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IDENTIFICATION AND CHARACTERIZATION OF THE PORE-FORMING PROTEIN IN THE OUTER MEMBRANE OF RAT LIVER MITOCHONDRIA

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The proteins of the outer membrane from rat liver mitochondria have been subfractionated by means of density gradient centrifugation. The different polypeptides of the membrane were incorporated into asolectin vesicles and black lipid membranes. It was observed that a polypeptide of M_r 32000 renders asolectin vesicles permeable to ADP and forms pores in bilayer membrane. These pores showed the same properties as the channels which are formed in the lipid membrane after addition of Triton X-100 solubilized complete outer membrane. The properties of the pore are as follows: (1) The formation of pores depends on the type of phospholipid used for the preparation of the black membranes. (2) The pore is inserted asymmetrically into the membrane. (3) The pore is voltage gated but does not switch off completely at higher voltages. The pore seems to show different conductance states decreasing conductance being observed at increasing voltage. The implications of these findings for the regulation of transport processes across the outer membrane are discussed.

Introduction

The permeability of the mitochondrial outer membrane has been studied in several laboratories [1,2]. Unlike most other membranes it seemed to be freely permeable to various small molecules, all these investigations did not however rule out membrane damage during isolation. Indeed more careful experiments suggest that the permeability of the outer mitochondrial membrane is limited, and regulated by some means. It has been shown that hexokinase, although bound to the mitochondrial surface, could utilize newly synthesized ATP without prior equilibration with the exogenous pool of ATP [3,4]. On the other hand mitochondria with a controlled rather low damage of the outer membrane exhibited only 50% of adenylate kinase activity, meaning that this enzyme, which is located in the outer mitochondrial compartment, did not have free access to ADP, ATP and AMP [5].

Recently it has been shown that total outer membrane from mitochondria treated with Triton X-100 is able to form ion-permeable pores in lipid bilayer membranes [6–8]. The pores are voltage gated and evidence has been presented that the pore radius is of the order of 2 nm [8]. Similar conclusions have been drawn from reconstituted vesicles in the presence of an M_r 32000 polypeptide derived from outer mitochondrial membrane of the mung bean and rat liver [9]. Thus the outer membrane contains a pore protein which is permeable for small molecules but in spite of that physiologically it has limited permeability. We would therefore predict that the outer membrane is exclusively permeable through the pore and that possibly transport across the pore is regulated.

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

In this investigation we have studied the permeability of reconstituted subfractions of the outer membrane from rat liver mitochondria and have used the lipid bilayer assay in order to characterize the pore-forming activity. By testing the different outer membrane fractions we could show that there is only one polypeptide of M_r 32000 which renders the outer membrane permeable and exhibits pore-forming activity. In contrast to the porins isolated from bacteria [10–12] the 'mitochondrial porin' appears to be voltage gated. Both the lifetime of the pore and the pore conductance seems to depend on the voltage. In addition, we present evidence that the protein is inserted asymmetrically into the membrane.

Experimental procedures

Materials

Octylglycoside was purchased from Calbiochem. Asolectin (L-α-phosphatidylcholine Type II-S from soybean) was from Sigma.

All other reagents were p.a. grade and were obtained from various commercial sources.

Methods

Preparation of mitochondria and mitochondrial outer membrane. Outer mitochondrial membrane was prepared as described by Sottocasa et al. [13] from rat liver mitochondria which had been isolated by differential centrifugation in a medium containing 0.25 M sucrose, 10 mM Hepes (pH 7.4), 0.1 mM EGTA and 0.1 mM phenylmethylsulfonylfluoride.

Subfractionation of the outer mitochondrial membrane. 15 mg of outer mitochondrial membrane suspended in a buffer containing 125 mM sucrose, 150 mM NaCl, 25 mM NaH_2PO_4 (pH 7.4), 0.5 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 0.02% (w/v) NaN_3 were incubated with 15 mg octylglycoside for 30 min at 25°C. The sample was centrifuged for 60 min at 200000 × *g* to yield a supernatant which contained mainly high molecular polypeptides and was discarded. The pellet fraction was suspended in the same medium as the outer membrane and incubated as described above with the addition of 30 mg octylglycoside. Subsequent centrifugation as above led to an enrichment of an M_r 32000 polypeptide in the supernatant. The supernatant was centrifuged for 17 h at 159000 × *g* on a linear 5–20% sucrose gradient containing 1% (w/v) octylglycoside. The gradient was fractionated into eight fractions which were used to investigate pore-forming activity.

Reconstitution and determination of permeability. The fractions from the gradient were dialysed together with 5 mg asolectin against 15 L 10 mM NaH_2PO_4 buffer (pH 7.4). The dialysed samples and a portion of 50 mg asolectin were dried as a film at the bottom of a glass tube. To each tube 0.2 ml of a 100 mM Hepes solution containing [U-^{14}C]ADP was added. Resuspension of the dried film was achieved by mixing the contents of the tube on a Vortex mixer for 10 min and subsequent incubation at room temperature for 1 h. The contents of the tubes were applied to a column (0.8 × 26 cm) of Sepahdex G-50 and then eluted with 0.1 M Hepes (pH 7.4) 0.75 ml fractions were collected and assayed for radioactivity in a liquid scintillation counter.

Incorporation into planar bilayers. Aliquots of the reconstituted vesicles without [U-^{14}C]ADP and pure outer membrane were used for these studies. Black lipid bilayer membranes were obtained [10] from a 1–2% (w/v) solution of asolectin, oxidized cholesterol or diphytanoylphosphatidylcholine. Oxidized cholesterol was prepared as described earlier [10] and diphytanoylphosphatidylcholine was synthesized according to standard methods in our own laboratory [14]. The chamber used for bilayer formation was made of Teflon. The circular holes in the wall between the two aqueous solutions have an area of either 2 mm^2 (in the case of the macroscopic measurements) or 0.1 mm^2 (for the single-channel experiments). The temperature was maintained at 25°C in all experiments. The pH of all aqueous solutions was adjusted to pH 6. Buffering the solutions with 5 mM phosphate KH_2PO_4 had no influence on the results, so it was used only in the presence of 1 M alkali chlorides. The protein was added to the aqueous phase prior to membrane formation or after the membrane had turned black. The protein containing salt solutions were prepared immediately before use in order to prevent protein inactivation. The protein solution was sonicated for 30 s in the presence of 1% w/v Triton X-100. Ag/AgCl or calomel elec-

trodes (with salt bridges) were inserted in both aqueous compartments. The macroscopic conductance measurements were performed using a 610 C Keithley electrometer. In the single-channel experiments the membrane current was measured with Keithley 427 current amplifier, monitored with a Tektronix 7633 storage oscilloscope and recorded with a strip chart recorder. The zero current potentials were measured as described elsewhere [15].

Enzyme assays. Acid phosphatase (EC 3.1.3.2) and urate oxidase (EC 1.7.3.3) were determined according to Bergmeyer [16,17]. Glucose-6-phosphatase (EC 3.1.3.9) was measured according to Swanson [18]. Monoamine oxidase (EC 1.4.3.4) and succinate dehydrogenase (EC 1.3.99.1) were assayed as described [19].

Other methods. SDS-polyacrylamide gel electrophoresis was performed as described by Lämmli [20]. Protein was determined by the method of Lowry et al. [21].

Results

Preparation and subfractionation of the outer mitochondrial membrane

Mitochondria were prepared in a medium which results in very low contamination especially by endoplasmic reticulum. From these mitochondria the outer membrane was stripped by swelling and shrinking. It was subsequently separated and purified by discontinuous gradient centrifugation. This method resulted in a very pure outer membrane fraction as can be seen from the activities of marker enzymes for other cellular membranes given in Table I.

The specific activity of monoamine oxidase in this preparation increased by a factor of 15–20 (Table II). A subfractionation of the outer membrane was performed by a stepwise increase of octylglycoside concentration. The membrane fraction which was solubilized by the addition of 3 mg octylglycoside per mg membrane protein was centrifuged on a linear sucrose density gradient as described in Methods. This resulted in the separation of high and low molecular weight polypeptides as shown by SDS-polyacrylamide gel electrophoresis in Fig. 1. The subfractions from the gradient were subsequently incorporated into asolectin vesicles (see Methods). The reconstituted vesicles were tested for ADP permeability by column chromatography and for pore forming activity in a planar bilayer. The vesicles containing the high molecular polypeptides retained ADP during chroamtography whereas the vesicles reonstituted with the fractions containing an M_r 32000 polypeptide did not. As shown in Fig. 2 the latter vesicles eluted from a Sephadex G-50 column con-

TABLE I

CONTAMINATION OF OUTER MITOCHONDRIAL MEMBRANE

The calculation of the protein amount of the contaminating membrane listed in column 4 is based on the following specific activities of the enzymes in the purified organelles taken from the literature. Glucose-6-phosphatase, 280 mU/mg [22,23]; uricase, 35.4 mU/mg [24]; acid phosphatase 2.83 U/mg [25]; succinate dehydrogenase 300 mU/mg (own data). The number of experiments is given in parenthesis.

Membrane system (organelle)	Marker enzyme	mU/mg	μg/mg *	% contamination
Endoplasmatic reticulum	Glucose-6-phosphatase	18 ±15 (6)	65	6.5
Peroxisomes	Uricase	0	0	0
Lysosomes	Acid phosphatase	1.7± 0.7 (7)	0.6	0.06
Inner mitochondrial membrane	Succinate dehydrogenase	21 ±11 (13)	70	7.0

* The values in column 4 are mean μg of organelle protein per mg of outer mitochondrial membrane protein.

TABLE II

PURIFICATION OF OUTER MITOCHONDRIAL MEMBRANE

The number of experiments is given in parenthesis.

	Monoamine oxidase (mU/mg)	Increase in specific activity	Succinate dehydrogenase (mU/mg)	Decrease in specific activity
Mitoblasts	4.1± 0.9 (7)	1	218 ±29 (5)	1
Mitochondria	4.8± 2.3 (13)	1.2	155.4±46 (5)	0.7
Outer membrane	68.8±33 (13)	16.7	21 ±11 (13)	0.096

tained a very low amount of ADP when compared to pure asolectin vesicles and to vesicles into which other outer membrane polypeptides were incorporated. In accordance to that the same subfractions of the outer membrane which turned out to be leaky for ADP had a high pore forming activity in planar bilayers (see Fig. 2). The channels displayed properties after insertion into the planar bilayers which are described below.

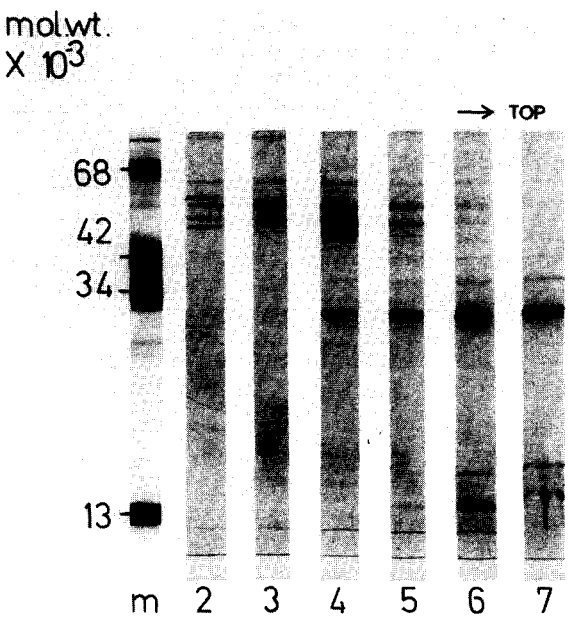

Fig. 1. SDS-polyacrylamide gel electrophoresis of fractions obtained after centrifugation of a outer membrane subfraction on a linear 5–20% (w/v) sucrose density gradient containing 1% (w/v) β-octylglycoside, 1 M KCl, 10 mM Hepes (pH 7.4), as described in Methods. As molecular weight standards bovine serum albumin (68000), aldolase (42000), lactate dehydrogenase (34000) and cytochrome *c* (13000) were used. Aliquots from each fraction were taken for measuring ADP permeability in reconstituted vesicles and pore forming activity in planar bilayer experiments as described above. The results of these experiments are summarized in Fig. 2.

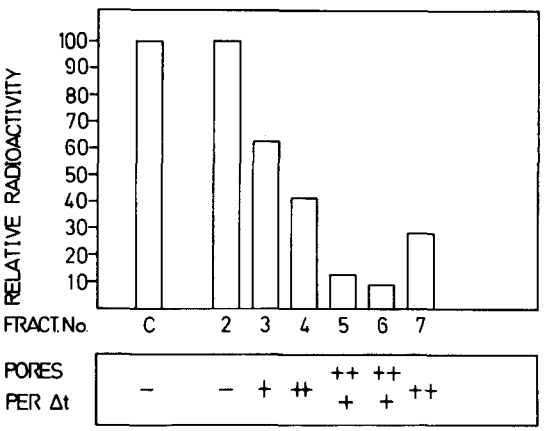

Fig. 2. [^{14}C]ADP permeability of reconstituted vesicles and pore forming activity in planar bilayers of different subfractions of the outer mitochondrial membrane separated on a linear sucrose density gradient. The polypeptide composition of the gradient fraction is shown in Fig. 1. The vesicles were reconstituted using the same amount of protein from the different fractions of the gradient as described in methods. [^{14}C]ADP was present during reconstitution of the vesicles. Free ADP was separated from the vesicles by column chromatography on Sephadex G-25. The radioactive ADP recovered in the vesicles fraction after elution was determined and refered to unpermeable vesicles as reconstituted from fraction 2. The latter vesicles retained the same amount of radioactivity as pure asolectin vesicles shown in column c. The different fractions from the sucrose gradient were tested also for pore-forming activity by adding the same amount of protein to the aqueous phase in the presence of $5 \cdot 10^{-8}$ g/ml Triton X-100 after blackening of the membranes. The number of pores formed in the first 15 min was measured.

Macroscopic conductance measurements

The conductance of lipid bilayer membranes increases by several orders of magnitude if outer mitochondrial membranes from rat liver are dissolved in Triton X-100, or mitochondrial subfractions containing the M_r 32000 polypeptide are added to the aqueous phase bathing a membrane. The conductance increase was a function of time similar to that found for the porins from outer bacterial membranes [10–12]. After blackening of the protein dotated membranes or after protein addition to black membranes the conductance increased rapidly for 10 to 20 min. After this time the increase was much slower. An almost stationary conductance level could be reached within about 40 min. The time-dependent conductance increase was more or less the same both for the total outer membrane and for the isolated protein. In addition, the same time course of conductance increase was observed whether the protein was added from one or from both sides of the membrane, although we found a considerable asymmetry of action of the voltage on the channel (see below).

Fig. 3 shows the concentration dependence of the macroscopic conductance measured in the presence of 1 M KCl and different concentrations of the isolated M_r 32000 polypeptide and the total outer mitochondrial membrane. The measurements were performed either 15 min after the addition of the protein or after the membranes had turned completely black. As can be seen from Fig. 3 there exists a linear relationship between membrane conductance and protein concentration in the aqueous phase for at least a 100-fold range in protein concentration. Whereas the kinetics of the conductance increase was approximately the same for different lipids, a considerable difference in the absolute conductance level was found for membranes made from different lipids. The pore formation probability was at least 100- to 1000-fold higher in membranes prepared from oxidized cholesterol than in diphytanoylphosphatidylcholine or in asolectin prepared membranes. The reason for this different behaviour remains unclear so far. It is interesting to note, however, that with a variety of porins from outer bacterial membranes a similar lipid specificity has been observed [10–12]. If the pore forming activity is referred to the

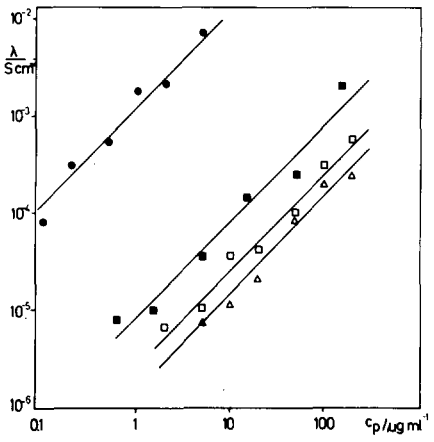

Fig. 3. Specific membrane conductance λ as a function of the protein concentration c_p of the M_r 32000 polypeptide from rat liver mitochondria in the aqueous phase. For complete outer mitochondrial membrane c_p refers also to the content of the M_r 32000 polypeptide in the membrane (approx. 7%) of the total membrane protein). The membranes were formed from different lipids dissolved in *n*-decane. The aqueous phase contained 1 M KCl and less than 5 μg/ml Triton X-100. Each point represents the mean value of at least three membranes 15 min after blackening of the membranes or after the addition of the protein; $T=25°C$; $V_m=5$ mV. ■———■, Outer membrane in asolectin bilayer ●———●, M_r 32000 polypeptide in oxidized cholesterol □———□, asolectin; △———△, diphytanoylphosphatidylcholine.

content of the M_r 32000 polypeptide in the different preparations, it is also obvious from Fig. 3 that the isolated M_r 32000 polypeptide is less active as the same polypeptide in the complete outer mitochondrial membrane. This does not seem to be caused by any difference in the pore formation or in the pore kinetics (see below). The reduced activity of the isolated protein may be due to partial damage of the protein during the isolation procedure.

The current-voltage behaviour of the mitochondrial pore in the asolectin membranes was approximately the same as has been reported by Colombini and co-workers [6–8]. However, if the polypeptide was added to one side of the membrane only, the current showed a strong asymmetry which depended upon the polarity of the voltage. Fig. 4 presents a representative experiment. The M_r 32000 polypeptide was added to one side of the membrane (*cis* side). After about 20 min a considerable conductance increase was ob-

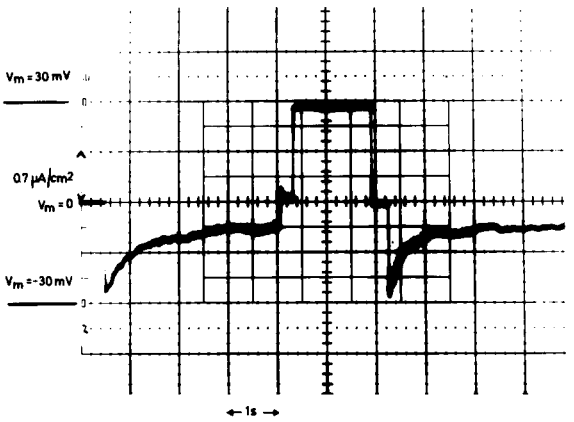

Fig. 4. Membrane current as a function of time after application of −30 mV (lower trace) and +30 mV (upper trace) to one side of the membrane (*cis* side). 20 min before the experiment was started 20 μg/ml of the M_r 32000 polypeptide and 1 μg/ml Triton X-100, was added to the *cis* side. The solution on both sides of the membrane contained 1 M KCl. The membrane was made from asolectin dissolved in *n*-decane; $T = 25°C$.

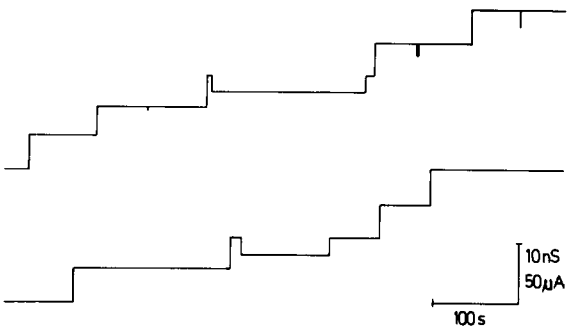

Fig. 5. Stepwise increase of the membrane current after the addition of 1 μg/ml of the M_r 32000 polypeptide from rat liver outer mitochondrial membrane together with $5 \cdot 10^{-8}$ g/ml Triton X-100 to the aqueous phase containing 1 M KCl; $T = 25°C$. The membrane was formed from a 1% (w/v) solution of asolectin in *n*-decane. The applied voltage was 5 mV. The current prior to the addition of the polypeptide was about 0.1 pA. The record starts at the left side of the lower trace and continues in the upper trace.

served. Then a voltage of 30 mV and different polarity was applied to the membrane. If the polarity was negative on the *cis* side (lower trace of the oscillographic record) the current decreased with a time constant in the order of 1 to 2 s. For inverted polarity (positive at the *cis* side) the conductance was fully recovered and no decay of the current was observed (upper trace of Fig. 4). This experiment was found to be reversible and reproducible. It indicates that the protein is inserted asymmetrically into the membranes.

Single-channel experiments

When the M_r 32000 polypeptide or the complete outer membrane after treatment with Triton X-100 was added in small quantities, to the aqueous solutions bathing the membrane, the membrane conductance increased in a stepwise fashion. A representative experiment is documented in Fig. 5. The occurrence of these steps was specific for the presence of the pore-forming protein and was not observed when the detergent concentration was the same as ($5 \cdot 10^{-5}$ mg/ml) or 100-times higher than that used in a single-channel experiment. The Triton X-100 channels, as described in the literature [26] were detected at a Triton X-100 concentration of 0.1 mg/ml in the aqueous phase. This concentration was never used in the experiments performed in this study. At a membrane potential of 5 mV most of the conductance steps observed in the single-channel experiment were directed upwards whereas terminating steps were rarely observed. This may be seen in Fig. 5. The lifetime of the pore at a membrane potential of 5 mV is at least 1 min as shown by records extending over long times. Changes in the salt concentration and in the lipid composition had no influence on the lifetime of the single conductance unit, irrespective of whether isolated M_r 32000 polypeptide or total outer membrane was used in the experiments. Both preparations showed much lower activity on phospholipid membranes than with membranes prepared from oxidized cholesterol and about 100-times more protein had to be added in order to obtain the same number of pores.

The single conductance increment was almost uniform in sizes for a membrane potential of 5 mV and smaller steps were only rarely observed (See Fig. 6). In addition, a basic difference does not exist between the isolated M_r 32000 polypeptide and the total outer mitochondrial membrane as the two histograms clearly indicate (Fig. 6).

The terminating steps at a given membrane potential of 5 mV were found to be much smaller in size (about 40–50%) than most of the steps

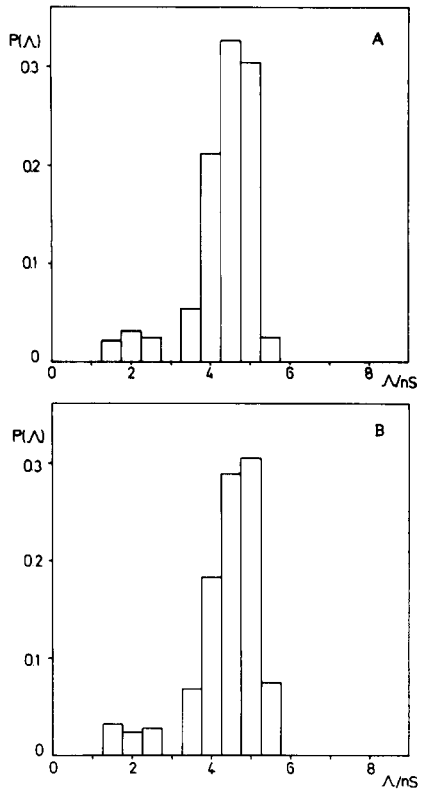

Fig. 6. Histogram of the conductance fluctuations observed with membranes from asolectin in the presence of: (A) total outer mitochondrial membrane from rat liver, $n=381$, $\bar{\Lambda}=4.3$ nS; (B) isolated M_r 32000 polypeptide from rat liver outer mitochondrial membrane, $n=322$; $\bar{\Lambda}=4.4$ nS. The aqueous phase contained 1 M KCl; $T=25°C$. The applied voltage was 5 mV.

directed upwards. However, at the time where small terminating steps occurred, small upward directed steps of the same amplitude could be observed. These smaller steps amount 5 to 10% of the total single conductance increment only at a membrane potential of 5 mV, but they became more and more prominent at higher voltages. Thus at 10 mV the average conductance of all the conductance increments directed upwards is much lower than at 5 mV and at higher voltages it became even lower. Simultaneously, the uniformity in size of the single conductance increments decreases at higher voltages (Fig. 7).

Single-channel experiments were performed with a variety of lipids and voltages. The results are summarized in Table III. Irrespective of the

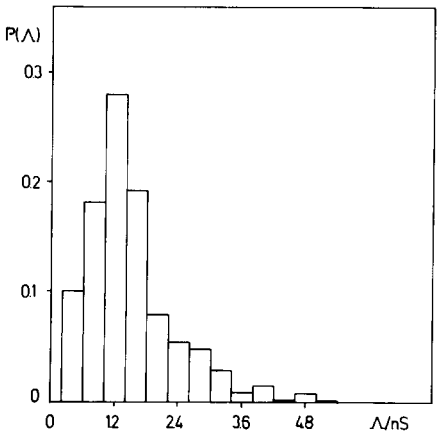

Fig. 7. Histogram of the conductance fluctuations observed with membranes from asolectin in the presence of isolated M_r 32000 polypeptide from rat liver outer mitochondrial membrane; $n=948$, $\bar{\Lambda}=1.5$ nS. The aqueous phase contained 1 M KCl; $T=25°C$. The applied voltage was 50 mV.

composition of the membrane, or if purified protein or the total outer mitochondrial membrane was used in the experiments, there exists a strong influence of the voltage upon the average conductance increment. The reason for this is not clear. However, it seems that the pore created by the 'mitochondrial porin' has different conductance states. Single-channel experiments were also performed with a variety of different eletrolytes and different concentrations at a membrane potential of 5 mV. From records similar to those given in Fig. 5 the average conductance increment was obtained by measuring a large number of individual events. In addition to $\bar{\Lambda}$, the specific conductance σ of the corresponding aqueous solution as well as the ratio $\bar{\Lambda}/\sigma$ are given in Table VI. These data show that a linear relationship exists between the conductance in the aqueous phase and the pore conductance. This indicates that even large ions such as $N(C_2H_5)_4^+$ or $Hepes^-$ permeate through the 'mitochondrial porin' pore with little or no interaction with the pore interior.

Zero-current membrane potentials

Information concerning the structure of the pore may be obtained from zero-current membrane potential measurements. In these experiments 10 μg/ml M_r 32000 polypeptide ($5 \cdot 10^{-7}$ g/ml Triton X-100) was added to a 30 mM solution of

TABLE III

AVERAGE CONDUCTANCE INCREMENT $\overline{\Lambda}_{on}$ AND $\overline{\Lambda}_{off}$ FOR TOTAL OUTER MITOCHONDRIAL MEMBRANE AND ISOLATED M_r 32000 POLYPEPTIDE AS A FUNCTION OF THE APPLIED MEMBRANE POTENTIAL

The membranes were formed from the lipids as indicated (dissolved in *n*-decane); $T=25°C$. The aqueous phase contained 1 M KCl. n_{on} or n_{off} are the numbers of events from which $\overline{\Lambda}_{on}$ and $\overline{\Lambda}_{off}$ have been calculated.

Lipid	V_m (mV)	$\overline{\Lambda}_{on}$ (nS)	n_{on}	$\overline{\Lambda}_{off}$ (nS)	n_{off}
Total outer mitochondrial membrane					
Asolectin	5	4.3	381	3.5	45
	10	4.0	914	3.3	524
	20	3.4	388	2.8	301
	50	1.5	948	1.5	909
	100	1.2	113	1.1	105
Diphytanoyl-	5	4.4	581	3.9	67
phosphatidylcholine	20	4.2	460	3.7	125
	100	2.0	204	1.2	150
Oxidized	10	4.8	259	5.1	45
cholesterol	20	4.0	161	3.2	65
	50	3.1	282	3.0	138
Isolated M_r 32000 polypeptide					
Asolectin	5	4.4	322	3.4	36
	10	3.9	415	3.4	197
	20	3.2	221	2.5	159
	50	1.7	637	1.5	583
	100	1.2	344	1.1	325
Diphytanoyl					
phosphatidyl-	5	4.5	477	4.2	55
choline	50	3.5	318	3.3	135
Oxidized					
cholesterol	5	4.7	439	4.9	36

TABLE IV

AVERAGE CONDUCTANCE INCREMENT $\overline{\Lambda}$ FORMED BY THE M_r 32000 POLYPEPTIDE FROM THE OUTER MEMBRANE OF RAT LIVER MITOCHONDRIA IN LIPID BILAYERS FROM ASOLECTIN (DISSOLVED IN *n*-DECANE) IN DIFFERENT SALT SOLUTIONS OF CONCENTRATION *c*

In all experiments the aqueous phase contained 1 μg/ml protein and $5 \cdot 10^{-8}$ g/ml Triton X-100; the pH was approx. 6 if not otherwise indicated; $T=25°C$; $V_m=5$ mV; σ is the specific conductance of the aqueous phase [15].

Salt	*c* (M)	$\overline{\Lambda}$ (nS)	σ (mS·cm^{-1})	$\overline{\Lambda}/\sigma$ (10^{-8} cm)	*n*
KCl	0.01	0.05	1.4	3.6	138
	0.03	0.15	3.9	3.8	265
	0.1	0.48	13	3.7	461
	0.3	1.3	36	3.6	179
	1	4.4	110	4.0	322
NaCl	1	3.8	84	4.5	357
LiCl	1	3.4	71	4.8	151
RbCl	1	4.2	120	3.5	467
$MgCl_2$	0.5	2.7	64	4.2	113
K_2SO_4	0.5	2.4	76	3.2	146
$Tris^+Hepes^-$ (pH 8)	0.5	0.23	7.2	3.2	189
$N(C_2H_5)^+Hepes^-$	0.5	0.17	4.8	3.5	177

either NaCl or KCl. Ten to twenty minutes after blackening of the membranes which were made from three different lipids the salt concentration was raised 10-fold on one side of the membrane. In most cases the more dilute side becomes negative within 10 min indicating a small anion selectivity of the incorporated M_r 32000 polypeptide (see Table V). This anion selectivity has already been described by Colombini and coworkers [6–8] for a pore protein inserted from complete outer mitochondrial membrane. These authors observed a potential difference of about 13 mV for a 10-fold gradient of KCl, while the asymmetry in our experiments was much smaller. Table V in addition shows the ratio of anion permeability P_a to cation permeability P_c, calculated according to the Goldman-Hodgkin-Katz equation as has been described previously [15]. The observed slight anionic selectivity may be explained by the presence of positively charged groups in or near the pore and/or by the higher mobility of chloride versus sodium ion.

TABLE V

Zero-current potentials V_m in the presence of a 10-fold salt gradient of NaCl or KCl. The membranes were formed from three different lipids dissolved in *n*-decane; $T=25°C$. V_m is the potential of the dilute side (0.03 M) minus the potential of the concentrated side (0.3 M). For further explanations see text. The data are mean values obtained from at least three membranes.

Salt	V_m (mV)	P_a/P_c
32000 mol. wt. polypeptide		
Asolectin		
NaCl	−7±2	1.4±0.16
KCl	−2±2	1.1±0.12
Diphytanoylphosphatidylcholine		
NaCl	−4±3	1.2±0.20
KCl	0±2	1.0±0.15
Oxidized cholesterol		
NaCl	−12±2	1.8±0.20
KCl	−7±2	1.4±0.15
Total outer mitochondrial membrane		
Asolectin		
NaCl	−5±2	1.3±0.12
KCl	0±3	1.0±0.15

Discussion

As shown by the experiments in this communication the outer mitochondrial membrane was purified to a high degree. The marker enzymes for the other cellular organelles showed a very low activity. Thus, we are sure that our results exclusively refer to components of the outer mitochondrial membrane. Both the results, obtained from reconstitution experiments and from lipid bilayer studies point to the existence of only one pore forming protein in the outer mitochondrial membrane. The protein could be isolated and characterized and was a polypeptide of a molecular weight of M_r 32000. The pore-forming properties of this polypeptide are identical to those observed with the complete outer membrane. No other polypeptide in the different subfractions of the outer membrane could either form channels in the lipid bilayer membranes or render the reconstituted vesicle membranes permeable to ADP. The M_r 32000 polypeptide exclusively increased the permeability of asolectin vesicles to ADP. The results obtained with lipid bilayer membranes indicate that the outer membrane pore shows some analogy to the pores formed by porins from the outer bacterial membrane from *Escherichia coli* [10,15], *Salmonella typhimurium* [11] and *Pseudomonas aeruginosa* [12]. In fact, the order of magnitude of the pore conductance for all these pores in 1 M KCl is the same (about 2 nS for the porins of *Salmonella* and *E. coli* and about 5 nS for the F-porin of *P. aeruginosa* [12]). This reflects that in principle the different pores are of the same size. The size is calculated under the assumption that the pores are cylinders with spherical cross-sections which are filled with an aqueous solution of the same conductance as the external bulk phase. According to the equation $\overline{\Lambda} = \sigma\pi r^2/l$, the average pore diameter ($d=2r$) may be calculated for a certain length of the pore. Assuming a pore length (l) in the outer mitochondrial membrane of 7.5 nm (corresponding to the thickness of the outer membrane) the pore diameter d can be calculated to be about 2 nm. This diameter is considerably larger than that of the porin pores from *E. coli* and *S. typhimurium* [10,11,15] but it is of the same size as the F-pore of *P. aeruginosa* [12]. Colombini [8] sug-

gested from swelling experiments in the presence of polyethylene glycol a radius for the outer membrane pore of 2 nm. It should, however, be considered that the calculations and the direct measurements employing polydisperse polyalcohols cannot give precise data on pore size. Permeability may depend on form and surface charge of the permeating substances. Pores in the outer mitochondrial membrane have also been studied by electron microscopy. Negative staining of isolated outer membranes from mung bean mitochondria have revealed stain filled pits of 2–3 nm diameter [27]. Investigation of the outer membrane from liver mitochondria by the same technique revealed a close-packed fine structure but no pits [28]. X-ray diffraction patterns of plant outer mitochondrial membranes suggest that a protein with an M_r of 30000 forms a prominent subunit structure, with an inner low-electron-density core of approx. 2 nm [29,30]. Therefore, in plant outer membrane may well be identity among the pore-forming component, the negative stain accumulating structures and the in-plane subunits inferred from X-ray diffraction experiments. It is, however, a question why a component of rat liver mitochondria which forms pores of a comparable diameter in planar bilayers is not visible in the respective outer membrane. One explanation might be that in rat liver kinases are bound to the outer surface of the pore as we have recently observed (Fiek, C., Benz, R., Roos, N. and Brdiczka, D., unpublished data).

The large size of the pore suggests that the M_r 32000 polypeptide is a subunit of an oligomer which forms the active pore. Colombini has determined a M_r 110000 as the size of the pore protein-Triton complex by column chromatography [31].

The pore-forming protein from the outer mitochondrial membrane exhibited in our hands a slight preference for anions. Whereas Colombini [7] found 13 mV for a 10-fold gradient of KCl we could find only about 4 mV for a 10-fold gradient of KCl and about 8 mV for a 10-fold gradient of NaCl (the more dilute side being negative). The somewhat higher value for NaCl is not all surprising, since the mobility of the chloride ion in the aqueous phase is larger than that of the sodium ion. Indeed the anion selectivity (4 or 13 mV) of the outer membrane pore is so poor that one can hardly speak of anion selective pore. However, the slight anion preference may suggest a positive charge near the entrance of the pore. This conclusion has also been drawn by Mannella et al. from the difference in the distribution of anionic and cationic stains outside the opaque centers of negatively stained *Neurospora* membranes (Manella, C.A. and Frank, J. (1982) Ref. 36). Both observations point to an asymmetric distribution of charges in the pore molecule. Generally each different conformation of a protein will have a different dipole moment. Not only does the distribution of charged groups depend on conformation, but the dissociation constants of ionizable groups also vary with protein configuration. Because each state of the protein has probably a different value for the dipole moment the energy associated with each state is differently affected by membrane voltage. Therefore, it seems not surprising that the pore formed by the M_r 32000 polypeptide is voltage gated a property which has been observed also by Colombini and coworkers [6–8]. However, since in our experiments the pore is not completely closed at high voltages we think it is more likely that the pore physiologically switches to a smaller diameter of the cross-section. This would result in a decrease in permeability for large molecules. On the other hand a voltage-dependent change in conformation could also alter the binding properties of the pore surface for cytosolic proteins. As mentioned above we have recently observed a binding of kinases to the pore protein (Fiek, C., Benz, R., Roos, N. and Brdiczka, D., unpublished data).

The asymmetric construction of the pore protein results also in particular orientation of the pore within the bilayer membrane. This is observed if the protein is added to one side (*cis* side) of the membrane. Under these conditions the conductance is significantly higher when the *cis* side is positive whereas the pore switches partly off, when the *cis* side is negative.

Because of the large number of pores in the outer mitochondrial membrane (the M_r 32000 polypeptide is a major protein) we do not believe that an ion gradient can be maintained across the membrane for longer than a few microseconds. It seems more plausible that an intrinsic potential in the outer membrane is caused by an asymmetry in

surface charges. It has indeed been described by Hackenbrock [32] from electron microscopic observations with cationic ferritin decoration that the inner surface of the outer membrane has more negative charges than the outer.

We do not know whether the observed voltage-dependent change in conductance of the pore protein is physiologically important. However, to imagine how transport across the pore can be regulated the observation of contacts between the outer membrane and the inner boundary membrane may be of relevance [33]. The number of contacts between both membranes is suggested to be regulated by the energetic state of the mitochondria [34]. In view of these results it seems probable that the pore protein forms contacts with the inner mitochondrial membrane depending on the metabolic states of the mitochondria. Thus allowing a direct exchange of metabolites between the mitochondrial surface and the inner compartment. A microcompartmentation like that for ATP and ADP has been postulated to exist between hexokinase bound to the mitochondrial surface and the oxidative phosphorylation at the cristamembranes [35]. The intrinsic membrane potential of the outer membrane may therefore on one hand serve to perform the contacts and on the other hand would be changed by the contacts. A consequence of the latter would be a difference in conformation and permeability properties of the pore as has been discussed above.

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