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Purification and Characterisation of a Pore Protein of the Outer Mitochondrial Membrane from *Neurospora crassa*

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The major protein of the outer mitochondrial membrane of *Neurospora* was purified. On dodecylsulfate-containing gels it displayed a single band with an apparent molecular weight of 31000. Reconstitution experiments with artificial lipid bilayers showed that this protein forms pores. Pore conductance was dependent on the voltage across the membrane. The protein inserted into the membrane in an oriented fashion, the membrane current being dependent on the sign of the voltage. Single pore conductance was 5nS, suggesting a diameter of 2 nm of the open pore. This mitochondrial protein shows a number of similarities to the outer membrane porins of gram-negative bacteria.

It is well documented that the outer mitochondrial membrane is freely permeable to low molecular weight components such as metal ions, substrates, nucleotides, etc. [1,2]. It is, however, impermeable to components with higher molecular weights [3]. The upper limit is not clearly defined and varies between apparent molecular weights of 2000–8000. Accordingly, unspecified channels or pores in the outer membrane have been postulated. Electron microscopic studies of mitochondrial outer membranes from different sources have shown characteristic pits in large abundance, which may represent such pores [4–6]. Recently, detergent extracts of mitochondrial outer membranes from rat liver, mung bean, and *Neurospora* were incorporated into liposomes or bilayers [7–9]. By this procedure the existence of non-specific pores could be verified.

In this paper we describe the purification of the major protein from the outer mitochondrial membrane of *Neurospora* and its insertion into lipid bilayers. This protein has an apparent molecular weight of 31000 and is firmly integrated into the membrane so that it is not digested by proteinase K added to intact or sonicated mitochondria. According to its amino acid composition, it is not particularly hydrophobic. The protein isolated from outer membranes spontaneously inserts into artificial lipid membranes. Conductance analysis indicates that this protein forms an asymmetric pore. From single-step conductance increments, an apparent pore diameter of 2 nm can be calculated. Finally, the conductance of the pore was found to depend on the membrane potential. In many respects this protein resembles the porins isolated from the outer membranes of gram-negative bacteria.

MATERIALS AND METHODS

Growth of Neurospora crassa *and Preparation of Mitochondria*

Growth of hyphae of *Neurospora crassa* (wild type 74A) for 17 h (inoculum 1×10^6 conidia/ml) and disruption of cells were carried out as described [10]. The medium for isolation of mitochondria was 0.44 M sucrose, 2 mM EDTA, 30 mM Tris/HCl pH 7.6 (adjusted at 4 °C) with the addition of phenylmethylsulfonyl fluoride (freshly dissolved in ethanol) to a final concentration of 0.5 mM. Mitochondria were isolated by centrifugation at 4 °C according to the following scheme: after spinning 20 min at $2000 \times g$, the resulting supernatant was centrifuged for 45 min at $17000 \times g$. The upper layer of the sediment (containing the mitochondria) was washed twice by resuspending and centrifuging for 45 min at $17000 \times g$.

Isolation of Outer Mitochondrial Membrane

The final mitochondrial pellet (330–400 mg mitochondrial protein, from 250–300 g wet mass of hyphae) was suspended in 300 ml of 2.5 mM K_2HPO_4, pH 7.3, 0.5 mM phenylmethylsulfonyl fluoride for 15 min at 4 °C. The suspension was stirred and 200 ml of 2.2 M sucrose, 8 mM $MgCl_2$, 8 mM ATP (pH 7.3) were added. After 10 min, the suspension was homogenized in a glass-teflon potter and the membranes were pelleted by centrifugation for 45 min at $98000 \times g$. The pellets were immediately suspended in isolation medium, homogenized, layered on 0.95 M sucrose, 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, and centrifuged for 60 min at $70000 \times g$. The red outer membranes on top of the 0.95 M sucrose solution were collected, diluted with 10 mM Tris/HCl, pH 7.5, and centrifuged for 30 min at $166000 \times g$ (see also [10]). The outer membrane pellets were suspended in 100 mM K_2HPO_4, pH 7.3, 8% dimethyl sulfoxide (v/v) at about 2 mg protein/ml and stored at −20 °C overnight. The yield of outer membrane on a protein basis was about 0.5% with respect to whole mitochondria.

Isolation of Mitochondrial Porin

The thawed suspension of outer membranes was adjusted to a protein concentration of 210 µg per 120 µl by adding 100 mM K_2HPO_4, pH 7.3, 8% dimethyl sulfoxide (v/v), and was mixed with 120 µl of a solution containing 60 mM octylglucoside, 5 mM Tris/HCl, pH 7.5, to obtain a final octylglucoside concentration of 30 mM and an octylglucoside to

Enzymes. Proteinase K (EC 3.4.21.14); trypsin (EC 3.4.21.4).

protein ratio of 10 (w/w). The mixture was kept for 45 min at 4 °C. After centrifugation for 30 min at 150000 × *g*, the membrane pellet was resuspended in 210 µl of a solution containing 1% Genapol X-100, 5 mM Tris/HCl, pH 7.5. Genapol X-100 was a gift from Farbwerke Hoechst, Frankfurt. The mixture was shaken at 4 °C for 45 min. After centrifugation in an Eppendorf centrifuge for 5 min, the supernatant was immediately subjected to chromatography on a DEAE-cellulose column. The column (15 ml, 1 cm diameter), equilibrated with 1% Genapol X-100, 5 mM Tris/HCl pH 7.5, was eluted with the same buffer at a flow rate of 10 ml/h. The protein peak which eluted with the void volume was collected and lyophilized. This fraction contained only the pore protein. The protein was stored in the lyophilized form at − 20 °C.

Preparation of Antibodies and Immunoprecipitation

The isolated protein (60 µg, dissolved in 0.2 ml 1% Genapol X-100, 5 mM Tris/HCl pH 7.5) was mixed with an equal volume of incomplete Freund's adjuvant and injected intradermally into the neck region of a rabbit. Three weeks later the procedure was repeated with 75 µg antigen. After another two weeks, 70 µg antigen were applied subcutaneously and one week later, the rabbit was bled from the ear vein. The immunoglobulin fraction was prepared by ammonium sulfate precipitation. Immunoprecipitation was carried out exactly as described previously [11], except that *p*-chloromercuribenzoate was omitted.

Gel Electrophoresis and Isoelectric Focusing

Gel electrophoresis was performed with slab gels (17.5% acrylamide) according to Laemmli [12]. When gels were sliced, electrophoresis was performed as described before [13]. Isoelectric focusing was carried out according to O'Farrell [14].

Amino Acid Analysis

The purified mitochondrial porin was dialysed against 100 mM ammonium bicarbonate, 0.1% Triton X-100 in Visking tubing 18/23 and subsequently hydrolysed for 20 h in 6 M HCl at 108 °C, with and without the addition of 7 mM mercaptoethanol. Amino acid analysis was performed with a Liquimat 3 (Kontron) by fluorescence and ninhydrin analysis.

Insertion of the 31000-M_r Protein into Lipid Bilayer Membranes

Artificial lipid bilayer membranes were obtained as described previously [15, 16] from a 1−2% (w/v) solution of two different lipids dissolved in *n*-decane (Fluka, Buchs, Switzerland, purum). Asolectin was purchased from Sigma Chemical Company and oxidized cholesterol was prepared as described earlier [16]. The chamber used for bilayer formation was made from Teflon. The circular hole in the wall between the two aqueous phases had an area of either 1.5 mm^2 (for the macroscopic conductance measurements) or 0.1 mm^2 (for the single channel experiments). The temperature was kept at 25 °C throughout. All salts were obtained from Merck (Darmstadt, FRG, analytical grade). The aqueous solutions were buffered with 5 mM Tris/HCl and were adjusted to pH 7.5. Variation of the pH between 6 and 7.5 had no influence on the results. The protein was added to the aqueous solution from a stock solution (66 ng protein/ml, 1% Genapol X-100, 5 mM Tris/HCl, pH 7.5) either immediately before membrane formation or after the membrane had completely turned black in order to prevent protein inactivation.

For the electrical measurements, Ag/AgCl electrodes were inserted in the aqueous compartments on both sides of the membranes. The stationary current measurements were performed with a Keithley 610 C electrometer. A Keithley 427 current amplifier was used for the single channel experiments. The amplified signal was monitored with a Tektronix 7633/7A22 storage oscilloscope and recorded with a strip chart recorder. The bandwidth of the single channel experiments was between 300 Hz and 3 kHz.

RESULTS

Purification of the Pore Protein

Outer membranes were prepared from *Neurospora* mitochondria according to a swelling-shrinking procedure developed for isolation of rat liver outer mitochondrial membranes. This procedure was modified for the separation of *Neurospora* mitochondrial membranes as described previously. Gel electrophoretic analysis of *Neurospora* outer mitochondrial membranes displays a prominent protein band with an apparent molecular weight of 31000 (see Fig. 1), as noted earlier [10]. A prominent band with such a molecular weight has also been observed in outer mitochondrial membranes from mung bean and potato [17], rat liver [9], and beef heart [18].

To isolate this protein, *Neurospora* outer mitochondrial membranes were first resuspended in 30 mM octylglucoside containing 5 mM Tris/HCl, pH 7.3. By this procedure, most of the minor protein components were extracted. The 31000-M_r protein was recovered after centrifugation in the membrane pellet (Fig. 1). In a subsequent step, this pellet was extracted with the nonionic detergent, Genapol X-100, which contains polyethoxy chains like Triton X-100, but has isotridecyl groups instead of aromatic rings and therefore does not absorb at 280 nm. As shown in Fig. 1, the Genapol extract is largely enriched in the 31000-M_r protein. Subsequent chromatography on DEAE-cellulose yields the pore protein which upon gel electrophoresis shows a single band without contaminating minor components (Fig. 1).

About 15% of total outer membrane was recovered as the purified 31000-M_r protein. The protein was analysed by isoelectric focusing according to the method of O'Farrell [14], employing ampholines in the pH range of 3−10. A double band was seen with isoelectric points at pH 7.7 (major band) and pH 7.8 (minor band).

Chemical Properties of the Pore Protein

Fig. 2 shows the ultraviolet spectrum of the isolated protein. This spectrum shows that the preparation contains approximately 25−30 mol ergosterol/mol of protein. Fig. 2 includes a spectrum of ergosterol in hexane. The outer mitochondrial membrane of *Neurospora* contains large amounts of ergosterol [19], which can be easily extracted into a hexane phase. Extraction of the isolated protein in Genapol X-100, 5 mM Tris/HCl, pH 7.5, with hexane failed to transfer ergosterol into the hexane phase.

In order to test if this protein was a glycoprotein, 200 µg of total outer membrane protein was subjected to electrophoresis in the presence of dodecylsulfate and subsequently stained for carbohydrate using the method of Fairbanks et

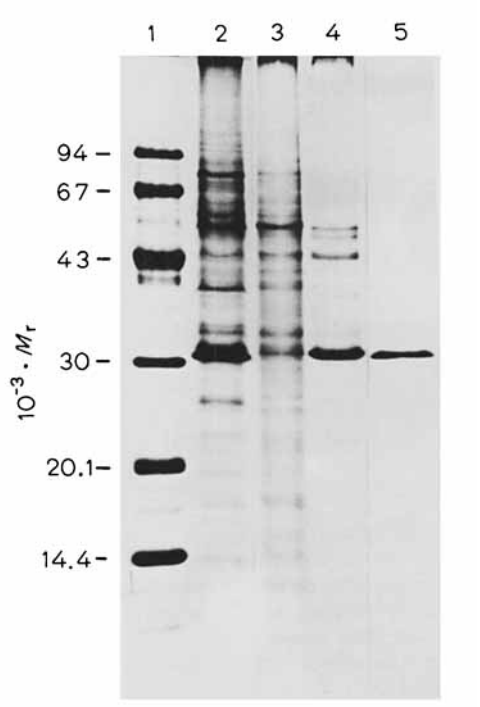

Fig. 1. *Isolation of the mitochondrial porin.* The various fractions obtained during the isolation procedure were analysed by gel electrophoresis in the presence of dodecylsulfate. Gels were stained with Coomassie blue. (1) Molecular weight standards; (2) total outer membrane; (3) proteins extracted with octylglucoside; (4) proteins extracted from the residual membrane with Genapol X-100; (5) purified protein obtained by DEAE-cellulose chromatography of the Genapol X-100 extract

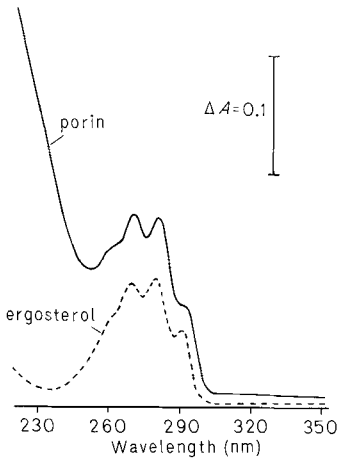

Fig. 2. *Ultraviolet spectrum of purified pore protein.* The purified 31000-M_r protein (12 µg/ml) in 1% Genapol X-100, 5 mM Tris/HCl, pH 7.5, was measured against 1% Genapol X-100, 5 mM Tris/HCl, pH 7.5 (——); ergosterol was dissolved in hexane (8.7 µM) and measured against hexane (– – – –)

al. [20]. The gel showed a few positive bands in the higher molecular weight range but no carbohydrate staining of the 31000-M_r band (comprised of at least 50 µg of protein) was seen. This observation suggests that the pore protein is not a glycoprotein.

Table 1 shows the amino acid composition of the protein. The minimum molecular weight calculated from the amino acid composition is 31800. Taking into account the possible

Table 1. *Amino acid analysis of the pore protein*
The values represent the mean of three determinations. Results are given as residues/molecule with the nearest integer in brackets. n.d., not determined

Amino acid	Amount present	
Aspartic acid	33.52	(34)
Threonine	29.67	(30)
Serine	23.32	(23)
Glutamic acid	8.82	(9)
Proline[a]	7.92	(8)
Glycine	28.40	(28)
Alanine	40.26	(40)
Valine	22.88	(23)
Methionine	6.15	(6)
Isoleucine	11.68	(12)
Leucine	17.94	(18)
Tyrosine	10.06	(10)
Phenylalanine	20.10	(20)
Lysine	25.87	(26)
Histidine	8.76	(9)
Xaa[b]		
Arginine	7.16	(7)
Tryptophan	n.d.	
Cysteine	n.d.	

[a] Single determination.
[b] The integral of this unknown peak is in the same range as those of histidine and arginine.

presence of tryptophan and cysteine as well as the presence of a modified amino acid, this value would be a little bit higher, but still agrees quite well with the apparent molecular weight of 31600 ± 400 derived from gel electrophoresis in the presence of dodecylsulfate. The content of hydrophobic amino acids was not particularly high. An unidentified component was found in the basic region (component X in Table 1) which may represent a modified basic amino acid. It should be noted that bacterial pore-forming outer membrane proteins also do not have an especially high content of hydrophobic amino acids, but do contain modified amino acids such as allysine [21].

Antibodies against the Pore Protein

Antisera against the purified protein were raised in rabbits (see Materials and Methods). Immunoprecipitation was performed employing isolated mitochondria from cells labelled with [^{3}H]leucine and the precipitate was analyzed by electrophoresis. When sufficient antibody was added to obtain complete precipitation, 0.4% of total mitochondrial protein was found in the band corresponding to porin.

The specificity of the precipitation is demonstrated in Fig. 3. The antibody precipitated a single radioactive polypeptide whose apparent molecular weight was identical to that of the isolated pore protein. Furthermore, excess unlabelled pore protein was able to compete with the radioactive protein from the mitochondria for binding to the antibody (Fig. 3). The specificity of the antibody was also shown by comparing the immunoprecipitated pore protein with isolated or immunoprecipitated ADP/ATP carrier and cytochrome c_1, two major mitochondrial membrane proteins with similar apparent molecular weights. These two proteins have migrations distinctly different from that of the pore protein on dodecylsulfate containing gels (Fig. 3).

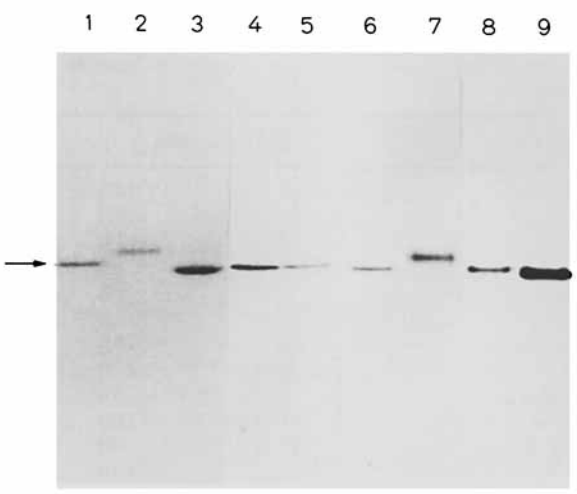

Fig. 3. *Specificity of immunoprecipitation of the pore protein from lysed mitochondria. Neurospora* cells were grown in the presence of [^{35}S]sulfate (0.5 mCi/l). The mitochondria were isolated, lysed with 1% Triton X-100, 0.3 M KCl, 10 mM Tris/HCl, pH 7.5, at a concentration of 1 mg protein/ml, then subjected to immunoprecipitation with antibodies against the mitochondrial porin, cytochrome c_1 and ADP/ATP carrier. Immunoprecipitates were analyzed by gel electrophoresis in the presence of dodecylsulfate and by autoradiography. Immunoprecipitation of the pore protein was also performed after addition of 7 µg of isolated pore protein to 1 mg of lysed mitochondria. For comparison, the isolated proteins were also subjected to gel electrophoresis and the gels were stained with Coomassie blue. (1–5) Immunoprecipitates: (1, 4) pore protein; (2) cytochrome c_1; (3) ADP/ATP carrier; (5) pore protein after addition of unlabelled isolated pore protein to the mitochondrial lysates. (6–9) Isolated and stained proteins: (6, 8) porin; (7) cytochrome c_1; (9) ADP/ATP carrier

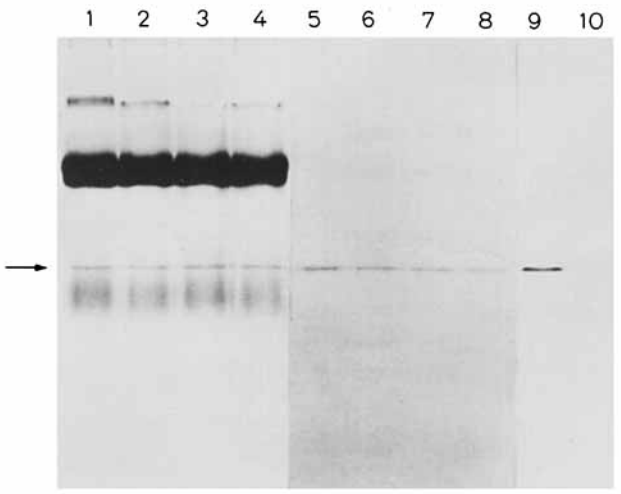

Fig. 4. *Sensitivity of the pore protein to proteases.* Mitochondria were isolated from cells grown in the presence of [^{35}S]sulfate (0.5 mCi/l). Protease digestion was performed with 100 µg proteinase K/ml for 45 min at 4 °C and stopped with the addition of 500 µM phenylmethylsulfonyl fluoride (final concentration). After protease treatment, the pore protein was immunoprecipitated and subjected to gel electrophoresis and autoradiography. (1–4) Stained gels; (5–10) autoradiographs. (1, 5) Control; (2, 6) mitochondria were incubated in 0.44 M sucrose, 10 mM Tris/HCl, pH 7.5, 2 mM EDTA with proteinase K; (3, 7) mitochondria were sonicated for 10 s in the presence of proteinase K; (4, 8) mitochondria were dissolved in 1% Triton X-100, 0.3 M KCl, 10 mM Tris/HCl, pH 7.5, and then incubated with proteinase K. 1 mg of mitochondria from [^{35}S]sulfate-labelled cells were dissolved in 1 ml 0.3% sodium dodecylsulfate, 10 mM Tris/HCl, pH 7.5. One sample was incubated for 30 min at 23 °C without further addition. A second sample was incubated for the same length of time after addition of 100 µg trypsin/ml and stopped with a 1.5-fold molar excess of soybean trypsin inhibitor. Triton X-100 and NaCl were added to both samples to a final concentration of 1% Triton X-100, 0.3 M NaCl, and the pore protein was immunoprecipitated. (9) Without trypsin treatment; (10) with trypsin treatment

Proteinase Sensitivity of the Pore Protein

In order to determine whether the pore protein in the membrane is susceptible to digestion by added protease, isolated mitochondria were incubated with proteinase K. After inactivating the protease with phenylmethylsulfonyl fluoride, the pore protein was immunoprecipitated from the mitochondria after lysis with Triton X-100. As shown in Fig. 4, this treatment did not lead to degradation of the assembled membrane protein. When mitochondria were sonicated after addition of proteinase K, the pore protein could still be immunoprecipitated.

When mitochondria were solubilized with Triton X-100 and then treated with proteinase K, 70% of the pore protein was recovered in the immunoprecipitate. In contrast, the precursor of the pore protein synthesized in a reticulocyte lysate was completely digested by added proteinase K (H. Freitag and W. Neupert, unpublished). When mitochondria were dissolved in buffer containing 0.3% sodium dodecylsulfate and then treated with proteinase K, the pore protein could no longer be immunoprecipitated. The same pattern of resistance and sensitivity to proteolytic attack was observed with trypsin.

The major outer membrane protein in mung bean mitochondria, which probably corresponds to mitochondrial porin, was reported by Mannella and Bonner to be resistant to added trypsin [22]. Together with their X-ray diffraction data, this led them to conclude that this protein does not extend far into the aqueous phase [23].

These results suggest that the protein is either not accessible to proteinase K when integrated into the membrane, or

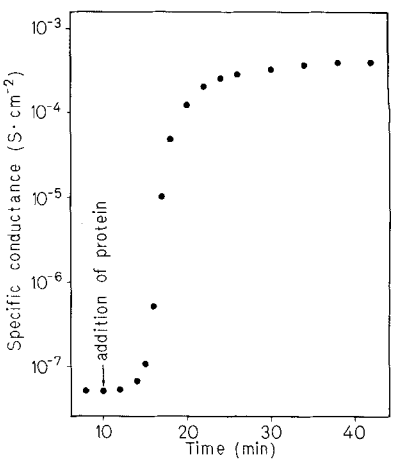

Fig. 5. *Insertion of the isolated pore protein into artificial membranes.* The specific membrane conductance λ increases after the addition of the 31000-M_r protein to the aqueous phase. The protein was added 10 min after blackening of the membrane. Concentration of the protein was 66 ng/ml. The aqueous phase contained 1 M KCl and 10 µg/ml Genapol X-100. The applied voltage was 5 mV

it does not have exposed sites sensitive to proteinase K. Solubilization with the nonionic detergent Triton does not lead to the exposure of sites sensitive to proteinase K, or alternatively, the detergent surrounds and shields the protein. Only

the precursor form and the dodecylsulfate-denatured form have exposed sites sensitive to proteinase K.

Macroscopic Conductance Measurements with Artificial Bilayers

The addition of the 31 000-M_r protein to the aqueous phase before or after membrane formation leads to a strong conductance increase of bilayer membranes. Fig. 5 shows such an experiment. A membrane was formed from asolectin and the protein was added to the aqueous phase while stirring. After an initial lag of 3 min, presumably due to diffusion of the protein through unstirred layers, the conductance increased by more than three orders of magnitude within about 20 min. Only a slight increase occurred after that time. The same reaction was observed when the protein was added to both aqueous compartments. However, asymmetrical addition caused an asymmetric current voltage relationship (see below). In control experiments, the membrane was formed in a solution of salt and detergent of the same concentration but without protein. No significant conductance increase was seen.

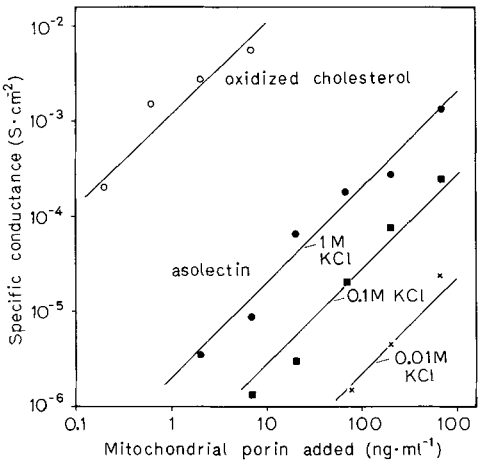

Fig. 6. *The specific membrane conductance λ depends on the concentration of the pore protein in the aqueous phase.* Membranes were formed from lipids dissolved in *n*-decane. The aqueous phase contained 0.01 – 1 M KCl and less than 100 µg/ml Genapol X-100. The conductance values were derived from at least three experiments 20 min after the addition of the protein or after blackening of the membranes (if the protein was present prior to membrane formation). The applied voltage was 5 mV

Fig. 6 shows the dependence of conductance on the protein concentration in the aqueous phase. A linear relationship was observed over a wide range. The conductance increase was strongly dependent on the lipid composition of the bilayer (Fig. 6). With membranes from oxidized cholesterol, the conductance was about two to three orders of magnitude larger than with membranes formed by asolectin. A similar lipid 'specificity' has been observed with porins from various outer membranes from bacteria. Surface tension or surface pressure may be different in membranes from oxidized cholesterol and phospholipids, thus allowing insertion of protein molecules at different rates.

Fig. 6 also shows that the conductance was a linear function of the KCl concentration within the limits of experimental error (see also Table 3). This indicates that the rate of protein incorporation was not dependent on the salt concentration in the aqueous phase. Accordingly, the interaction between protein and membrane appears to be of hydrophobic nature.

The asymmetric addition of the protein to only one side of the membrane results in an asymmetric current response to voltage of opposite sign. This is illustrated in Fig. 7. A membrane was formed from asolectin, and the 31 000-M_r protein was added to one side (*cis* side). The conductance increased within 20 min as described above. Then a voltage of 25 mV with opposite polarity was applied. At first, the polarity was negative at the *cis* side (Fig. 7, lower trace). The initial current decreased exponentially with a time constant of 1 – 2 s. Then the voltage was removed for about 1 s and switched to the opposite sign (positive at the *cis* side) (Fig. 7, upper trace). The conductance was completely recovered. No decay of the membrane current was observed when the membrane potential was positive at the *cis* side. This effect was reversible. Only at higher voltages above about 50 mV positive at the *cis* side would the membrane current decrease. These results indicate that the protein is inserted asymmetrically into the membrane.

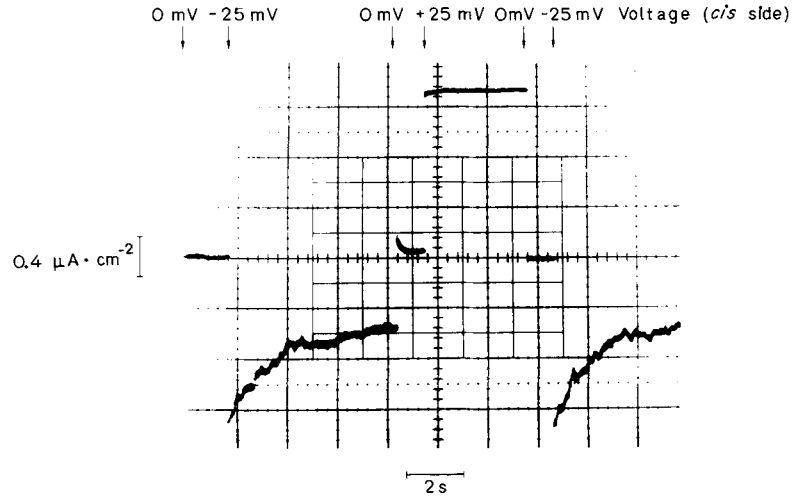

Fig. 7. *Asymmetric membrane current response after application of voltage of different sign.* A membrane was made from asolectin/*n*-decane. The protein concentration in the aqueous phase containing 1 M KCl was 33 ng at the *cis* side. A voltage of – 25 mV and + 25 mV was applied to the membrane (as referred to the *cis* side) about 20 min after addition of the protein. Note that the membrane current decreases only if the *cis* side is switched to a negative potential

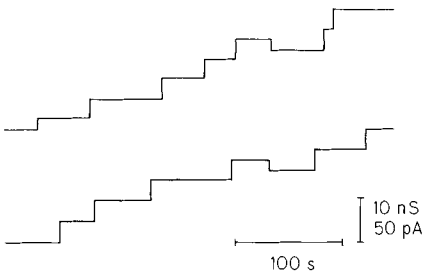

Fig. 8. *Stepwise increase of the membrane current after the addition of the pore protein.* The aqueous phase contained 3.3 ng/ml protein, 1 M KCl, and 0.5 μg/ml Genapol. The membrane was formed from asolectin dissolved in *n*-decane. The applied voltage was 5 mV; the current prior to the addition of the protein was less than 0.2 pA. The record starts at the left end of the lower trace and continues in the upper trace

Single Channel Analysis

When the 31000-M_r protein was added to the aqueous phase at low concentrations (1 – 5 ng/ml), the membrane current at a given voltage started to increase in a stepwise fashion. These current fluctuations were not observed when only the detergent Genapol was added. Fig. 8 shows an experiment in which the membrane potential was set at 5 mV. In the beginning, most of the steps were directed upwards, whereas terminating steps were rarely observed. In most cases, the terminating steps had only about half the amplitude of the steps directed upwards. Later, when terminating steps became more frequent, fluctuations directed upwards also appeared which had about half the amplitude of the large earlier steps. These small steps were only a small fraction of all the conductance fluctuations at a membrane potential of 5 mV.

Fig. 9A presents a histogram of all conductance fluctuations recorded at a membrane potential of 5 mV with membranes formed from asolectin. The large steps (8 – 10 nS) were presumably caused by the insertion of the protein aggregates containing more than one pore. Steps with a larger amplitude than two single units were only rarely observed (not included in Fig. 9A). The mean of all conductance fluctuations was 5 nS. If only fluctuations up to 6 nS are considered, the mean value decreases to 4.6 nS.

At higher membrane potentials, the pores did not switch off completely. The pore may have different conductance states depending on the magnitude of the membrane potential. Fig. 9B shows a histogram of the conductance fluctuations directed upwards at a membrane potential of 20 mV. The most frequently assumed Λ value is 2.8 nS and is thus considerably smaller than the Λ value assumed at 5 mV (5 nS).

The decrease in pore conductance with increasing voltage was only observed when the *cis* side was switched to a negative potential. Terminating steps were only rarely observed when the *cis* side had a positive polarity, thus pointing to a strong asymmetry of the pore. Table 2 contains the values of the single channel conductance $\bar{\Lambda}$ measured at various membrane potentials. Besides the average conductance increment $\bar{\Lambda}_{on}$, the $\bar{\Lambda}_{off}$ values are also given. n_{on} and n_{off}, respectively, are the numbers of events from which Λ_{on} and Λ_{off} have been calculated. Apparently, a difference exists between the average conductance of opening and closing pores, at least at low voltage. This may be caused by the voltage dependence of the conductance states and the fact that the first opening of a pore always has a conductance value between 4 nS and 6 nS.

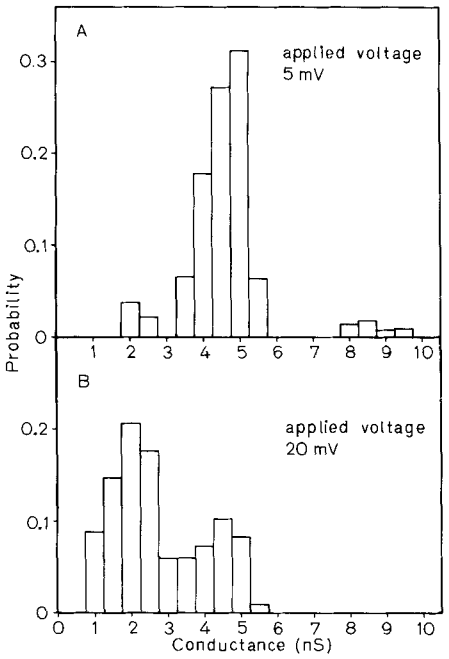

Fig. 9. *Histogram of conductance fluctuations.* Membranes were prepared from asolectin dissolved in *n*-decane. The protein concentration was 3.3 ng/ml. The aqueous phase contained 1 M KCl. (A) Applied voltage, 5 mV; the mean value of all observed conductance fluctuations was 5 nS for $n = 489$ single events. (B) Applied voltage, 20 mV; the mean value of all upward directed conductance fluctuations was 2.8 nS for $n = 525$ single events

Table 2. *Average conductance increments $\bar{\Lambda}_{on}$ and $\bar{\Lambda}_{off}$ as a function of the applied membrane potential* V_m
The aqueous phase contained 1 M KCl, 1 – 5 ng/ml pore protein and less than 0.8 μg/ml Genapol X-100; $t = 25$ °C. The membranes were formed from asolectin dissolved in *n*-decane. n_{on} and n_{off} are the numbers of events from which $\bar{\Lambda}_{on}$ and $\bar{\Lambda}_{off}$ have been calculated

V_m	$\bar{\Lambda}_{on}$	n_{on}	$\bar{\Lambda}_{off}$	n_{off}
mV	nS		nS	
5	5.0	489	4.7	54
10	3.5	231	2.8	175
20	2.8	525	1.7	295
30	1.4	160	1.2	133
50	0.72	347	0.70	211
100	0.48	441	0.45	327

Table 3. *Dependence of the average conductance increments $\bar{\Lambda}$ on the concentration and type of salt solution*
The aqueous phase contained 1 – 5 ng/ml pore protein and less than 0.8 μg/ml Genapol X-100. Membranes were formed from asolectin dissolved in *n*-decane. The applied membrane potential was 5 mV. *n* is the number of events from which $\bar{\Lambda}$ has been calculated

Salt	Concentration	$\bar{\Lambda}$	*n*
	M	[nS]	
NaCl	1	4.0	174
KCl	1	5.0	489
KCl	0.1	0.55	241
KCl	0.01	0.053	145
RbCl	1	5.2	255
$MgCl_2$	0.5	2.4	126
K_2SO_4	0.5	2.9	159

Table 3 gives the average conductance increments for different salts and concentrations. A hundred-fold decrease of the KCl concentration (from 1 M to 10^{-2} M) led to the same decrease of the conductance of the pore. This indicates that the pore cannot be saturated. The same conclusion can be drawn from the experiments in the presence of divalent cations and anions. The pore conductance follows roughly the conductance sequence of the salts in aqueous solution.

DISCUSSION

We have isolated and purified the major protein component of the outer mitochondrial membrane of *Neurospora*. This protein is firmly integrated into the outer membrane. The isolated protein contains considerable amounts of ergosterol, a major lipid component of the outer mitochondrial membrane of *Neurospora* [19]. This supports the view that the protein has a hydrophobic outside. The amino acid composition does not group this protein among the very hydrophobic proteins. This may indicate that the major outer mitochondrial membrane protein has hydrophilic as well as hydrophobic domains.

The studies on the incorporation of the purified protein into artificial membranes define its function as a pore or channel-forming component. Reconstitution at very low protein concentrations in the aqueous phase show an increase of the membrane conductance in distinct steps; this single conductance unit most probably corresponds to a reconstituted channel. The single channel conductance is about 4.5 nS in 1 M KCl. Assuming a pore length l of 7 nm (corresponding to a similar thickness of the outer mitochondrial membrane), and assuming that the pore is filled with a solution of the same specific conductance σ as the external solution, the average pore may be calculated according to $\Lambda = \sigma\pi r^2/l$ as being on the order of 2 nm in diameter. An interesting result of these studies is the asymmetric insertion of the porin into the membrane. It may indicate that the porin is asymmetric in nature and prefers one direction when it incorporates into the bilayer membrane. Another result of our study is that the pore conductance is dependent on the applied membrane potential. Between 5 mV and 100 mV the average conductance increment Λ decreases by a factor of about 10. We conclude that the pore has several different states as a function of the applied voltage rather than only two states (open and closed). These reconstitution studies do not tell whether *in vivo* the permeability of the outer membrane is also regulated by a potential across the membrane. A Donnan potential or an intrinsic voltage generated by an asymmetry in surface potential could be involved in regulating the permeability of the outer membrane.

Taken together, the information from these studies allows us to conclude that the major protein from *Neurospora* outer mitochondrial membrane is a pore or channel-forming entity.

The presence of channels allowing the diffusion of small molecules has been inferred from permeability studies of whole mitochondria [1,2]. Colombini [7,8] has shown that insertion of detergent-solubilized proteins from various mitochondrial sources (*Paramecium*, rat liver, *Neurospora*, and yeast) into planar bilayers produces channels for small ions. The channel-forming activity was enriched when outer membrane fractions were used. It was proposed that this pore protein might be a glycoprotein [24]. Our experiments, however, strongly suggest that the pore-forming protein is not a glycoprotein. In agreement with the data presented here, these channels were found to be voltage-dependent. Also, a conductance around 4.5 nS was found with these mitochondrial detergent extracts. Liposomes produced from mixtures of phospholipids and mitochondrial membrane material from *Neurospora* were found to be permeable to polyethylenglycol up to an M_r of 3400. This would correspond to a channel diameter of 4 nm. Membrane vesicles were also prepared from fragments of outer membranes of rat liver mitochondria and mung bean mitochondria by Zalman et al. [9]. A detergent extract enriched in a 30000-M_r protein had the highest pore-forming activity. In this case, the exclusion limit of the channels was found around an M_r of 2000 – 8000. This could be consistent with the pore diameter calculated from our data. It should, however, be kept in mind that the calculations and the direct measurements employing polydisperse polysaccharides or polyalcohols cannot give precise data on pore size. Furthermore, permeability may depend on the form of the permeating substances and on their surface charge.

Pores in the outer mitochondrial membranes have also been investigated using a morphological approach. Negative staining of isolated outer membranes from mitochondria of various species have revealed abundant stain filled pits, 2 – 3 nm in diameter [4 – 6]. X-ray diffraction studies on pellets of plant outer mitochondrial membranes suggest that a protein with an M_r of 30000 forms a prominent subunit structure with an outer diameter of 5.0 nm and an inner low-electron-density core of 1.8 – 2 nm [22,23]. This inner core may correspond to the channel diameter and could thus agree with the data discussed above.

In summary, all these studies support the view that even small proteins such as cytochrome *c* (12000 M_r) cannot penetrate through outer mitochondrial membranes. This conclusion has also been reached using a different approach [3].

Finally, freeze-fracture studies have presented some evidence that the particle clustering of the outer membrane is dependent on the energetisation of the mitochondria [25]. This phenomena may be related to the voltage dependence and asymmetry of the porin described in this report.

The mitochondrial porin shares a number of chemical and biophysical properties with the bacterial porins, which have been extensively studied [26 – 34]. Its apparent molecular weight is similar to those of the bacterial porins, which range between 30000 and 38000. The mitochondrial porin is composed of 45.5% polar amino acids according to the definition by Capaldi and Vanderkooi [35]. Similar amounts are found in porins of *Salmonella* (48.5%) [26] and *Escherichia coli* (matrix protein, 45.3%) [36]. The bacterial porins are relatively rich in threonine, which is also true for the mitochondrial porin. Furthermore, both bacterial and mitochondrial porins contain very little or no cysteine. Also, both types of porin contain modified amino acids. A difference between bacterial and mitochondrial porins, however, is that the bacterial porins are rather acidic with pI values around 4.8 [26] and 6 [36], while the *Neurospora* porin has a pI around 7.7.

Porins from outer bacterial membranes show a similar conductance if reconstituted in lipid bilayer membranes. The reported conductance in the presence of 1 M KCl varies between 1.8 nS with *E. coli* [32] and 5.6 nS with *Pseudomonas aeruginosa* [28]. A voltage-dependence of the channels in planar bilayers which were formed from *E. coli* outer membranes has recently been described [34].

The quantitative immunoprecipitation of the mitochondrial porin makes it possible to calculate the number of particles in the outer mitochondrial membrane. Assuming

the dry weight of a single mitochondrion to be 0.1 pg [37] with a diameter of 1 μm, approximately 4×10^{11} porin molecules are present per cm^2. This is comparable to the density of porins in the *E. coli* outer membrane of $10^{12}/cm^2$ [28].

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REFERENCES

1. O'Brien, R. L. & Brierley, G. (1965) *J. Biol. Chem. 240*, 4527–4531.
2. Pfaff, E., Klingenberg, M., Ritt, E. & Vogell, W. (1968) *Eur. J. Biochem. 5*, 222–232.
3. Wojtczak, L. & Zaluska, H. (1969) *Biochim. Biophys. Acta. 193*, 64–72.
4. Parsons, D. F., Bonner, W. D. & Verboon, J. G. (1965) *Can. J. Bot. 43*, 647–655.
5. Parsons, D. F., Williams, G. R. & Chance, B. (1966) *Ann. N.Y. Acad. Sci. 137*, 643–666.
6. Stoeckenius, W. (1970) in *Membranes of Mitochondria and Chloroplasts* (Racker, E., ed.) pp. 53–90, von Nostrand Reinhold Co., New York.
7. Colombini, M. (1979) *Nature (Lond.) 279*, 643–645.
8. Colombini, M. (1980) *J. Membr. Biol. 53*, 79–84.
9. Zalman, L. S., Nikaido, H. & Kagawa, Y. (1980) *J. Biol. Chem. 255*, 1771–1774.
10. Neupert, W. & Ludwig, G. D. (1971) *Eur. J. Biochem. 19*, 523–532.
11. Zimmermann, R. & Neupert, W. (1980) *Eur. J. Biochem. 109*, 217–229.
12. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
13. Korb, H. & Neupert, W. (1978) *Eur. J. Biochem. 91*, 609–620.
14. O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
15. Benz, R., Stark, G., Janko, K. & Läuger, P. (1973) *J. Membr. Biol. 14*, 339–364.
16. Benz, R., Janko, K., Boos, W. & Läuger, P. (1978) *Biochim. Biophys. Acta, 511*, 305–319.
17. Mannella, C. A. & Bonner, W. D. (1975) *Biochim. Biophys. Acta, 413*, 213–225.
18. Hayashi, H. & Capaldi, R. A. (1972) *Biochim. Biophys. Acta, 282*, 166–173.
19. Hallermayer, G. & Neupert, W. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 1–10.
20. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry, 10*, 2606–2617.
21. Diedrich, D. L. & Schnaitman, C. A. (1978) *Proc. Natl Acad. Sci. USA, 75*, 3708–3712.
22. Mannella, C. A. & Bonner, W. D. (1975) *Biochim. Biophys. Acta, 413*, 226–233.
23. Mannella, C. A. (1981) *Biochim. Biophys. Acta, 645*, 33–40.
24. Colombini, M. (1980) *Ann. N.Y. Acad. Sci. 341*, 552–563.
25. Hackenbrock, C. R. (1972) *Ann. N.Y. Acad. Sci. 195*, 492–505.
26. Tokunaga, H., Tokunaga, M. & Nakae, T. (1979) *Eur. J. Biochem. 95*, 433–439.
27. Tokunaga, M., Tokunaga, H., Okajima, Y. & Nakae, T. (1979) *Eur. J. Biochem. 95*, 441–448.
28. Benz, R., Ishii, J. & Nakae, T. (1980) *J. Membr. Biol. 56*, 19–29.
29. Hancock, R. E. W., Decad, G. M. & Nikaido, H. (1979) *Biochim. Biophys. Acta, 554*, 323–331.
30. Benz, R. & Hancock, R. E. W. (1981) *Biochim. Biophys. Acta, 646*, 298–308.
31. Schindler, H. & Rosenbusch, J. P. (1978) *Proc. Natl Acad. Sci. USA, 75*, 3751–3755.
32. Benz, R., Janko, K. & Läuger, P. (1979) *Biochim. Biophys. Acta, 551*, 238–247.
33. Nakae, T., Ishii, J. & Tokunaga, M. (1979) *J. Biol. Chem. 254*, 1457–1461.
34. Schindler, H. & Rosenbusch, J. P. (1981) *Proc. Natl Acad. Sci. USA, 78*, 2302–2306.
35. Capaldi, R. A. & Vanderkooi, G. (1972) *Proc. Natl Acad. Sci. USA, 69*, 930–932.
36. Rosenbusch, J. P. (1974) *J. Biol. Chem. 249*, 8019–8029.
37. Bahr, G. F. & Zeitler, E. (1962) *J. Cell Biol. 15*, 489–501.

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