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A Water-soluble Form of Porin from the Mitochondrial Outer Membrane of *Neurospora crassa*

PROPERTIES AND RELATIONSHIP TO THE BIOSYNTHETIC PRECURSOR FORM*

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Mitochondrial porin, the outer membrane pore-forming protein, was isolated in the presence of detergents and converted into a water-soluble form. This water-soluble porin existed under nondenaturing conditions as a mixture of dimers and oligomers. The proportion of dimers increased with decreasing porin concentration during conversion.

Water-soluble porin inserted spontaneously into artificial bilayers as did detergent-solubilized porin. Whereas the latter form had no specific requirements for the lipid composition of the bilayer, water-soluble porin inserted only into membranes containing a sterol, and only in the presence of very low concentrations of Triton X-100 (0.001% w/v) in the solution bathing the bilayer. The channels formed by water-soluble porin were indistinguishable from those formed by detergent-purified porin with respect to specific conductance and voltage dependence of conductance.

Water-soluble porin bound tightly in a saturable fashion to isolated mitochondria. The bound form was readily accessible to added protease, indicating its presence on the mitochondrial surface. The number of binding sites was in the range of 5–10 pmol/mg of mitochondrial protein. Water-soluble porin apparently binds to a site on the assembly pathway of the porin precursor, since mitochondria whose binding sites were saturated with the water-soluble form did not import porin precursor synthesized in a cell-free system.

Mitochondrial porin is an integral protein of the outer mitochondrial membrane. Combined electron microscopic, biochemical, and biophysical studies demonstrated its ability to form channels through which small molecules can diffuse (1–13). The diameter of the channels was estimated to be around 2 nm (3, 5–8, 13) and the upper molecular weight limit of permeants was estimated to be in the range of 2000–8000 (3, 9, 13). Purified porin could be inserted into artificial bilayers (4–6, 10). Insertion occurred in an asymmetric fashion and the channels were found to be voltage dependent. The molecular weight of the porin from *Neurospora* in the presence of SDS[1] is 31,000 (3–5, 8, 9). In the membrane, porin may form dimers or trimers (9, 11). In these and many other respects mitochondrial porin resembles the porins of the outer membrane of Gram-negative bacteria (12, 13).

Porin is synthesized on free polysomes and post-translationally imported into the mitochondria (14–16). The precursor form of this protein has the same apparent molecular weight as the mature protein—a feature which distinguishes it from the majority of mitochondrial proteins which are synthesized as precursors with a molecular weight greater than the mature protein (for review, see Refs. 17–21). The precursor form of porin has been shown to insert into the outer membrane of mitochondria *in vitro* (14–16). The transfer of porin from a postribosomal supernatant of a reticulocyte lysate programed with *Neurospora* mRNA into mitochondria did not depend on a membrane potential, in contrast to the transfer of many mitochondrial precursor proteins (17–21). Transfer of the precursor porin can be regarded as a two-stage process. The first involves recognition and binding to the outer membrane involving receptor proteins (22). In this form the bound protein is accessible to externally added protease as is the free precursor. The second stage involves the insertion of the precursor into the mitochondrial membrane and is accompanied by the acquisition of resistance or inaccessibility to added proteases.

We have investigated how the integral membrane protein porin can exist in a soluble form in the cytosol and have converted the isolated mature protein into a form in which it behaves in essentially the same manner as the precursor protein. The soluble form of porin binds to isolated mitochondria in a saturable fashion and competes with *in vitro* synthesized precursor for binding to and uptake into mitochondria and thus provides a model system to study precursor recognition and import into the outer mitochondrial membrane.

EXPERIMENTAL PROCEDURES

Growth of Neurospora crassa and Preparation of Mitochondria—*N. crassa* hyphae (wild type 74 A) were grown in Vogel's minimal medium (23) in the presence of 2% sucrose. Mitochondria from *N. crassa* hyphae were isolated either by using a grind mill (24) for large scale isolation in case of porin purification, or by grinding with sand (25) for binding studies, or by preparing protoplasts (26) for competition experiments.

Isolation of Mitochondrial Porin—Porin was purified from isolated mitochondria according to the procedure described by Freitag *et al.* (27) with several modifications. A mitochondrial pellet (700 mg of protein) was treated with 45 ml of buffer A (2.5% Genapol X-100, 50 mM KCl, 10 mM KP_i, 1 mM EDTA, 10 mM Tris-HCl, pH 7.0) at 4 °C

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate.

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under continuous stirring. The suspension was then centrifuged for 20 min at 48,400 × *g* (Beckman rotor JA-20, 4 °C). The supernatant was applied to a hydroxylapatite/Celite (1:1, w/w) column (40 × 3 cm, 4 °C) and eluted with the same buffer at a flow rate of 80 ml/h. Protein-containing fractions were collected at 6-min intervals. Fractions were pooled and dialyzed overnight against 30 volumes of buffer B (1% Genapol X-100, 10 mM Tris-HCl, pH 7.5, dialysis tubing Union Carbide 36/18). The dialyzed sample was applied to a DE52 column (9 × 1.5 cm, 4 °C) and eluted with the same buffer at a flow rate of 30 ml/h, and fractions were collected at 12-min intervals. Protein-containing fractions were tested for purity by SDS-gel electrophoresis according to Laemmli (28) and pooled. Protein was determined by the method of Lowry *et al.* (29).

Preparation of Water-soluble Porin—After addition of 1/10 volume of 3 M trichloroacetic acid and 1/3 volume of methanol, purified porin was precipitated for 1 h at 0 °C, and the precipitate was pelleted by a 20-min centrifugation at 48,800 × *g* (rotor JA-20, 4 °C) and washed twice with ethanol. The resulting protein pellet was resolubilized by vortexing with 1 volume of 0.1 M NaOH for 60 s and immediately neutralized by an equal volume of 0.2 M NaH_2PO_4. This procedure yielded water-soluble porin in 100 mM NaP_i, pH 6.8.

Sucrose Density Gradient Centrifugation—Linear sucrose gradients were prepared by mixing 2.25 ml of 5% and 2.25 ml of 20% (w/v) sucrose, both dissolved in 100 mM NaP_i, pH 6.8. After the samples (0.5 ml) were layered on top of the gradients, centrifugation was performed in a Beckman L8-70 M ultracentrifuge at 4 °C and 300,000 × *g* using a SW 65 rotor. Centrifugation time was usually 3 or 4 h. After centrifugation, fractions of 550 μl were collected. Protein at the bottom of the tube was dissolved in 550 μl of 100 mM NaP_i, pH 6.8, containing 1% (w/v) SDS. 45 μl of each fraction were analyzed by gel electrophoresis and subjected to silver staining according to Merril *et al.* (30) for detection of protein bands.

Cross-linking of Water-soluble Porin—Cross-linking of water-soluble porin was carried out with DSS according to a procedure described by Pilch and Czech (31). Water-soluble porin (30 μg/ml) was incubated for 15 min at 25 °C with a 1 mM DSS solution (prepared before use by diluting a 0.1 M DSS solution in dimethyl sulfoxide with 100 mM NaP_i, pH 6.8). The reaction was stopped by adding 1/3 volume of electrophoresis sample buffer (28) and heating the resulting solution at 95 °C for 3 min. 30-μl aliquots of each sample were analyzed by SDS-gel electrophoresis and silver staining.

Reductive Methylation of Water-soluble Porin—Reductive methylation was carried out according to Jentoft and Dearborn (32) employing [^{14}C]formaldehyde as radioactive label and sodium cyanoborohydride as reducing agent. [^{14}C]Formaldehyde (specific activity of 52.5 μCi/μmol, New England Nuclear) at a concentration of 270 nmol/nmol of porin in 100 mM NaP_i, pH 6.8, and sodium cyanoborohydride (334 nmol/nmol of formaldehyde; 48 mM stock solution in 100 mM NaP_i, pH 6.8) were consecutively added to a sample of water-soluble porin (40 μg/ml). After a 30-min incubation at 25 °C, the reaction was stopped by adding 100 mM Tris-HCl, pH 7.0. Then water-soluble porin was precipitated with 3 M trichloroacetic acid and methanol for 1 h at 0 °C and centrifuged, and the pellet was washed once with acetone. The protein pellet was resolubilized as described to a final protein concentration of 40 μg/ml.

Lipid Bilayer Experiments—Artificial lipid bilayer membranes were obtained as described previously (5, 33) from a solution of asolectin (L-α-phosphatidylcholine, Type IV-S from soybean, Sigma) or of an asolectin/cholesterol mixture (Eastman, molar ratio 1:1) in *n*-decane. The cell used for membrane formation was made from Teflon. The circular hole in the wall separating the two aqueous compartments had an area of 0.1 mm^2. The membrane current was measured through Ag/AgCl electrodes with a current amplifier (Keithley 427). The amplified signal was monitored with a Tektronix 5115 storage oscilloscope and recorded with a strip chart or a tape recorder.

Other Methods—*In vitro* protein synthesis and immunoprecipitation were carried out as described (26, 34). Phospholipid determination was performed as described earlier (35) using a colorimetric phosphate assay.

RESULTS

Preparation and Properties of Water-soluble Porin—Porin isolated from mitochondria with the nonionic detergent Genapol X-100 was first precipitated with trichloroacetic acid in the presence of methanol. To further remove detergent and phospholipids the precipitated protein was washed twice with ethanol. It was then redissolved by a short exposure (60 s) to NaOH, and the solution was neutralized with NaH_2PO_4. The recovery of porin was approximately 90%. It was not degraded by the brief alkaline treatment as shown by SDS-gel electrophoresis (Fig. 1). The water-soluble porin was apparently free of ergosterol and of phospholipids present in the initial detergent-solubilized form. The UV spectrum lacked the typical ergosterol absorption bands. Phospholipid phosphorus determination yielded a value of 0.08 mol of phosphate/mol of porin. Also, the detergent content was less than 0.5 mol/mol of porin, which was the lower limit of detection, when porin was isolated in the presence of radioactively labeled Triton X-100.

Molecular Weight of Water-soluble Porin—In order to analyze the molecular size of the water-soluble porin sucrose density gradient centrifugation and cross-linking experiments were performed. Fig. 2 shows the analysis of a density gradient by SDS-gel electrophoresis and silver staining. Marker proteins and porin were run on parallel gradients, since the sedimentation of porin was influenced by the presence of other proteins. Part of the porin sedimented with an apparent molecular weight of 60,000–70,000 in a number of experiments with different centrifugation times. It co-migrated with the marker bovine serum albumin and could be resolved from carbonic anhydrase (M_r 30,000) and phosphorylase B (M_r 96,000). This suggests a dimeric form of this fraction of water-

FIG. 1. **Gel electrophoretic analysis of water-soluble porin.** Detergent-purified porin was precipitated from a 1% Genapol solution and water-soluble porin was precipitated from 0.1 M sodium phosphate solution with 0.3 M trichloroacetic acid and 35% methanol. The precipitates were analyzed by SDS-gel electrophoresis. *Lane 1*, detergent-purified porin (0.6 μg); *lane 2*, water-soluble porin (0.6 μg); *lane 3*, molecular weight standards (bovine serum albumin, 66,000; ovalbumin, 48,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,000; lysozyme, 14,000).

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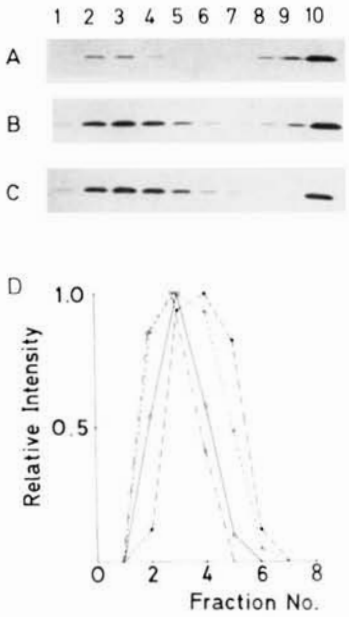

FIG. 2. **Sucrose gradient centrifugation of water-soluble porin.** *A–C*, aggregation of water-soluble porin is dependent on its concentration during resolubilization. A pellet of porin containing 28.5 μg was redissolved at concentrations of 171, 81, and 56.7 μg/ml, respectively, as described under "Experimental Procedures." The samples were then adjusted to a final volume of 0.5 ml and applied to sucrose gradients which were centrifuged for 3 h at 300,000 × *g*. Fractions were subjected to SDS-gel electrophoresis and silver staining. *Fraction 1* is the top of the gradient and *fraction 10* is the pellet. *D*, molecular weight determination. Water-soluble porin and marker proteins were centrifuged in parallel tubes. The gradients were fractionated and analyzed by SDS-gel electrophoresis and silver staining. The protein bands were quantified by densitometry. □——□, water-soluble porin; ○·—○, carbonic anhydrase (M_r 30,000); △– – –△, bovine serum albumin (M_r 66,000); ●— —●, phosphorylase *b* (M_r 96,000).

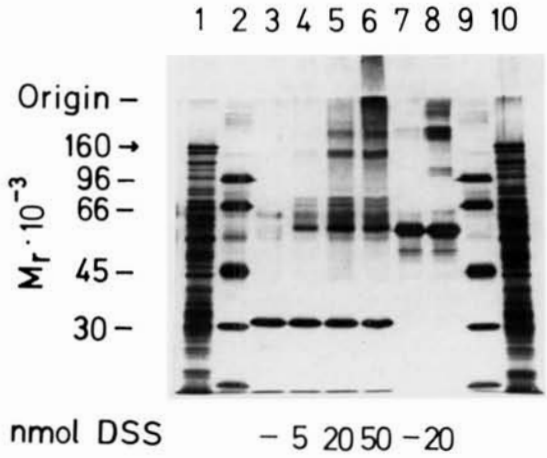

FIG. 3. **Cross-linking of water-soluble porin with DSS.** Water-soluble porin and malate synthase, respectively, in 100 mM NaP_i, pH 6.8, were incubated for 15 min at 25 °C with various concentrations of DSS in a volume of 450 μl. The amounts of water-soluble porin and malate synthase were 15.3 and 22 μg, respectively. The reaction was stopped by adding 150 μl of SDS-gel electrophoresis sample buffer (28) and heating the mixture for 3 min at 95 °C. Aliquots of 30 μl were subjected to gel electrophoresis and silver staining. *Lanes 1* and *10*, rat liver mitochondria, containing the 160,000-dalton protein carbamoylphosphate synthase (see *arrow*); *lanes 2* and *9*, molecular weight markers; *lanes 3–6*, water-soluble porin, addition of 0, 5, 20, and 50 nmol of DSS, respectively; *lanes 7* and *8*, malate synthase, addition of 0 and 20 nmol of DSS, respectively.

soluble porin. A second part of the porin was detected in the pellet fraction. An apparent molecular weight of higher than 280,000 was determined by co-sedimentation of marker protein pyruvate kinase. The proportion of the dimeric form was found to depend on the concentration at which re-solubilization of porin was carried out. At 30 μg/ml roughly 80% was in the dimeric form. Molecular sieving of water-soluble porin, prepared from cells which had been metabolically labeled with [^{35}S]sulfate, yielded essentially the same results (not shown). Dilution of the aggregated form did not lead to disaggregation. The tendency to aggregate was a salient feature of water-soluble porin, *e.g.* storage at 4 °C for longer than 4–6 h led to almost complete disappearance of the dimeric form. Also, the water-soluble porin had a strong tendency to adhere to walls of centrifuge tubes and plastic pipette tips.

Cross-linking of water-soluble porin was carried out with the bifunctional reagent disuccinimidyl suberate (Fig. 3). Upon SDS-gel electrophoresis, aggregates were found with apparent molecular weights of 60,000, 130,000, and 190,000. This suggests the formation of dimers, tetramers, and hexamers. No material was found in the region of the gel, where a trimeric form was to be expected. As a control, malate synthase from *Neurospora* was subjected to cross-linking in a parallel reaction. This enzyme consists of three identical subunits[2] as does the enzyme from yeast (36). It yielded very little cross-linked dimer and a strong cross-linked trimer. These data support the conclusion drawn from density gradient analysis that the low molecular weight form of water-soluble porin is a dimer.

Insertion of Water-soluble Porin into Artificial Lipid Bilayers—Porin isolated in the presence of nonionic detergents inserts into lipid bilayers prepared from asolectin. It forms channels whose conductivity is dependent on the potential across the bilayer with a maximal value around 4.6 nS (4, 5, 6, 10).

Water-soluble porin did not form channels when applied to black lipid bilayer membranes from asolectin/*n*-decane under the same conditions as detergent-solubilized porin (Fig. 4, *trace B*). However, channel formation did occur when two requirements were fulfilled. Firstly, the bilayer had to contain a sterol, and secondly, a detergent like Triton-X 100 or Genapol X-100 had to be present in very low concentrations (about 0.001% w/v). In the presence of these two components channel formation occurred in discrete steps (Fig. 4, *trace A*). In the absence of water-soluble porin, the detergent alone did not lead to conductivity changes. The same was found in the absence of sterol even for long duration of the experiments (Fig. 4, *trace C*). The average increment of conductance steps measured with bilayer membranes from asolectin/cholesterol was 4.5 nS (Fig. 5*A*). This value is quite similar to that observed with the detergent-solubilized protein. The latter form, however, was found to have a somewhat broader distribution of channel conductances (Fig. 5*B*) (5, 10).

The channels formed by water-soluble porin were found to be voltage-dependent in a similar way as those formed by detergent-purified porin. This is shown in Fig. 6, where voltages of +50 mV and −50 mV were applied to a membrane containing one channel from water-soluble porin. The open channel switches to different sublevels as a result of the applied voltage. The response is asymmetric with respect to the sign of the transmembrane potential. This indicates an asymmetric structure of the channel in agreement with what has been postulated for the detergent-purified porin (4–6, 10).

[2] H. Desel and W. Neupert, unpublished observations.

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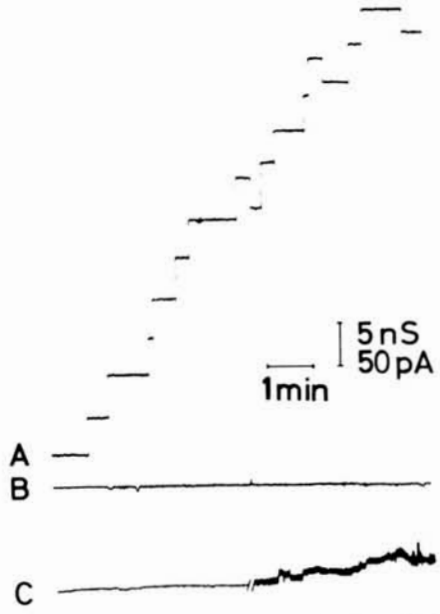

FIG. 4. **Insertion of water-soluble mitochondrial porin into lipid bilayer membranes.** In all experiments the applied voltage was 10 mV and the temperature 25 °C. *A*, the lipid bilayer membrane was made from asolectin/cholesterol (molar ratio 1:1) dissolved in *n*-decane. The aqueous phase contained 1 M KCl, 5 mM Tris-HCl, pH 7.5, 0.5 μg/ml of Triton X-100; 1.5 μg/ml of water-soluble porin was added to the aqueous phase. *B*, the lipid bilayer membrane was made from asolectin/*n*-decane. Water-soluble porin was added to the aqueous phase in a final concentration of 1.5 μg/ml 5 min before the start of the recording. *C*, conditions were the same as in *B* but with 0.5 μg/ml of Triton X-100 and 15 μg/ml of water-soluble porin. The *break* indicates a time interval of 40 min.

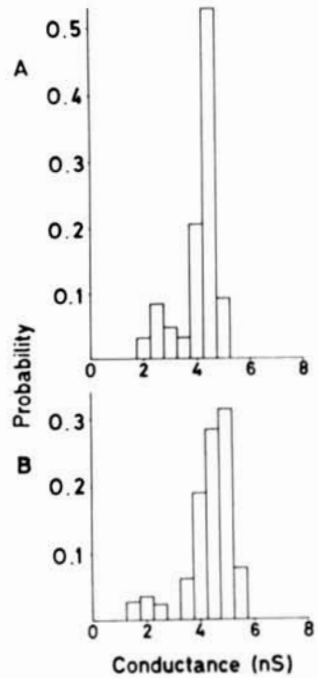

FIG. 5. **Pore size distribution of water-soluble and detergent-solubilized mitochondrial porin in lipid bilayer membranes.** *A*, membranes were made from asolectin/cholesterol (molar ratio 1:1) dissolved in *n*-decane. Water-soluble porin was added in a final concentration of 1.5 μg/ml in 1 M KCl, 5 mM Tris-HCl, pH 7.5, 0.5 μg/ml of Triton X-100. The average single channel conductance was 4.5 nS for 127 single events. *B*, membranes were made from asolectin/*n*-decane. 0.6 μg/ml of detergent-solubilized mitochondrial porin was added to the same solution as above. The average single channel conductance was 4.4 nS for 195 single events.

The frequency of pore formation in the presence of water-soluble porin was less defined than with the detergent-solubilized form. In the latter case, a distinct concentration led to a conductance which was reproducible within a factor of 3. With water-soluble porin the dose-response was only reproducible within a factor of 50. Table I shows the maximum and minimum conductances observed with the two different porin preparations. Interestingly, in many experiments the same or an even larger conductance increase was seen with identical concentrations of water-soluble as compared to detergent-purified porin. The reason for this variability may lie in the variability of the preparation of water-soluble porin with respect to its dimer-polymer relation and in the high tendency of water-soluble porin to adhere to plastic surfaces.

In summary, the reconstitution of water-soluble porin into artificial bilayers shows that: (i) the detergent and lipid-free porin can resume its functional conformation under appropriate conditions; and (ii) the insertion into a lipid bilayer and/or the formation of the original pores does not occur in an unspecific manner but has certain well-defined requirements. The requirement for sterols in the lipid bilayer may be a reflection of the presence of high amounts of ergosterol in the outer mitochondrial membrane of *Neurospora* and in the detergent-isolated porin (5, 35).

Precursor Nature of Water-soluble Porin—A major aim of this study was to find conditions under which mature porin could be converted to its extramitochondrial precursor form. Two criteria were employed to assay conversion: (i) binding of water-soluble porin to isolated mitochondria, and (ii) competition of water-soluble porin with porin synthesized in a reticulocyte lysate for binding to and uptake into mitochondria.

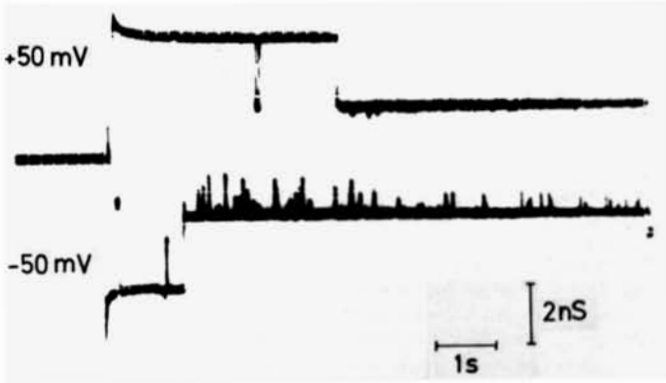

FIG. 6. **Asymmetric current response of a single channel formed by water-soluble mitochondrial porin to voltages of opposite sign.** The membrane was made from asolectin/cholesterol (molar ratio 1:1) dissolved in *n*-decane. The aqueous phase contained 1 M KCl, 5 mM Tris-HCl, pH 7.5. *Upper trace*, 50 mV were applied to the cis-side (where the mitochondrial porin was added). *Lower trace*, −50 mV were applied to the cis-side.

For the binding studies, water-soluble porin was radioactively labeled either by reductive methylation with [^{14}C]formaldehyde and cyanoborohydride or by growth of cells in the presence of [^{35}S]sulfate. With these two preparations essentially the same results were obtained, although reductive methylation yielded a higher specific radioactivity. Fig. 7 shows an SDS-gel electrophoretic analysis of mitochondria after incubation with ^{14}C-labeled porin. Various concentrations of mitochondria were employed, and as controls, binding assays were performed (i) in the absence of mitochondria, to

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TABLE I

Lowest and highest specific conductances of membranes formed from asolectin/cholesterol (molar ratio 1:1) with 100 ng/ml of detergent-purified porin or with 100 ng/ml of water soluble porin

The aqueous phase contained 1 M KCl, pH 6. The applied voltage was 10 mV; temperature 25 °C. The results were taken from 6 experiments with each porin preparation.

	Detergent-purified porin	Water-soluble porin
	S/cm^2	S/cm^2
Lowest conductance	1.2×10^{-4}	1.5×10^{-5}
Highest conductance	4.0×10^{-4}	6.0×10^{-4}

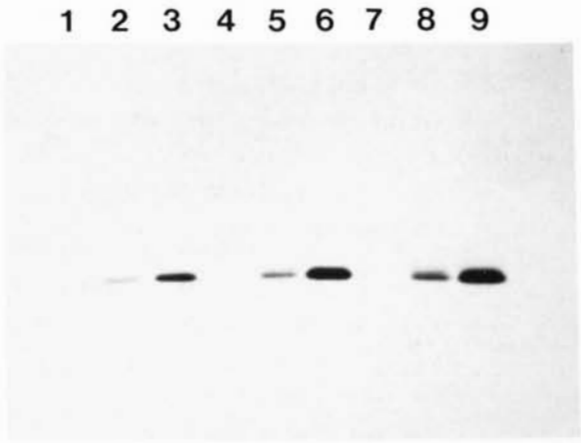

FIG. 7. **Association of water-soluble porin with isolated mitochondria.** A mitochondrial preparation (2 mg of protein) in 1 ml of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.2 (SET buffer) was divided into two halves. To one half 100 μg of trypsin inhibitor was added together with 5 μg of trypsin and incubation was performed for 15 min at 25 °C. To the second half, first, 5 μg of trypsin were added, the sample was incubated for 15 min at 25 °C and then 100 μg of trypsin inhibitor were added. Both samples were incubated for 3 min at 25 °C and then for 10 min at 0 °C. Mitochondria were reisolated, washed once in SET buffer containing 100 μg/ml of soybean trypsin inhibitor and resuspended in SET buffer at a final concentration of 2 mg/ml. Binding of water-soluble porin to mitochondria was determined in samples which were prepared by mixing 125 μl of reticulocyte lysate, 125 μl of SET buffer, and 18 μl of 0.1 M NaP_i, pH 6.8, containing 22 pmol of water-soluble porin labeled with ^{14}C by reductive methylation (specific radioactivity, 82 μCi/μmol). Three different amounts of mitochondria were employed: *samples 2* and *3*, 50 μg; *samples 5* and *6*, 100 μg; *samples 8* and *9*, 250 μg. *Samples 1*, *4*, and *7* did not contain mitochondria and served to control binding to tube walls. *Samples 2*, *5*, and *8* contained mitochondria pretreated with trypsin. *Samples 3*, *6*, and *9* contained mitochondria pretreated with trypsin in the presence of trypsin inhibitor. After incubation for 20 min at 25 °C samples were centrifuged, and the pellets were resuspended, transferred to new tubes, and centrifuged again. All were then dissolved in SDS-containing buffer and subjected to gel electrophoresis. The dried gel was fluorographed.

determine unspecific binding to tube walls and (ii) after pretreatment of mitochondria with trypsin. The latter procedure has been shown to lead to reduction of specific binding sites for porin synthesized in a cell-free system (22). It is clear from Fig. 7 that water-soluble porin associates with mitochondria in a concentration-dependent manner. Controls without mitochondria contain negligible amounts of water-soluble porin, and protease-pretreated mitochondria have a stongly reduced capacity for binding. When mitochondria were incubated with labeled porin and were then treated with proteinase K, a large part of the porin (80–90% in different experiments) proved to be proteinase-sensitive (not shown here). The water-soluble porin present in the supernatant of

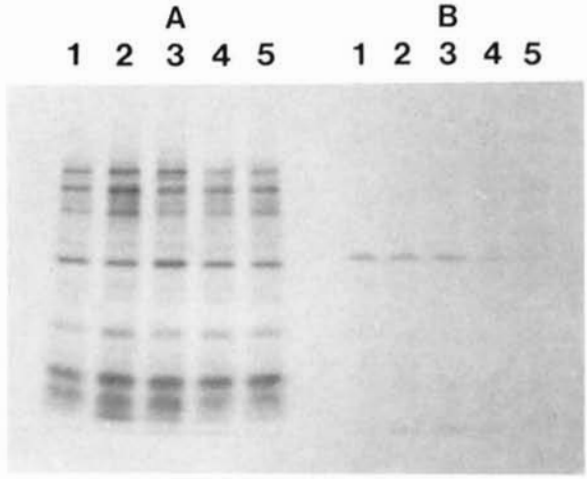

FIG. 8. **Transfer of biosynthetic porin into mitochondria after preincubation of mitochondria with water-soluble porin.** Mitochondria were incubated for 10 min at 25 °C in 110 μl of 0.1 M NaP_i buffer in the absence and presence of water-soluble porin. The mitochondria were reisolated and then incubated with 0.1 ml of reticulocyte lysate supernatant containing radioactive biosynthetic porin at 25 °C for 15 min. Mitochondria were reisolated and lysed with 200 μl of 1% Triton, 10 mM Tris-HCl, 0.3 M KCl, pH 7.4. Aliquots of 10 μl were directly analyzed by SDS-gel electrophoresis. The rest was subjected to immunoprecipitation and electrophoretic analysis as above. *A*, total mitochondria; *B*, immunoprecipitated porin. *Lane 1*, 25 μg of mitochondria, no porin added; *lane 2*, 12.5 μg of mitochondria, no porin added; *lane 3*, 25 μg of mitochondria, 15 pmol of porin; *lane 4*, 25 μg of mitochondria, 45 pmol of porin; *lane 5*, 25 μg of mitochondria, 135 pmol of porin.

the assay and biosynthetic porin precursor were found to be completely proteinase K-sensitive. These data suggest that water-soluble porin mainly binds to the surface of the mitochondria. Since this binding is saturable and of high affinity, it can be interpreted as binding to specific sites. A preliminary determination of the number of binding sites[3] yielded roughly 5–10 pmol of porin bound/mg of mitochondrial protein with a K_d of about 10^{-10} M. The portion of water-soluble porin which becomes protease-resistant appears to be inserted into the mitochondrial outer membrane in a manner similar to that shown for the biosynthetic porin precursor (14). There may be several reasons for the low rate of insertion of water-soluble porin, such as nonoptimal composition of the medium used, lack of a proteinaceous factor in the reconstituted system that is required for insertion, or modification of porin by the reductive methylation.

The result of an experiment in which water-soluble porin was employed to compete for import of porin synthesized in a reticulocyte lysate is shown in Fig. 8. Increasing amounts of water-soluble porin were added to isolated *Neurospora* mitochondria. The mitochondria were reisolated and incubated with reticulocyte lysate containing [^{35}S]methionine-labeled porin precursor. The water-soluble porin inhibited uptake of *in vitro* synthesized porin. It affected uptake of bulk precursor proteins only to a small degree. Also, incubation of mitochondria with labeled reticulocyte lysate in the presence of water-soluble porin led to inhibition of uptake of *in vitro* synthesized porin (not shown). The data in Figs. 7 and 8 suggest that water-soluble porin shares some salient features with the extramitochondrial precursor form of porin.

DISCUSSION

We describe the conversion of detergent-purified porin into a water-soluble form. Several lines of evidence strongly sug-

[3] R. Pfaller and W. Neupert, unpublished observations.

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gest that this water-soluble porin does not represent a denatured form of the membrane protein. It can be inserted into artificial bilayers under defined conditions and the inserted protein forms channels. These channels are indistinguishable from those formed by detergent-purified porin. The soluble porin competes with the biosynthetic porin for binding and uptake into the outer mitochondrial membrane. It binds to mitochondria in a saturable fashion and becomes inserted into the membrane. Thus, it has characteristics of a precursor form. In summary, it appears that the mature assembled porin can be converted into a form which is very similar to its precursor form, and this again can insert into the outer mitochondrial membrane and become a membrane protein.

Porin belongs to the small group of mitochondrial proteins whose precursors do not have a polypeptide extension (17–21). It seems reasonable to assume that with these proteins the essential step in membrane insertion is a conformational change of the precursor form. Another protein which can be converted into its precursor form and which is not synthesized as a larger precursor, is cytochrome *c*. In this case, conversion requires the removal of the covalently bound heme group by chemical means and careful renaturation (25, 37–39).

Amino acid analysis of mature porin showed the presence of a modified amino acid (5). This modification apparently does not interfere with the ability of the water-soluble form to act like a precursor. So far it is not known what the function of the modification is. It may be speculated that it has a role in the assembly of porin subunits in the outer membrane.

The binding sites on mitochondria titrated with water-soluble porin show the characteristics of receptor sites: (i) they are saturable and their number is in a range to be expected for a receptor site; (ii) they are of high affinity, comparable to that of the receptor for apocytochrome *c* (39) or to that of polypeptide hormone receptors on cell surfaces; and (iii) they are related to the uptake of porin precursor into the outer membrane, since when occupied by water-soluble porin, uptake of biosynthetic labeled precursor is inhibited. These sites may function in the recognition of the precursor and in the triggering of membrane insertion. The number of binding sites determined here would suggest that the mature porin in the outer membrane does not operate as its own receptor since its amount is much higher than that of the putative receptors (130 and 5–10 pmol, respectively, per mg of mitochondrial protein). In agreement with that, the binding sites for the porin precursor were found to be protease-sensitive (22), whereas the membrane-integrated porin is extremely resistant to added proteases (5).

The solubility of water-soluble porin as a dimer is quite low. It seems also possible that the extramitochondrial precursor pool of porin consists partly of oligomers and polymers. High molecular weight forms of precursors have been found in the case of the ADP/ATP carrier and ATPase subunit 9 (40, 41).

Formation of channels by insertion of water-soluble porin into artificial bilayers shows a peculiar requirement for sterols in the lipid phase. This may be related to the presence of large amounts of ergosterol in the outer membrane of *Neurospora* mitochondria (35). Moreover, as reported earlier (5), porin isolated in the presence of detergents contains considerable amounts of ergosterol. Water-soluble porin also requires the addition of very small amounts of nonionic detergent such as Triton X-100 or Genapol X-100 for channel formation in bilayer membranes. The role of this detergent is not known; it may be necessary for the insertion of the molecule into the bilayer or for porin to assume a conformation in the bilayer which enables channel formation. Insertion of porin into the natural target membrane, the outer mitochondrial membrane, seems to follow quite a different pathway since binding to specific proteinaceous sites is required as a first step. Clearly, the cell must have mechanisms to avoid the insertion of the porin precursor into other membranes than the outer mitochondrial membrane.

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