provide a starting point for the reconstitution of protein translocation reactions with purified components.

Acknowledgments

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[18] Purified and Protein-Loaded Mitochondrial Outer Membrane Vesicles for Functional Analysis of Preprotein Transport

By ANDREAS MAYER, ARNOLD DRIESSEN, WALTER NEUPERT, and ROLAND LILL

Introduction

The translocation of proteins into and across biological membranes is a complex, multistep process that requires the coordinated interplay of many integral membrane components and of soluble factors from both sides of the membrane (for a review see articles in Neupert and Lill[1]). The functional analysis of the translocation process depends on the availability of defined biochemical *in vitro* systems. Many important mechanistic questions can only be answered conclusively, if purified biochemicals are utilized. This applies especially for protein translocation into and across the double-membrane system of mitochondria. Detailed mechanistic dissection of the translocation processes across the individual membranes is often hampered by the fact that usually the preproteins pass simultaneously across the

[1] W. Neupert and R. Lill, "Membrane Biogenesis and Protein Targeting," Elsevier Science Publishers, Amsterdam, 1992.

two membranes.[2,3] Here, we describe the purification of outer membrane vesicles (OMV) derived from *Neurospora crassa* mitochondria. These OMV have proven to be an invaluable tool for the description of the endogenous protein translocation machinery, which can act independently of that in the mitochondrial inner membrane.[4,5] Furthermore, OMV are useful to study mechanistic aspects of protein translocation such as the specific binding of preproteins to surface receptors and the unfolding of the polypeptide chain before membrane transit.[6]

Biochemical investigations of protein transport require the manipulation of participating components. Whereas it is experimentally easy to manipulate factors on the *cis* side of the membrane, the membrane-embedded translocation machinery has to be reconstituted into proteoliposomes to allow variation of individual components. Manipulation of proteins on the *trans* side, however, comprises a considerable experimental problem, thus complicating the examination of the functional role of such components. So far, soluble proteinaceous factors of the *trans* side of a biological membrane can be varied either by entrapping these proteins into the lumen of proteoliposomes during the reconstitution of the translocation process,[7] by sonication of the vesicles,[8] or by alkaline treatment of the membranes in the presence of the protein to be entrapped.[9] All of these procedures have some important shortcomings. The first method is difficult, time consuming, and often yields low efficiencies, the second tends to inactivate the membranes, and the third involves extreme pH shifts restricting its applicability to alkali-stable proteins. Furthermore, the first two procedures require high amounts of protein, since the sample volumes have to be large.

In this contribution, we describe a simple method to introduce soluble proteins into the lumen of purified mitochondrial OMV by using a gentle freeze–thaw technique. To achieve this we adapted a procedure that originally had been developed for the reconstitution of membrane proteins.[10] The lumen of these vesicles corresponds to the intermembrane space of intact mitochondria. Thus, enclosing soluble proteins from this submitochondrial compartment may help to identify soluble components involved in protein translocation across the outer membrane and to study their

[2] B. G. Glick, C. Wachter, and G. Schatz, *Trends Cell Biol.* **1,** 99 (1991).
[3] N. Pfanner, J. Rassow, I. J. van der Klei, and W. Neupert, *Cell* **68,** 999 (1992).
[4] A. Mayer, R. Lill, and W. Neupert, *J. Cell Biol.* **121,** 1233 (1993).
[5] B. Segui-Real, G. Kispal, R. Lill, and W. Neupert, *EMBO J.* **12,** 2211 (1993).
[6] A. Mayer, W. Neupert, and R. Lill, *Cell* **80,** 127 (1995).
[7] J. L. Brodsky, S. Hamamoto, D. Feldheim, and R. Schekman, *J. Cell Biol.* **120,** 95 (1993).
[8] A. Economou and W. Wickner, *Cell* **78,** 835 (1994).
[9] N. J. Bulleid and R. B. Freedman, *Nature* **335,** 649 (1988).
[10] A. J. M. Driessen and W. N. Konings, *Methods Enzymol.* **221,** 394 (1993).

functional role. Furthermore, foreign proteins may be enclosed for analytical purposes. This latter application has been used successfully for the enclosure of matrix-processing peptidase (MPP) in the lumen of the OMV to demonstrate the translocation of a mitochondrial presequence across the outer membrane[6] and for entrapping apocytochrome-*c*-specific antibodies to analyze the reversible membrane passage of apocytochrome *c*.[11] The procedure can be used in an analogous way to introduce soluble proteins into vesicles derived from other cellular membranes rendering it possible to identify and functionally characterize lumenal proteins involved in various transmembrane processes.

Materials

Sucrose in the Rotipuran quality is purchased from Roth (Karlsruhe, FRG), fluorescein isothiocyanate (FITC)-dextran from Sigma (Deisenhofen, FRG), and R18 fluorescence dye (octadecylrhodamine B) from Molecular Probes (Eugene, OR). Published procedures are followed for the purification of various proteins: MPP,[12] holocytochrome *c*,[13] and immunoglobulin G (IgG).[14] Protein concentrations are determined by using the Bio-Rad dye binding assay (Bio-Rad, München, FRG). The glass–Teflon homogenizer is from Braun (Melsungen, FRG). Centrifugations are done in L8/50 ME and TL-100 ultracentrifuges from Beckman (München, FRG). The refractometer is from Leitz (Wetzlar, FRG), the fluorimeter Fluoromax from Instruments S.A. (Grasbrunn, FRG).

Procedures

Isolation of Mitochondrial Outer Membrane Vesicles from Neurospora crassa

Mitochondria freshly prepared from *N. crassa* strain 74A according to Mayer *et al.*[4] are pelleted at 17,000*g* in a Beckman JA20 rotor for 12 min at 2°. The mitochondrial pellet (500 mg protein) is resuspended at a protein concentration of 6 mg/ml in swelling buffer [5 m*M* potassium phosphate, pH 7.2, 5 m*M* EDTA, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)] and incubated for 10 min on ice to promote swelling of the mitochondria. The suspension is transferred into a glass–Teflon homogenizer and homoge-

[11] A. Mayer, C. Hergersberg, W. Neupert, and R. Lill, *J. Biol. Chem.* **270,** 12390 (1995).
[12] M. Arretz, H. Schneider, B. Guiard, M. Brunner, and W. Neupert, *J. Biol. Chem.* **269,** 4959 (1994).
[13] B. Hennig and W. Neupert, *Methods Enzymol.* **97,** 261 (1983).
[14] T. Söllner, G. Griffith, R. Pfaller, N. Pfanner, and W. Neupert, *Cell* **59,** 1061 (1989).

nized by 20 strokes to dislodge the mitochondrial outer membrane from the mitoplasts. Fifteen milliliters each of the homogenate is layered on top of six sucrose gradients prepared by overlaying 12 ml of 0.9 *M* sucrose in EMP buffer (2.5 m*M* EDTA, 10 m*M* MOPS–KOH, pH 7.2, and 1 m*M* PMSF) with 9 ml of 0.25 *M* sucrose in EMP buffer in tubes for a Beckman SW28 ultracentrifugation rotor (Fig. 1A, left). The tubes are spun for 1 hr at 141,000*g* and 2° (sedimentation centrifugation).

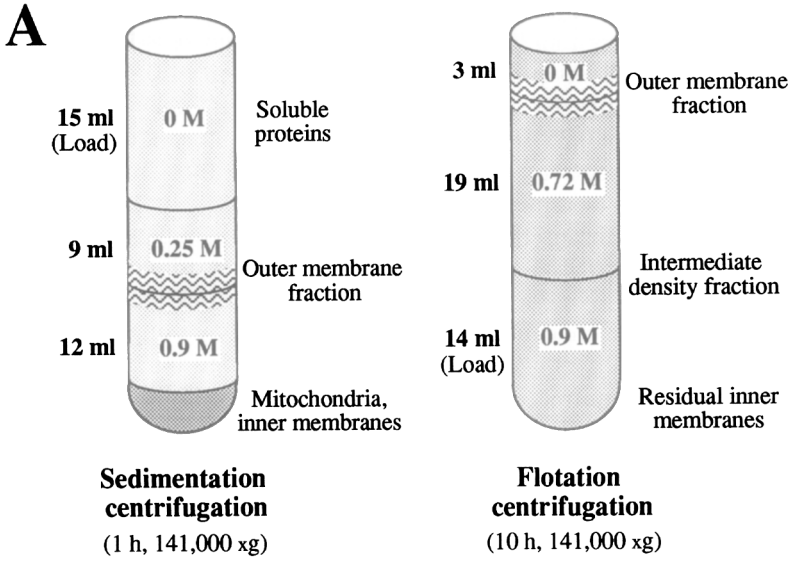
The outer membrane fraction can be harvested as a light-orange band from the 0.25 and 0.9 *M* sucrose interface. The sucrose concentration is adjusted to 0.9 *M* by adding sucrose from a 2 *M* stock solution in EMP buffer. The sucrose concentration may be measured by using a refractometer. Fourteen milliliters each of this solution is loaded into four tubes for a Beckman SW28 ultracentrifugation rotor and overlayed with 19 ml of 0.72 *M* sucrose in EMP buffer and 3 ml EMP buffer (Fig. 1A, right). The tubes are centrifuged for 10 hr at 141,000*g* and 2° (flotation centrifugation). The purified outer membrane fraction is harvested from the 0 and 0.72 *M* sucrose interface. Aliquots are frozen in liquid nitrogen and stored −70° until use. The typical yield is 2.5 mg outer membrane protein per gram of mitochondrial protein, which corresponds to a 4% overall recovery of outer membrane protein (Table I).

The purity of the OMV preparation can be assessed from the enrichment of outer over inner membranes. This can be analyzed by immunostaining for relevant marker proteins, for example, MOM38 for the outer membrane and ADP/ATP carrier (AAC) for the inner membrane. In both centrifugation steps, the enrichment of MOM38 over AAC is about 35-fold, resulting in an overall purification of more than 1000-fold (Table I). As judged from the protease susceptibility of MOM38 and MOM19, practically all of the vesicles are sealed and in a right-side-out orientation.[4] In electron micrographs the isolated OMVs are visible as large and spherical vesicles (200 to 500 mm in diameter; Fig. 1B). The OMV fraction appears to be homogenous, whereas the intermediate density fraction separated in the last step of purification (cf. Fig. 1A, right) is highly heterogenous and contains a large number of small vesicles. This latter fraction is known to contain both mitochondrial outer and inner membranes.[15]

Inclusion of Proteins in Lumen of Isolated Mitochondrial Outer Membrane Vesicles

Standard Inclusion Procedure. Isolated OMV (50 μg) is quickly thawed at 25° and transferred to an ice bath. The OMVs are diluted with one

[15] L. Pon, T. Moll, D. Vestweber, B. Marshallsay, and G. Schatz, *J. Cell Biol.* **109,** 2603 (1989).



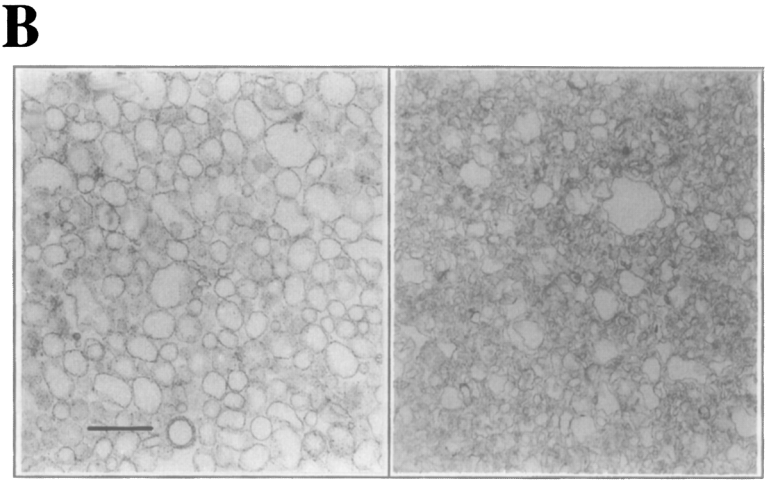


FIG. 1. Purification of mitochondrial outer membrane vesicles. (A) Sucrose gradients used for sedimentation and flotation centrifugation. For details see text. (B) Electron micrographs of the isolated OMVs (left) and the intermediate density fraction (right) harvested from the flotation centrifugation. Preparation of the samples for electron microscopy was as described in Ref. 18. Bar: 1 μm.

TABLE I
PURIFICATION OF MITOCHONDRIAL OUTER MEMBRANE VESICLES[a]

Purification step	Enrichment of MOM38 over AAC	Protein (mg)	Yield of OM protein (%)
Mitochondria	=1	500	=100
Sedimentation centrifugation	32	5.0	16.7
Flotation centrifugation	1050	1.3	4.2

[a] From the purification steps the amounts of MOM38 and ADP/ATP carrier (AAC) were analyzed by SDS–PAGE and immunostaining using the enhanced chemiluminescence (ECL) detection system (Amersham). Resulting bands were quantitated by laser densitometry. It is essential to analyze various amounts of protein to ensure that the signals used for quantitation are in the linear range of detection. Purification is given as the fraction of MOM38 and AAC that is set to one for isolated mitochondria. The calculation of the yield of outer membrane (OM) protein is based on the finding that the outer membrane comprises 6% of total mitochondrial protein.[4]

volume of EM buffer (EMP buffer without PMSF) and reisolated by ultracentrifugation (260,000*g* for 15 min at 2° in a Beckman TLA100.3 rotor). After resuspending the OMVs in EM buffer, they are centrifuged as before, and resuspended in 12.5 μl inclusion buffer (10 m*M* MOPS–KOH, pH 6.5). The protein to be enclosed (e.g., cytochrome *c* as a model protein) is mixed with the OMV. We routinely use a final protein concentration of 1 to 6 mg/ml, but, depending on the biochemical problem studied, lower concentrations might be sufficient. The volume is adjusted to 25 μl with inclusion buffer. The sample is snap-frozen in liquid nitrogen, and then placed in an ice-water bath to allow slow thawing, which can take up to 30 min. During the slow thawing period the vesicles are transiently opened permitting almost quantitative equilibration of the vesicle lumen with the surrounding solution (see below). Then, 5 μl of 100 m*M* MOPS–KOH, pH 7.2, is added, and the sample is incubated for 5 min at 25°.

Nonenclosed protein is now removed by flotation in a sucrose step gradient. The sample is diluted with wash Buffer A (EM buffer containing 1.4 *M* sucrose and 150 m*M* KCl) to a final volume of 500 μl. The vesicle suspension is overlayed with a sucrose step gradient consisting of three 500-μl steps of 1.15 *M* sucrose plus 150 m*M* KCl, 0.9 and 0.25 *M* sucrose in EM buffer. Centrifugation in a Beckman SW60 rotor is for 30 min at 150,000*g* and 2°. The vesicles are harvested from the 0.25 and 0.9 *M* sucrose interphase, and can now be used for subsequent biochemical studies. From the protease sensitivity of MOM38 and MOM19 it follows that the majority of the vesicles regain both their sealed character and right-side-out orientation (data not shown). Extensive passages through narrow pipette tips can

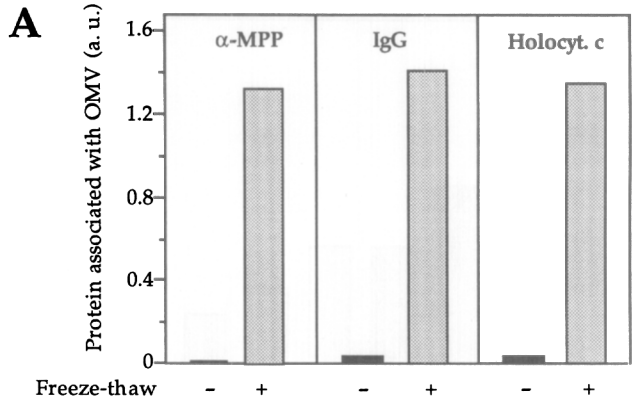
cause transient opening of the vesicles and release of their contents into the medium. Therefore, 5 mm of the yellow pipette tips should be cut off before pipetting the protein-loaded vesicles.

The freeze–thaw procedure is applicable to any soluble protein or protein mixture. Background binding of the protein to the outer face of the membrane should be negligible to avoid complications in subsequent biochemical examinations. In the case of the α subunit of matrix-processing peptidase (α-MPP), IgG, or holocytochrome *c*, the membrane association of these proteins in the absence of a freeze–thaw step was less than 3% (Fig. 2A). The enclosed material was soluble in the lumen of the OMV. On opening of the OMV by sonication, the proteins were released into the surrounding medium and stayed in the supernatant after pelleting the OMV by centrifugation (Fig. 2B).

Parameters Affecting Inclusion Efficiency. Parameters such as pH and the composition of the solution used during the freeze–thaw step may influence the efficiency of the inclusion. For establishing, controlling, and optimizing the procedure, the amount of vesicle-enclosed protein can be followed through immunoblotting. A very convenient alternative is the use of FITC-dextran (average molecular mass 70 kDa) as a model substrate. Its inclusion can be traced in a sensitive and rapid fashion by measuring the fluorophor retained in the vesicles. The inclusion procedure should be performed in a pH range between 6 and 7. Higher pH will significantly decrease the efficiency of inclusion (Fig. 3A), whereas lower pH causes aggregation of the vesicles. Best results were obtained in solutions buffered with Tris–HCl or MOPS–KOH, whereas in potassium phosphate the entrapment was poor. It is important to note that the solution used during inclusion is of low ionic strength and does not contain any cryopreservatives such as glycerol or sucrose, because these chemicals severely interfere with the transient opening of the vesicles.

As shown in Fig. 3B, one freeze–thaw cycle is sufficient for maximal enclosure. Further cycles do not increase the inclusion efficiency. However, we have noted that the biochemical activity of the OMVs in subsequent experiments is compromised by more than one freeze–thaw step. For some applications the presence of fatty acid-free BSA may help preserve the activity of the OMVs and of the protein to be enclosed. Protein concentrations higher than 10 mg/ml may significantly decrease the efficiency of inclusion (data not shown) possibly because the membranes are protected from perturbation by the freeze–thaw step.

Quantitation of Inclusion Efficiency. To be useful in biochemical studies, the majority of the OMVs should be filled with the protein of interest. For quantitation of the portion of OMVs that were opened during the freeze–thaw step, we take advantage of the protease degradation behavior of



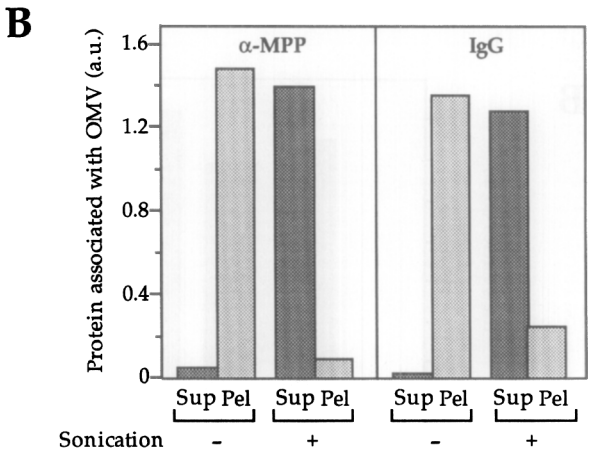
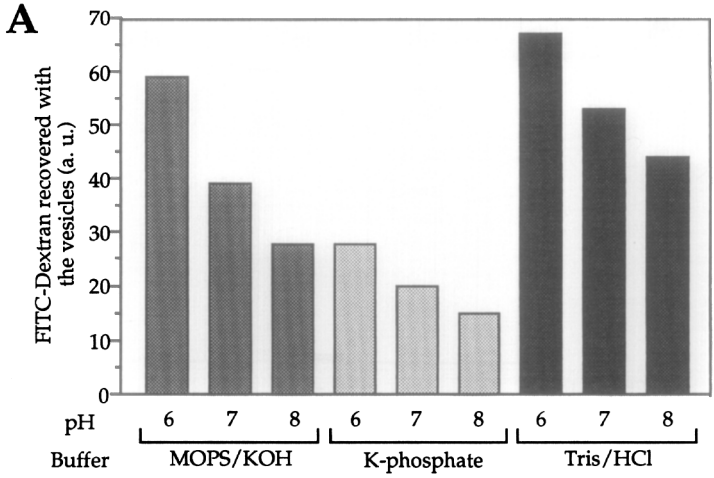


FIG. 2. Purified proteins can be enclosed in soluble form in the lumen of OMVs by a freeze–thaw treatment. (A) OMVs (10 μg/sample) were incubated with the indicated proteins and subjected to a freeze–thaw treatment or left on ice. OMVs were reisolated by flotation centrifugation and precipitated with TCA. Samples were analyzed by SDS–PAGE and immunostaining for the amount of the respective protein recovered with the vesicles. (B) After a freeze–thaw step in the presence of α-MPP or IgG as in Part (A), the vesicles were reisolated and diluted with 5 volumes SEMK buffer (250 m*M* sucrose, 1 m*M* EDTA, 10 m*M* MOPS–KOH, pH 7.2, and 100 m*M* KCl). Samples were left on ice or sonicated for 1 min in an ice–water bath (Branson Sonifier 250 with a microtip, intensity 4, 30% duty cycle). The membranes were reisolated (45 min, 125,000*g*) and the supernatants (Sup) and pellets (Pel) were precipitated with TCA and analyzed as in Part (A). a.u., arbitrary units; α-MPP, α-subunit of the matrix-processing peptidase; IgG, immunoglobulin G; Holocyt. c, holocytochrome *c*.



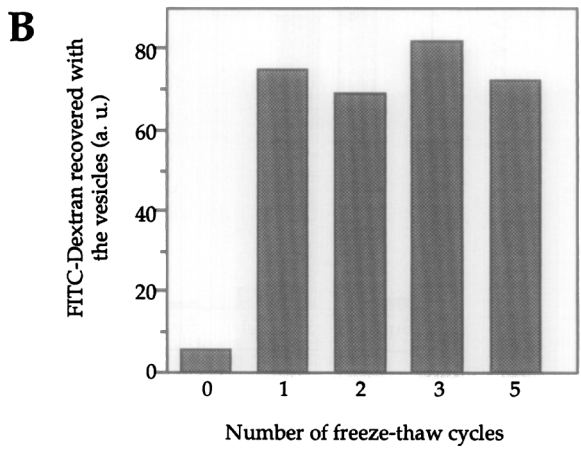


FIG. 3. Parameters influencing the efficiency of the inclusion procedure. (A) For testing the influence of buffer substance and pH, OMVs (15 μg/sample) were incubated with 10 m*M* FITC-dextran in the indicated buffer solution (10 m*M*) and subjected to a freeze–thaw treatment. OMVs were reisolated by flotation centrifugation and solubilized in 0.5 ml EM buffer containing 0.5% (v/v) Triton X-100. FITC-dextran in these solutions was determined by fluorimetry. Excitation was at 490 nm, emission was recorded at 530 nm. Control samples that were not subjected to a freeze–thaw treatment yielded fluorescence signals of less than 3 units. (B) OMVs were subjected to different numbers of freeze–thaw cycles in 10 m*M* MOPS–KOH, pH 6.5. Further treatment was as in part (A). a.u., arbitrary units.

MOM38. Proteases (e.g., thermolysin or proteinase K) that are added externally to the OMV cleave the protein to generate an N-terminal 26-kDa fragment, termed MOM38*. Proteolysis from the internal face of the outer membrane, however, results in complete degradation of the protein.[4] Addition of thermolysin to OMV was accompanied by almost quantitative generation of MOM38* showing that the OMV were tightly sealed and in a right-side-out orientation (Fig. 4). When a freeze–thaw step was performed in the presence of thermolysin, 85% of MOM38* was degraded, indicating that the protease had gained access to MOM38 from the intermembrane space side of the outer membrane. This suggests that the inclusion procedure results in loading of more than 80% of the vesicles. The results are corroborated by examining FITC-dextran-loaded OMVs under the fluorescence microscope after staining the membranes with the fluorescence dye R18. Most of the vesicles appeared to be charged with the fluorescein probe (data not shown). A semiquantitative estimation of the amount of FITC-dextran entrapped inside the OMV revealed that the concentration of enclosed material is similar to the concentration present during the inclusion step. Taken together, the transient opening of the outer membrane

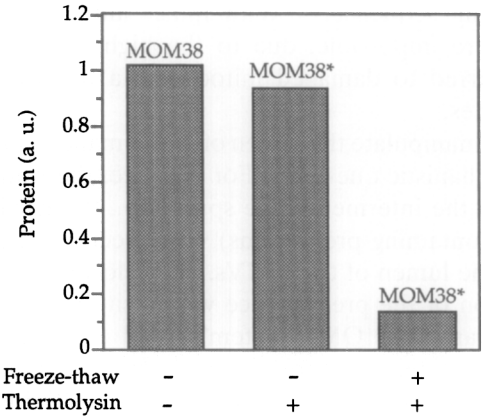


FIG. 4. Efficiency of the transient opening of the outer membrane. OMVs (30 μg) were incubated with 80 μg/ml thermolysin in EM buffer (pH 6.5). One aliquot of the sample was supplemented with 20 m*M* MOPS–KOH, pH 7.5, incubated for 5 min at 25°, and precipitated with TCA. A second aliquot was treated equally, but received 2 m*M* $ZnCl_2$ prior to the 25° incubation to activate thermolysin. A third aliquot was subjected to the freeze–thaw procedure and then treated like the second aliquot. Proteins were precipitated with TCA, separated by SDS–PAGE, transferred to nitrocellulose, and immunodecorated with antibodies raised against an N-terminal peptide of MOM38. Inclusion of thermolysin was assessed by the degradation behavior of MOM38. This protein yields a 26-kDa N-terminal fragment (MOM38*), if the protease has access only from outside, but is completely degraded from the internal face of the outer membrane.[4]

allows almost quantitative equilibration of the internal volume with the external medium.

Conclusions and Perspectives

Utilization of purified mitochondrial OMVs resulted in a number of discoveries that have advanced our knowledge of protein transport into mitochondria. First, purified outer membranes have led to the identification of the first components of the mitochondrial protein import apparatus, namely, MOM19 and MOM72, which are involved in the initial stages of protein import.[14,16] Second, the competence of the outer membrane for protein transport independent of the inner membrane was directly demonstrated by using purified OMVs.[4] Interestingly, the machinery is specific in that only outer membrane proteins are inserted and proteins such as cytochrome-*c* heme lyase (holocytochrome-*c* synthase) are translocated. Proteins with cleavable presequences, however, were not translocated into the OMV. Third, the purified OMV system was useful for studying preprotein binding to the surface receptors of the outer membrane. These investigations led to the identification of the components deciphering the mitochondrial targeting sequence.[16a] With intact mitochondria, comparable investigations were impossible, due to the high unspecific binding that presumably occurred to damaged mitochondria or other contaminating cellular membranes.

The ability to manipulate the lumen of OMV made it possible to address a number of mechanistic questions. For instance, the translocation of the presequence into the intermembrane space (i.e., the partial translocation of presequence-containing preproteins) was directly demonstrated by enclosing MPP in the lumen of the OMVs.[6] Translocation was driven by the specific interaction of the presequence with a site on the internal face of the outer membrane. The OMV system should now prove useful for the elucidation of the chemical nature of this binding site. Furthermore, inclusion of IgG directed against apocytochrome *c* resulted in import of this protein into OMVs.[11] Apparently, the IgG mimicked the function of cytochrome-*c* heme lyase by rendering translocation irreversible through tight complex formation.[17] It will be interesting to see whether inclusion of appropriate binding partners for presequence-containing preproteins will cause their complete translocation across the outer membrane.

[16] T. Söllner, R. Pfaller, G. Griffith, N. Pfanner, and W. Neupert, *Cell* **62,** 107 (1990).
[16a] Mayer *et al.*, *J. Biol. Chem.* **270,** 12390 (1995).
[17] D. W. Nicholson, C. Hergersberg, and W. Neupert, *J. Biol. Chem.* **263,** 19,034 (1988).

The experimental systems introduced in this contribution could result in further insights that would not be gained with intact mitochondria. Pressing questions are centered around the identification of the yet ill-defined translocation pore and the description of its chemical character. A recent breakthrough in *Neurospora* genetics introducing gene disruption and the generation of specifically designed mutants in this species should now allow the deliberate alteration of the protein import complex.[18,19] Thus, experiments using OMVs derived from such mutants may lead to an assignment of specific functions to individual components or even domains within these proteins. Finally, OMVs should be an excellent source for the purification and functional reconstitution of the protein import machinery.

Acknowledgments

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[18] T. A. A. Harkness, F. E. Nargang, I. van der Klei, W. Neupert, and R. Lill, *J. Cell Biol.* **124,** 637 (1994).
[19] F. E. Nargang, K.-P. Künkele, A. Mayer, R. G. Ritzel, W. Neuert, and R. Lill, *EMBO J.* **14,** 1099 (1995).

[19] Mitochondrial Receptor Complex from *Neurospora crassa* and *Saccharomyces cerevisiae*

By AGUSTÍN ALCONADA, FRANK GÄRTNER, ANGELIKA HÖNLINGER, MICHAEL KÜBRICH, and NIKOLAUS PFANNER

Introduction

Most mitochondrial proteins are synthesized as precursor proteins in the cytosol and must be subsequently imported into the mitochondria. In most cases, this process requires the precursor polypeptide to cross two biological membranes (outer and inner mitochondrial membranes) before it reaches its final destination. A specialized machinery exists in each of these membranes in order to accomplish this event. Major advances have been made in our understanding of the function and structure of the proteinaceous components of the mitochondrial outer membrane (MOM) that are