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Biomembranes

Part K

*Membrane Biogenesis: Assembly and Targeting
(Prokaryotes, Mitochondria, and Chloroplasts)*

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[27] Isolation and Properties of the Porin of the Outer Mitochondrial Membrane from *Neurospora crassa*

By HELMUT FREITAG, ROLAND BENZ, and WALTER NEUPERT

The outer mitochondrial membrane is freely permeable to low molecular weight components such as metal ions, substrates, and nucleotides.^{1,2} Therefore pores in the outer membrane have been postulated. The size of these pores is not clearly defined. Molecules with apparent molecular weights up to 2000–8000 were found to permeate through these channels. Detergent extracts of mitochondrial outer membranes or purified pore proteins were incorporated into liposomes or bilayers. Formation of non-specific pores with a diameter of about 20 Å was observed.^{3–8} The pore-

¹ R. L. O'Brien and G. Brierley, *J. Biol. Chem.* **240**, 4527 (1965).

² E. Pfaff, M. Klingenberg, E. Ritt, and W. Vogell, *Eur. J. Biochem.* **5**, 222 (1968).

³ M. Colombini, *Nature (London)* **279**, 643 (1979).

⁴ M. Colombini, *J. Membr. Biol.* **53**, 79 (1980).

⁵ L. S. Zalman, H. Nikaido, and Y. Kagawa, *J. Biol. Chem.* **255**, 1771 (1980).

⁶ H. Freitag, W. Neupert, and R. Benz, *Eur. J. Biochem.* **123**, 629 (1982).

⁷ N. Roos, R. Benz, and D. Brdiczka, *Biochim. Biophys. Acta* **686**, 204 (1982).

⁸ H. Freitag, G. Genchi, R. Benz, F. Palmieri, and W. Neupert, *FEBS Lett.* **145**, 72 (1982).

forming protein, called mitochondrial porin, is a major component of outer mitochondrial membranes. It has an apparent molecular weight of about 31,000. In many respects the mitochondrial porin resembles the porins of outer membranes from gram-negative bacteria.

Here we describe two different isolation procedures for this protein and discuss some of its properties and its reconstitution into artificial bilayers.

Isolation of Mitochondrial Porin

Growth of *Neurospora crassa* hyphae (wild-type 74A) for 17 hr in 8-liter cultures (inoculum 1×10^6 conidia/ml) in the presence of 2% sucrose and disruption of cells are carried out according to standard procedures.⁹ Mitochondria are isolated in isolation medium (0.44 M sucrose, 2 mM EDTA, 30 mM Tris-HCl, pH 7.6, adjusted at 4°) with the addition of 0.5 mM phenylmethylsulfonyl fluoride (PMSF; final concentration), freshly dissolved in ethanol. The following centrifugation steps are carried out at 4°, employing a Sorvall RC 5B centrifuge and a GS-3 rotor. The cell debris are removed by centrifugation for 20 min at 2000 g. The resulting supernatant is centrifuged for 45 min at 17,000 g. The upper layers of the resulting sediment (containing the mitochondria) are washed twice by resuspending them in a glass-Teflon potter and centrifuging for 45 min at 17,000 g. The final mitochondrial pellet is the starting material for the porin isolation. Under typical conditions, from an 8-liter culture of *Neurospora crassa* 180 g of hyphae (wet weight) are obtained and the yield of mitochondria is 230 mg protein.

Porin Isolation from Outer Membranes⁶

Principle

Mitochondrial outer membranes are isolated by swelling-shrinking of the mitochondria and subsequent separation by a sucrose step centrifugation. Differential detergent extraction of the outer membrane followed by DEAE-cellulose chromatography gives pure porin, which forms active pores after reconstitution into artificial bilayers.

Reagents

Swelling medium: 2.5 mM potassium H_2PO_4 , adjusted to pH 7.3 with KOH

PMSF stock solution: 100 mM PMSF in ethanol, freshly prepared

Shrinking medium: 2.2 M sucrose, 8 mM $MgCl_2$, 8 mM ATP, adjusted to pH 7.3 with KOH

⁹ W. Neupert and G. D. Ludwig, *Eur. J. Biochem.* **19**, 523 (1971).

Isolation medium: 0.44 M sucrose, 2 mM EDTA, 30 mM Tris-HCl, pH 7.5

Sucrose step medium: 0.95 M sucrose, 10 mM Tris-HCl, pH 7.5

Tris buffer: 10 mM Tris-HCl, pH 7.5

Phosphate buffer: 100 mM potassium H_2PO_4 , adjusted to pH 7.3 with KOH

Octyl glucoside solution: 60 mM octyl- β -D-glucoside, 5 mM Tris-HCl, pH 7.5 (17.5 mg of octyl glucoside per milliliter)

Genapol solution: 1% Genapol X-100 (Farbwerke Hoechst, Frankfurt, FRG), 5 mM Tris-HCl, pH 7.5. Genapol X-100 contains polyethoxy chains like Triton X-100, but has isotridecyl groups instead of aromatic rings and therefore shows no absorption at 280 nm

DEAE-cellulose column: A column (15 ml, 1 cm diameter) is filled with DEAE-cellulose (DE-52, Whatman, Maidstone, England) and equilibrated with the Genapol solution. The flow rate was 10 ml/hr. In all solutions, the pH was adjusted at 4°.

Procedure

Isolation of Outer Membranes. Swelling solution, 100 ml containing 0.5 mM PMSF is used for 100 mg of mitochondrial protein. The mitochondrial pellet is suspended in the swelling medium with a glass-Teflon potter. The suspension is stirred for 15 min at 4°, and then 70 ml of the shrinking medium are added while stirring. During shrinking the suspension becomes more turbid. It is further stirred for 10 min. Then 60-ml portions of the suspension are transferred into a glass-TEFLON potter, and the pestle, driven by a motor at 500–700 rpm, is moved up and down five times. The membranes are then pelleted by centrifugation for 45 min at 98,000 g in a Beckman ultracentrifuge using a 45 Ti rotor. The pellets are suspended in isolation medium with a glass-Teflon potter, then 35 ml of the sucrose step medium are filled into a Beckman SW 25.2 centrifuge tube and overlaid with 20 ml of the mitochondrial suspension, containing the membranes from about 100 mg of mitochondria. Centrifugation is performed for 60 min at 70,000 g. The small band of red outer membranes on top of the 0.95 M sucrose solution is collected with a banded Pasteur pipette. The suspension is diluted with an equal volume of Tris buffer and centrifuged for 30 min at 166,000 g in a Beckman 50 Ti rotor. The outer membrane pellets are suspended in phosphate buffer with the addition of 8% dimethyl sulfoxide (v/v) at a protein concentration of 3.5 mg/ml and stored in –20°. The yield of outer membranes is about 0.5% of protein with respect to whole mitochondria, which is about 25% of the theoretical amount. Samples should be stored not longer than 1 week, because prolonged storage alters the behavior of the membranes during the subsequent extraction procedures.

Isolation of Porin. The thawed suspension of outer membranes is first extracted with octyl glucoside, which removes most of the outer membrane proteins, except the porin.⁶ For this extraction it is important to adjust the octyl glucoside concentration to 30 mM, and the octyl glucoside-to-protein ratio to 10 (w/w), to obtain reproducible results. One milliliter of outer membrane suspension is first diluted with phosphate buffer to 2 ml, then 2 ml of 60 mM octyl glucoside solution are added. The mixture is kept at 4° for 45 min. Then it is transferred into Eppendorf tubes, which are placed in water-filled plastic adaptors and centrifuged for 30 min at 150,000 g in a Beckman 50 Ti rotor. The membrane pellets are suspended in the Genapol solution by adding 1 ml per milligram of the original outer membrane protein. They are shaken for 30 min at 4°. After centrifugation in an Eppendorf centrifuge for 5 min, the supernatant is subjected to chromatography on a DEAE-cellulose column, equilibrated with the Genapol solution. Elution is followed by monitoring the absorbance at 280 nm. The protein that elutes with the void volume is pure porin and can be stored for several months at -20°.

Porin Isolation from Whole Mitochondria⁸

Principle

Whole mitochondria are lysed with Genapol X-100 and chromatographed on hydroxyapatite, Celite, and DEAE-cellulose. This procedure gives pure and functionally active porin in high yield and can be easily scaled up.

Reagents

Buffer A: 2.5% Genapol X-100 (Farbwerke Hoechst, Frankfurt, Federal Republic of Germany), 50 mM KCl, 10 mM potassium H₂PO₄, 1 mM EDTA, 10 mM Tris adjusted to pH 7.0 with HCl

Genapol solution: 1% Genapol X-100, 5 mM Tris-HCl, pH 7.5

Dry hydroxyapatite (BioGel HTP, Bio-Rad, Richmond, California)

Dry Celite (Celite 535, Roth, Karlsruhe, Federal Republic of Germany).

Procedure

For this isolation procedure, frozen mitochondria can be used. Two Pasteur pipettes plugged with cotton wool, one filled with 0.6 g of hydroxyapatite and the other filled with 0.6 g of a dry mixture of equal weights of hydroxyapatite and Celite, are prepared. To 4-6 mg of protein of a mitochondrial pellet, 0.7 ml of buffer A are added, mixed, and kept for 30 min at 4°, followed by centrifugation for 15 min at 27,000 g. The supernatant is applied to the hydroxyapatite pipette. When the solution has entered the

column, further buffer is applied until the eluate has reached the original volume (0.7 ml). This eluate is transferred to the hydroxyapatite–Celite pipette. Again 0.7 ml of the eluate is collected, which already contains the largely enriched porin.⁸ The eluate is dialyzed overnight against 20 volumes of the Genapol solution and subjected to chromatography on DEAE-cellulose as described before.

There are two ways to scale up the isolation procedure. For up to 200 μg of porin it is recommended to use 10 Pasteur pipettes in parallel and to combine the eluates for one DEAE-cellulose column of the size described above. In order to prepare porin in larger amounts, larger columns should be used. Mitochondria (1.2 g of protein) are dissolved in 130 ml of buffer A and chromatographed on columns containing 600 g of hydroxyapatite or hydroxyapatite–Celite mixture (diameter 3 cm) and over a DEAE-cellulose column of 40 \times 3 cm. The yield is 3–4 mg of porin.

Comment

The porin contains an acid-labile bond.⁸ Precipitation of porin from detergent-containing solutions with acids should be carried out in the cold. Add one-tenth volume of 3 *M* trichloroacetic acid and one-third volume of methanol (to prevent precipitation of the detergent). After 1 hr at 0–4° the porin can be separated by centrifugation.

Properties of the Mitochondrial Porin

General Properties

The mitochondrial porin (M_r 31,000) is the major protein of the outer membrane. It represents about 20% of total outer membrane protein and 0.4% of total mitochondrial protein. Upon isoelectric focusing, and purified porin gives two bands with *pI*s of 7.7 (major band) and 7.8.⁸ The reason for this heterogeneity is not known, but porins from other sources also show this behavior.^{10,11}

From protease digestion experiments⁶ and from x-ray diffraction data,¹² it was concluded that the porin is deeply embedded into the membrane and does not extend into the aqueous phase.

It has been demonstrated that the mitochondrial hexokinase binding protein, which binds brain hexokinase^{12a} or hexokinase from fast growing tumor cells,¹³ is identical with the mitochondrial porin.^{11,14}

¹⁰ H. Tokunaga, M. Tokunaga, and T. Nakae, *Eur. J. Biochem.* **95**, 433 (1979).

¹¹ M. Linden, P. Gellerfors, and B. D. Nelson, *FEBS Lett.* **141**, 189 (1982).

¹² C. A. Mannella, *Biochim. Biophys. Acta* **645**, 33 (1981).

^{12a} J. E. Wilson, J. L. Messer, and P. L. Felgner, this volume [42].

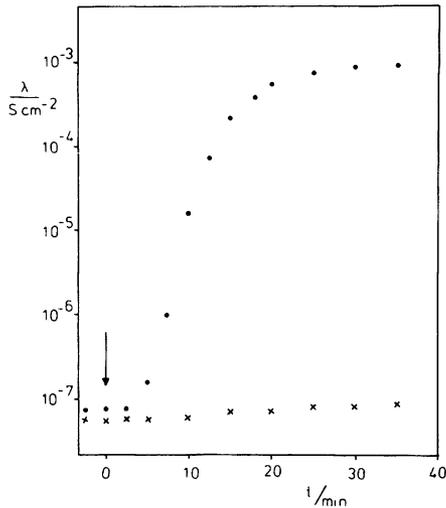


FIG. 1. Specific membrane conductance λ as a function of time after addition of 100 ng of mitochondrial porin from *Neurospora crassa* per milliliter to a black lipid bilayer membrane from asolectin-*n*-decane (arrow). The crosses represent a control experiment where only 10 μ g of Genapol X-100 per milliliter were added to another membrane; 1 M KCl, pH 6; $T = 25^\circ$.

Biosynthesis of the Porin¹⁵

Mitochondrial porin is synthesized on cytoplasmic free polysomes without an additional presequence. In contrast to the biogenesis of mitochondrial inner membrane or matrix proteins,¹⁶ assembly of the porin is not dependent on energization of the mitochondria. By digitonin treatment it could be shown that the porin precursor in an *in vitro* transfer assay is incorporated into the outer membrane. Binding to the outer membrane precedes the incorporation into this membrane.¹⁵

Reconstitution of the Porin in Lipid Bilayer Membranes

The addition of small quantities of mitochondrial porin to the aqueous phase bathing a black lipid bilayer membrane prepared according to established procedures¹⁷ results in a strong increase of the membrane conductance. A typical experiment is given in Fig. 1. Mitochondrial porin was added in a final concentration of 100 ng/ml to a black membrane from

¹³ E. Bustamente, H. P. Morris, and P. L. Pedersen, *J. Biol. Chem.* **254**, 8699 (1981).

¹⁴ C. Fiek, R. Benz, N. Roos, and D. Brdiczka, *Biochem. Biophys. Acta* **688**, 429 (1982).

¹⁵ H. Freitag, M. Janes, and W. Neupert, *Eur. J. Biochem.* **126**, 197 (1982).

¹⁶ M. Schleyer, B. Schmidt, and W. Neupert, *Eur. J. Biochem.* **125**, 109 (1982).

¹⁷ R. Benz, K. Janko, W. Boos, and P. Lauser, *Biochim. Biophys. Acta* **511**, 305 (1978).

asolectin-*n*-decane. After an initial lag of about 5 min, presumably caused by the diffusion of the protein through unstirred layers, the conductance increased by about four orders of magnitude within about 30 min. Only a slight further increase occurred after that time. The conductance increase was approximately linearly dependent on the protein concentration in the aqueous phase if the same times after addition of the protein were considered (usually 20–30 min).

A considerable asymmetry of the action of the voltage on the single conductive unit was observed, if the protein was added to only one side of the membrane.^{6,7} This indicates an asymmetric insertion of the protein into the membrane.

Figure 2 shows fluctuations of current observed with mitochondrial porin inserted into a asolectin membrane in the presence of 1 M KCl. Most of the fluctuations were directed upward, and terminating steps were only rarely observed at a membrane voltage of 5–10 mV membrane potential.⁶ This indicated that the lifetime of the pores was long at low voltage and usually exceeded 5 min.

The single conductance increment was fairly uniform in size at a membrane voltage of 5–10 mV (Fig. 3).

Lifetime and single-channel conductance were strongly dependent on the membrane potential. Whereas the single channel conductance Λ was about 4.5 nS at 5 mV (1 M KCl), it decreased at 50 mV to about 0.7 nS.⁶

The conductance of the mitochondrial porin is comparable to that of the bacterial porins under otherwise identical conditions. Assuming that the pores are filled with a solution of the same specific conductivity σ as the

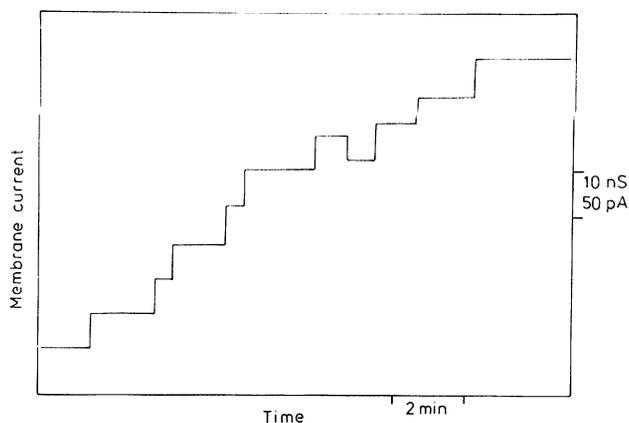


FIG. 2. Stepwise increase of the membrane current in the presence of 5 ng of mitochondrial porin per milliliter from *Neurospora crassa* added to the aqueous phase containing 1 M KCl; $T = 25^\circ$. The membrane was formed from asolectin-*n*-decane; $V_m = 5$ mV.

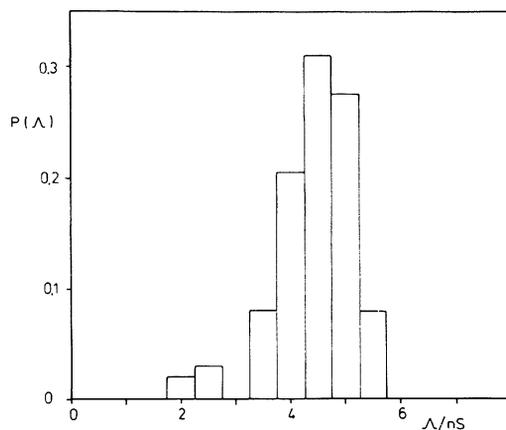


FIG. 3. Histogram of the conductance fluctuations observed with membranes from asolectin-*n*-decane in the presence of mitochondrial porin from *Neurospora crassa*. The aqueous phase contained 1 M KCl; pH 6; $T = 25^\circ$. Applied voltage was 5 mV; the mean value of all observed conductance fluctuations was 4.5 nS for 253 single events.

external solution and assuming a pore length 7.5 nm (corresponding to the thickness of the outer membranes of bacteria and mitochondria), the average pore diameter $d (= 2r)$ and the cross section can be calculated according to the equation $\Lambda = \sigma\pi r^2/l$. The table shows the diameter and the cross section for mitochondrial porins from *Neurospora crassa* and rat liver, as well as for the bacterial porins from different gram-negative bacteria.^{6,7,17-21}

¹⁸ R. Benz and R. E. W. Hancock, *Biochim. Biophys. Acta* **646**, 298 (1981).

¹⁹ R. E. W. Hancock, G. M. Decad, and H. Nikaido, *Biochim. Biophys. Acta* **554**, 323 (1979).

²⁰ H. Nikaido and T. Nakae, *Adv. Microbiol. Physiol.* **20**, 163 (1979).

²¹ R. Benz, J. Ishii, and T. Nakae, *J. Membr. Biol.* **56**, 19 (1980).

COMPARISON OF THE PORES FORMED BY MITOCHONDRIAL AND BACTERIAL PORINS IN LIPID BILAYER MEMBRANE^a

Porin	Λ (nS)	d (nm)	Area (nm ²)	Reference footnote
Mitochondrial				
<i>Neurospora crassa</i>	4.5	2.0	3.1	6
Rat liver	4.3	1.9	2.9	7
Bacterial				
<i>Escherichia coli</i>	1.9	1.3	1.3	17
<i>Salmonella typhimurium</i>	2.4	1.4	1.6	21
<i>Pseudomonas aeruginosa</i>	5.6	2.2	3.8	18

^a The pore diameter d was calculated from the pore conductance in 1 M KCl according to $\Lambda = \sigma\pi r^2/l$ (using $\sigma = 110 \text{ mS cm}^{-1}$ and length (l) = 7.5 nm).

The diameter of the pore from *Pseudomonas aeruginosa* protein F is about the same as that of the mitochondrial porin pores.¹⁸ This is consistent with the observations on the pores in reconstituted vesicles, which in both cases were permeable to hydrophilic solutes of molecular weights up to 6000.^{5,19}