CELL BIOLOGY

A LABORATORY HANDBOOK

Edited by

JULIO E. CELIS

Danish Centre for Human Genome Research
Aarhus, Denmark

VOLUME 1

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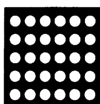

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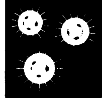

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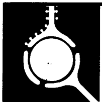

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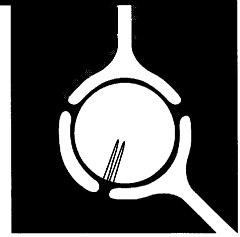
Inclusion of Proteins into Isolated Mitochondrial Outer Membrane Vesicles



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

I. Introduction

The translocation of proteins into and across biological membranes requires the coordinated action of multiple components (for a review, see articles in Neupert and Lill, 1992). In addition to a membrane-embedded translocation machinery, further components on both the *cis* and the *trans* sides of the membrane are needed to achieve translocation. While it is experimentally easy to manipulate factors on the *cis* side of a membrane, it is a considerable problem to gain access to proteins on the *trans* side, thus precluding the investigation 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 the proteins into the lumen of proteoliposomes reconstituted from detergent-solubilized vesicles or by alkaline treatment of the membranes in the presence of the protein to be entrapped. Whereas the first procedure is difficult, sometimes yields low efficiencies, and has been developed only for *Escherichia coli* inner membranes and microsomal membranes (Driessen and Wickner, 1990; Nicchitta *et al.*, 1991; Brodsky *et al.*, 1993), the second procedure involves extreme pH shifts, restricting its applicability to alkali-stable proteins (Bulleid and Freedman, 1988).

Here we describe a simple method to introduce soluble proteins into the lumen of membrane vesicles using a freeze–thaw technique developed by adapting a procedure originally described for the reconstitution of membrane proteins (Driessen and Konings, 1993). As a model system, we use vesicles derived from purified outer membranes of *Neurospora crassa* mitochondria (Mayer *et al.*, 1993). The lumen of these vesicles corresponds to the intermembrane space of intact mitochondria. Thus, enclosing soluble proteins from this submitochondrial compartment may help to elucidate the role of these components in protein translocation across the outer membrane. The procedure may be used in an analogous way for inclusion of soluble proteins into vesicles derived from any other isolated cellular membrane, rendering it possible to identify and characterize luminal proteins involved in protein translocation. Moreover, the role of luminal proteins in other membrane-related processes, e.g., signal transduction, may be investigated.

II. Materials and Instrumentation

PMSF (Cat. No. P-7626), Mops (Cat. No. M-1254), and fatty acid-free BSA (Cat. No. A-6003) were obtained from Sigma. K_2HPO_4 (Cat. No. 5104), KH_2PO_4 (Cat.

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No. 4873), KCl (Cat. No. 4936), KOH (Cat. No. 5033), and EDTA (Cat. No. 8418) were obtained from Merck. Sucrose (Cat. No. 4621) was purchased from Roth. Protein concentrations were determined by using the Bio-Rad protein assay method (Bio-Rad, Cat. No. 500-0006). Mitochondria were prepared according to Mayer *et al.* (1993) and were centrifuged at 17,000 *g* in a Beckman JA20 rotor for 12 min at 2°C to give a mitochondrial pellet.

The glass–Teflon homogenizer was from Braun. Centrifugations were done in Beckman L8/50 ME and TL-100 ultracentrifuges. The refractometer was from Leitz.

III. Procedures

A. ISOLATION OF MITOCHONDRIAL OUTER MEMBRANE VESICLES

Solutions

1. *200 mM PMSF:* To make 1 ml, dissolve 34.5 mg PMSF in 1 ml ethanol. Prepare fresh each time.

2. *Swelling buffer:* 5 m*M* potassium phosphate, pH 7.2, 5 m*M* EDTA, 1 m*M* PMSF. To make 500 ml, dissolve 0.435 g K_2HPO_4 and 0.340 g KH_2PO_4 in 250 ml each in H_2O, and adjust the pH of the KH_2PO_4 solution by adding the K_2HPO_4 solution to pH 7.2. Dissolve 0.931 g EDTA in 250 ml of this solution, adjust to 497.5 ml, and store at 4°C. Before use add 2.5 ml 0.2 *M* PMSF.

3. *2 M sucrose:* To prepare 100 ml, dissolve 68.4 g sucrose in H_2O and bring to 100 ml.

4. *EM buffer:* 2.5 m*M* EDTA, 10 m*M* Mops–KOH, pH 7.2. To make 100 ml, dissolve 93 mg EDTA and 0.21 g Mops in H_2O, adjust pH to 7.2 with 5 *M* KOH, and bring to 100 ml with H_2O.

5. *EMP buffer containing various amounts of sucrose:* To make 100 ml, dissolve 93 mg EDTA, 0.21 g Mops in H_2O, adjust pH to 7.2 with 5 *M* KOH, and bring to 50 ml with H_2O. To make EMP buffer containing (i) 0 *M*, (ii) 0.25 *M*, (iii) 0.72 *M*, and (iv) 0.9 *M* sucrose, add (i) 0 ml, (ii) 12.5 ml, (iii) 36 ml, and (iv) 45 ml of 2 *M* sucrose. Adjust the volume of each solution to 99.5 ml and add 0.5 ml 0.2 *M* PMSF just before use.

Steps

Samples should be kept on ice throughout the procedure.

1. Resuspend the mitochondrial pellet (550 mg protein) at a protein concentration of 6 mg/ml in EMP buffer and incubate for 10 min on ice to promote swelling of the mitochondria.

2. Transfer the suspension into a glass–Teflon homogenizer and homogenize (20 strokes) to dislodge the mitochondrial outer membrane from the remaining mitoplasts and intact mitochondria.

3. Prepare six sucrose step gradients by loading 12 ml of 0.9 *M* sucrose in EMP buffer into tubes for a Beckman SW 28 ultracentrifugation rotor (Fig. 1A). Carefully overlay with 9 ml of 0.25 *M* sucrose in EMP buffer using a glass pipette with a wide opening to avoid mixing of the two layers.

4. Load 15 ml of the homogenate from step 2 on top of each gradient by using the same glass pipette. Avoid mixing of the load and the top sucrose solution.

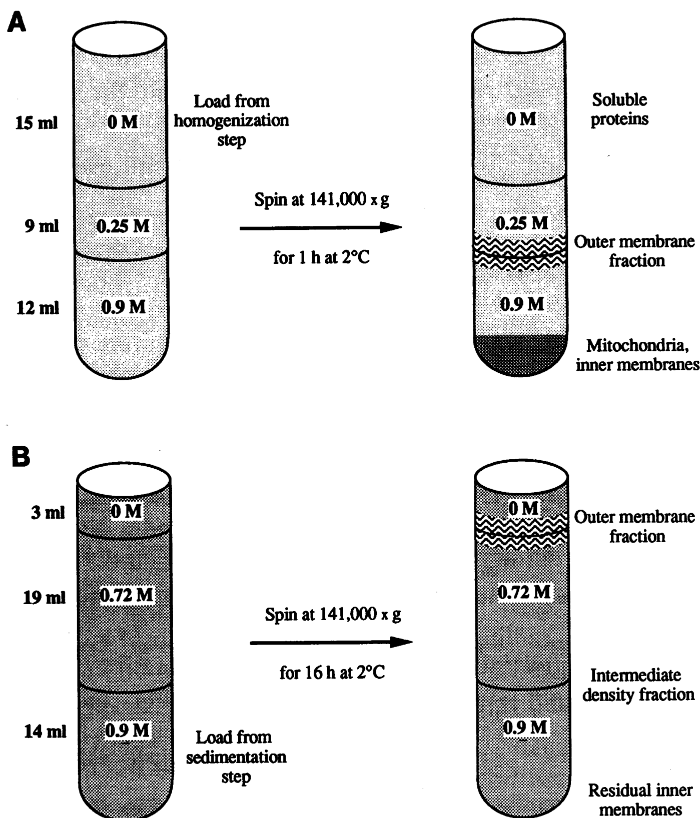

FIGURE 1 Purification of outer membrane vesicles from *Neurospora crassa* mitochondria by sucrose step gradient centrifugation. Sucrose step gradients for (A) sedimentation centrifugation and (B) flotation centrifugation are formed in tubes for a Beckman SW 28 ultracentrifugation rotor using the indicated molarities of sucrose in EMP buffer (left side). The positions of the various fractions after centrifugation are shown on the right side of the figure.

5. Spin for 1 hr at 141,000 *g* and 2°C in a Beckman SW 28 rotor (sedimentation centrifugation).

6. The outer membrane fraction is visible as a light-orange band (cf. Fig. 1A). Harvest the outer membrane fraction from the 0.25 and 0.9 *M* sucrose interface after removing most of the top layer by aspiration. The total volume of the harvested outer membrane fraction is usually about 25 ml.

7. Determine the sucrose concentration of the outer membrane fraction using a refractometer. If a refractometer is not available, it suffices to assume the sucrose concentration to be 0.55 *M*. Adjust the sucrose concentration to 0.9 *M* by adding sucrose from a 2 *M* stock solution.

8. Load 14 ml each of this solution into tubes for a Beckman SW 28 ultracentrifugation rotor. Carefully overlay the outer membrane suspension with 19 ml of 0.72 *M* sucrose in EMP buffer and 3 ml EMP buffer. Avoid mixing of the layers (see step 3, Fig. 1B).

9. Spin for 10 hr at 141,000 *g* and 2°C in a Beckman SW 28 rotor (flotation centrifugation).

10. Harvest the purified outer membrane fraction from the 0 and 0.72 *M* sucrose interface (see Fig. 1B).

11. Dilute the sample fivefold with EM buffer and concentrate the membrane vesicles by centrifugation for another 3 hr at 141,000 *g* and 2°C in a Beckman SW 28 rotor.

12. Resuspend the pellet in 0.5 ml EM buffer and determine the protein concentration using the Bio-Rad protein assay method. Adjust the protein concentration to 0.5 mg/ml. The typical yield is 1–2.5 mg outer membrane protein per gram of mitochondrial protein.

13. Freeze 100-μl aliquots in liquid nitrogen and store at −70°C until use.

B. INCLUSION OF PROTEINS INTO THE LUMEN OF ISOLATED MITOCHONDRIAL OUTER MEMBRANE VESICLES

Solutions

1. *Inclusion buffer:* 10 m*M* Mops–KOH, pH 6.5, and 5 mg/ml BSA. To make 10 ml, dissolve 21 mg Mops and 50 mg fatty acid-free BSA in H_2O, titrate pH to 6.5 with 1 *M* KOH, and adjust the volume to 10 ml.

2. *Wash buffer A:* EM buffer containing 0.25 *M* sucrose and 100 m*M* KCl. To make 100 ml, dissolve 8 g sucrose, 93 mg EDTA and 0.21 g Mops, and 0.75 g KCl, adjust pH to 7.2 with 5 *M* KOH, and bring to 100 ml with H_2O.

3. *Wash buffer B:* EM buffer containing 0.43 *M* sucrose and 100 m*M* KCl. To make 100 ml, dissolve 15 g sucrose, 93 mg EDTA, 0.21 g Mops, and 0.75 g KCl, adjust pH to 7.2 with 5 *M* KOH, and bring to 100 ml with H_2O.

4. *100 mM Mops, pH 7.2:* To make 10 ml, dissolve 0.21 g Mops in H_2O, adjust pH to 7.2 with 5 *M* KOH, and bring to 10 ml.

Steps

Samples and buffers should always be kept at 0–4°C if not stated otherwise.

1. Quickly thaw 50 μg isolated outer membrane vesicles at 25°C and transfer to an ice bath. Reisolate the vesicles by ultracentrifugation (260,000 *g* for 15 min at 2°C in a Beckman TLA 100 rotor).

2. Resuspend the vesicles in 12.5 μl inclusion buffer by gently pipetting up and down through a yellow pipette tip. Transfer to a 1.5-ml Eppendorf tube.

3. Add the protein to be included (e.g., cytochrome c as a model protein) to a final protein concentration of up to 30 mg/ml. We routinely use a final concentration of 3 mg/ml. Adjust the total volume of the sample to 20 μl with inclusion buffer.

4. Snap-freeze the solution in liquid nitrogen. Place the tube in an ice water bath and let the sample thaw slowly, which may take up to 1 hr.

5. Add 4 μl of 100 m*M* Mops–KOH, pH 7.2, and incubate for 5 min at 25°C. Dilute with wash buffer A to a final volume of 80 μl.

6. Pipette 100 μl wash buffer B into a tube for a Beckman TLA 100 rotor and carefully overlay with the vesicle suspension.

7. Spin for 15 min at 260,000 *g* at 2°C in a TLA 100 rotor.

8. Aspirate 150 μl of the supernatant using an injection needle connected to a suction hose. Spin again for 2 min at 260,000 *g* and aspirate the remaining supernatant. Carefully resuspend the pellet in EM buffer containing 0.25 *M* sucrose.

9. The vesicles may now be used for further biochemical analyses (e.g., protein translocation).

IV. Comments

The vesicles produced by the isolation procedure described in Section IIIA are relatively large (average diameter approximately 300 μm) and should be handled with care. Extensive passages through narrow pipette tips can cause a transient opening of the membrane and release of the contents into the medium. Therefore, we routinely cut off 5 mm of the yellow pipette tips before use for pipetting the loaded vesicles.

Repeated freeze–thaw steps do not increase the efficiency of inclusion and rather diminish the competence of the vesicles for protein translocation.

The inclusion of proteins can be checked by immunoblotting. A very convenient alternative used successfully for establishing the procedure is FITC–dextran (average molecular weight 70,000, Sigma Cat. No. FD-70S) as a model substrate. Its inclusion can be traced fast and easily by measuring the fluorophore retained by the vesicles.

After the inclusion of proteins the vesicles cannot be frozen before use in further experiments.

V. Pitfalls

1. To obtain an outer membrane preparation that is free of contaminants such as endoplasmic reticulum and mitochondrial inner membranes, it is essential to keep all equipment as clean as possible. It is necessary to use double-distilled water for all solutions.

2. The inclusion procedure should be performed in the pH range 6.0 to 7.2. Higher pH will decrease the efficiency of inclusion, whereas lower pH may cause aggregation of the vesicles.

3. It is crucial to freeze the vesicles very rapidly and leave them undisturbed during the (slow) thawing period.

4. The buffer containing the protein to be included should be of low ionic strength and must not contain any cryopreservatives, e.g., glycerol, as these chemicals interfere with the inclusion.

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