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Porin of *Paramecium* mitochondria isolation, characterization and ion selectivity of the closed state

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Porin was isolated and purified from mitochondria of *Paramecium tetraurelia*. The protein showed a single band of apparent M_r 37000 on sodium dodecyl sulfate polyacrylamide electrophoretograms. The reconstitution of the protein into artificial lipid bilayer membranes revealed it to be a porin giving pores with an average single-channel conductance of 0.26 nS in 0.1 M KCl. This conductance is about half of that of other eukaryotic porins studied to date. The pore formed by the mitochondrial porin of *Paramecium* was found to be voltage-dependent and switched to a defined substrate at membrane voltages larger than 20 mV. In the open state the pore exhibited the characteristics of a general diffusion pore because the mobility sequence of the ions inside the pore was similar to that in the bulk aqueous phase. The effective diameter was estimated to be about 1.3 nm. The properties of the low conductance state of the pore were studied in detail. In this state the pore favored the passage of cations, in contrast to the open state which favored anions slightly. The possible role of the low-conductance state in the regulation of transport processes across the outer mitochondrial membrane and in mitochondrial metabolism is discussed.

Introduction

The mitochondrial outer membrane acts as a molecular filter for hydrophilic solutes because it is freely permeable to a variety of different small solutes [1,2], but retains molecules with higher molecular weights [3,4]. This indicates that the outer mitochondrial membrane contains a defined passage for mitochondrial substrates. Pore-forming proteins are present in crude extracts of *Paramecium* mitochondria [5] and in the mitochondrial outer membranes of a variety of mammalian cells [6–10], *Neurospora crassa* [6,11,12], yeast [13], and mung beans [14]. The mitochondrial porins (also known as voltage-dependent anion channel (VDAC) [5,6]) have several similarities to the bacterial porins [15]: they give general diffusion pores in the open state [8–10,12–14] and contain mostly β-pleated sheet structure with no indication of α-helical regions [16,17]. These mitochondrial porins characterized to date have molecular masses between 30 and 35 kDa [7–10, 12–14]. They are encoded in the nucleus without a leader sequence and are synthesized on free cytoplasmic ribosomes [18–20]. Rat liver porin binds dicyclohexylcarbodiimide (DCCD) with high affinity, which indicates that the pore-forming complex contains a negatively charged group in a hydrophobic environment [21]. Experiments with water-soluble porin from *N. crassa* and cross-linking experiments with yeast porin suggest that the channel-forming unit is a complex composed of one or several polypeptide chains, lipids and sterols [22,23].

The role of the mitochondrial pore in the metabolism and physiology of mitochondria is not yet understood. There is an indication that ATP and ADP cannot penetrate the closed state of the mitochondrial porin because adenylate kinase located in the intermembrane space of mitochondria is separated from external ATP if the pore is closed [24]. This means that the outer membrane permeability could be controlled by a transmembrane potential, or an intrinsic membrane potential, because all mitochondrial porins studied so far are voltage-dependent and switch to substates when the transmembrane potential is larger than 10–20 mV

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; SDS, sodium dodecylsulfate.

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[5,6,9–13]. The binding of hexokinase and glycerokinase to the mitochondrial porin (i.e., the hexokinase binding protein) could represent another control of the channel permeability. This binding of kinases to the pore could have the advantage that these enzymes directly utilize the mitochondrial ATP pool [25–28].

In this publication we describe the isolation and purification of porin from *P. tetraurelia*. The porin was reconstituted in lipid bilayer membranes and the properties of the pores were studied in detail. The *Paramecium* porin formed general diffusion pores with a small preference for Cl^- over K^+ at small transmembrane potential differences. The basic difference to other mitochondrial porins is the smaller single-channel conductance and the defined substate of the pore. The substate is at least 10-times more permeable to cations than to anions of the same size and valency.

Materials and Methods

Cell cultures

P. tetraurelia wild-type strain 51s cells were grown axenically at 25°C in 20 l airlift bioreactors with 250 mg/l L-glycerophosphocholine from soy bean (Sigma type II S) as lipid source [29]. At stationary phase the cultures contained around 30000 cells/ml [30].

Preparation of cell bodies

Stationary cells from 20 l medium were harvested at room temperature with a cream separator at $300 \times g$. The cells (about 70 ml packed cells) were washed once with 2.4 l of Dryl's solution (2 mM sodium phosphate/2 mM sodium citrate/1.5 mM $CaCl_2$ (pH 7.2)) and with 1.2 l solution A (150 mM sucrose/4 mM KCl/1 mM $CaCl_2$/1 mM Mops (pH 7.2)) and centrifuged for 5 min at $380 \times g$. Finally, the cell pellet was resuspended in 240 ml of ice-cold solution A and deciliated as described previously [31]. Detachment of somatic cilia from the cells was complete within 3 min. Phase-contrast microscopy showed no cell lysis. The bulk of the cell bodies was obtained by centrifugation in a Sorvall RC 5B centrifuge at $160 \times g$ for 10 min at 4°C [30].

Isolation of mitochondria from Paramecium

The cell bodies were washed once with 1 l Dryl's solution (10 min at $160 \times g$). The cells, resuspended in 200 ml ice-cold solution B (containing 0.2 M sucrose/1 mM potassium phosphate (pH 6.2)) were disrupted by passing them once through a hand French-press. The mitochondria were isolated by differential centrifugation. Initially, the homogenate was centrifuged for 5 min at $500 \times g$ and the pellet was discarded. The supernatant was now centrifuged for 2 min at $1500 \times g$. From the supernatant of this centrifugation, mitochondria were collected by spinning for 5 min at $5000 \times g$. The pellet was resuspended in solution B and centrifuged for 2 min at $1000 \times g$ to remove trace contaminations of cell bodies. The supernatant was further centrifuged for 5 min at $5000 \times g$. The final pellet contained essentially pure mitochondria [32,33]. The different steps of this procedure were followed by phase contrast microscopy of all fractions and were performed at 4°C throughout.

Purification of porin from Paramecium mitochondria

The mitochondrial porin was isolated from whole mitochondria essentially as published previously [10]. The mitochondrial pellet (about 15 mg protein) was resuspended to give a concentration of 3 mg protein per ml solution C (20 mM Hepes (pH 6.8) containing 20 mM Na_2SO_4/1 mM EDTA/0.05% Triton X-100) and placed on ice for 30 minutes. Following a 30 min centrifugation at $100000 \times g$, the pellet was resuspended in solution C and 2.4 mg Triton X-100 per mg of starting protein was added. After another 30 min on ice and a second centrifugation at $100000 \times g$ for 30 min, the resulting supernatant was applied to a hydroxyapatite column (1 ml Bio-Gel hydroxyapatite (Bio-Rad, 8000 München, F.R.G.)) per 2 mg of starting protein). The column was equilibrated and eluted with solution C and 1 ml fractions were collected. The first ten fractions were applied to a hydroxyapatite/Celite (1 : 1; w/w)-column and eluted with solution C (Celite 535 was obtained from Roth, Karlsruhe, F.R.G.). 1 ml fractions were collected and monitored by the absorbance at 280 nm. The protein-containing fractions were pooled and stored at −25°C.

SDS-gel electrophoresis

The protein composition was examined by electrophoresis on a polyacrylamide-SDS gel. The protein was precipitated according to the method of Wessel and Flügge [34] to remove the lipid associated with the protein. For SDS-gel electrophoresis the pellet was dissolved in 20 µl SDS-sample buffer (containing 5% SDS and 2% mercaptoethanol) and kept for 10 min at 97°C. Gel electrophoresis was carried out on 12% acrylamide gels according to Laemmli [35]. Staining was performed with Coomassie blue.

Membrane experiments

The method used for black lipid bilayer experiments has been described previously in detail [36]. The apparatus consisted of a Teflon chamber with two aqueous compartments. The circular holes in the wall between the two compartments had an area of either 2 mm^2 (for the macroscopic conductance measurements) or about 0.1 mm^2 (for the single-channel experiments). Membranes were formed across the holes by painting on a 1% (w/v) solution of diphytanoylphosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. The temperature was kept at 25°C

throughout. All salts and buffers were analytical grade and obtained from Merck (Darmstadt, F.R.G.). The aqueous solutions were unbuffered and had a pH of approx. 6. Voltage was applied using a pair of calomel electrodes with salt bridges, which were inserted into the aqueous solutions on both sides of the membrane. In the case of single-channel measurements, the membrane current was amplified by a Model 427 amplifier (Keithley, Cleveland, OH), monitored on a storage oscilloscope (Tektronix, Beaverton OR, Model 5115) and recorded on a strip chart on a tape recorder. The macroscopic conductance measurements were performed with a Keithley 610 C electrometer. Zero-current membrane potentials were measured with the same instrument 5–10 min after the application of a salt gradient across the membranes [37].

Results

Purification of the Paramecium porin

The isolation procedure of the mitochondrial porin from *Paramecium* was similar to that used for the isolation of the mitochondrial porins from yeast [13] and mammalian cells [10]. The second column (hydroxyapatite/celite) was necessary to eliminate contaminations of other mitochondrial proteins, especially the nucleotide carrier from mitochondrial inner membrane [10]. The *Paramecium* porin had an electrophoretic mobility on the SDS-polyacrylamide gel electrophoresis corresponding to a mass of 37 kDa (Fig. 1). This is about 20% larger than that of yeast porin [13,16] and about 6% larger than that of mitochondrial porins from other eukaryotic cells [7–10,12,14].

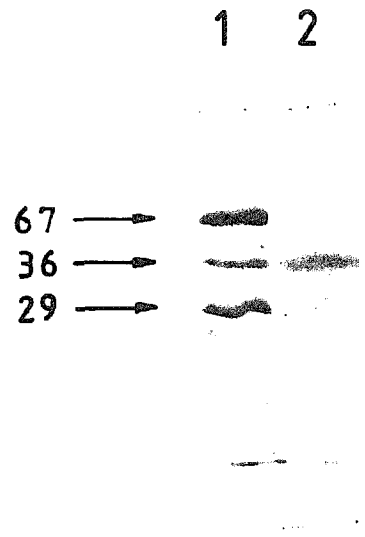

Fig. 1. SDS-polyacrylamide gel of *Paramecium* porin stained with Coomassie blue. 10 μg of the purified protein were applied to a 12% gel. The apparent molecular mass of the porin was about 37000 Da (lane 2). Lane 1, molecular mass standards.

Macroscopic conductance

When *Paramecium* porin was added in small concentrations (30–100 ng/ml) to the aqueous solution bathing a lipid bilayer membrane, the specific conductance of the membrane increased by several orders of magnitude. The time-course of the conductance increase was similar to that measured with other mitochondrial porins, such as those from rat liver [7] and *N. crassa* [12], and is not shown here. After an initial rapid rise for 15–20 min, the membrane conductance continued to increase at a much lower rate. The insertion of the protein usually continued until membrane breakage. The conductance increase could be observed ragardless of whether the protein was added to only one or to both sides of the membrane. The addition of detergent alone at the same concentration as present in the protein solution did not lead to any appreciable increase in the specific membrane conductance above that in the absence of protein and detergent (10^{-8}–10^{-7} S/cm^2).

Single-channel analysis

Experiments with low concentrations of the *Paramecium* porin (1–6 ng/ml) using membranes of small surface area and high current resolution showed that the membrane conductance increased in discrete steps. Fig. 2 shows an experiment in which *Paramecium* porin was added to give a final concentration of 4 ng/ml to the solutions bathing a black lipid bilayer membrane of diphytanoylphosphatidylcholine/*n*-decane. The membrane potential was set at 10 mV. Most of the steps were directed upwards, whereas terminating steps were only rarely observed at this voltage. A histogram of 181 conductance events at 10 mV is given in Fig. 3A. The most frequent value (more than 50% of all events) of the single-channel conductance was 2.5 nS in 1 M KCl, which was about half of that of yeast [13] and of other eukaryotic porins studied to date [10,15,40]. The difference is definitely not caused by the purification procedure, because the single-channel conductance of pure protein and that of a crude Triton X-100 extract were found to be very similar. Fig. 3B shows for comparison a histogram measured under identical experimental conditions with yeast mitochondrial porin. A variety of different ions could traverse the *Paramecium* porin. Table I shows the dependence of the average single-channel conductance Λ of the *Paramecium* porin on different salt solutions. Although there existed a considerable influence of the different salt solutions on Λ, the ratio between single-channel conductance and the specific conductance, σ, of the aqueous phase (i.e., the ion mobility) varied only little. This result indicated that the mechanism of ion movement inside the pore is similar to that in the bulk aqueous phase (i.e., binding plays no significant role).

Selectivity of Paramecium porin

Further information about the structure of the pore formed by *Paramecium* porin may be obtained from

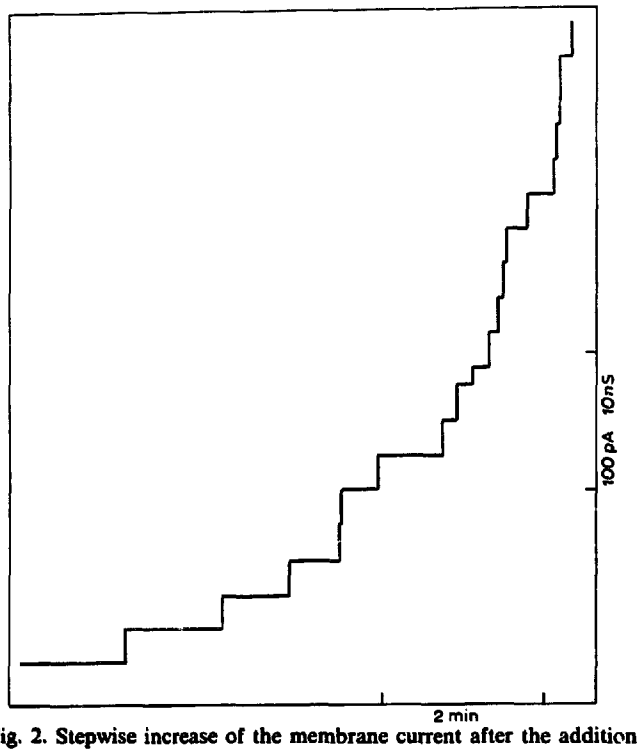

Fig. 2. Stepwise increase of the membrane current after the addition of the porin from *Paramecium* mitochondria. The aqueous phase contained 4 ng/ml porin and 1 M KCl. The membrane was formed from diphytanoylphosphatidylcholine/*n*-decane. The applied voltage was 10 mV; *T*, 25 °C.

zero-current potential measurements in the presence of salt gradients. A 10-fold KCl gradient across a lipid bilayer membrane in which porin had been reconstituted, resulted in an asymmetry potential of about −11 mV on the more dilute side. This result indicated some preferential movement of anions over cations through the pore. The zero-current membrane potentials were analyzed using the Goldman-Hodgkin-Katz equation [37]. The ratio of the anion permeability P_a divided by the cation permeability P_c was about 1.7 for the mitochondrial pore of *Paramecium* in the case of K^+

TABLE I

Average single-channel conductance of Paramecium porin in different salt solutions

The solutions contained 5–30 ng/ml *Paramecium* porin and less than 0.1 μg/ml Triton X-100; the pH value was between 6.0 and 7.0. The membranes were made of diphytanoylphosphatidylcholine/*n*-decane; *T*, 25 °C; V_m, 10 mV. Λ was determined by recording at least 70 conductance steps and averaging over all values. *c* is the concentration of the salt solution and σ is its specific conductance.

Salt	*c* (M)	Λ (nS)	Λ/σ (10^{-8} cm)
KCl	0.03	0.078	2.40
KCl	0.1	0.26	2.17
KCl	0.3	0.85	2.5
KCl	1	2.4	2.16
KCl	3	5.0	2.00
KF	1	1.9	1.94
KBr	1	2.3	2.13
K_2SO_4	0.5	1.5	2.03
LiCl	1	2.8	2.52
$MgCl_2$	0.5	1.9	3.00
Tris-Cl	0.5	1.1	4.90
Tris-Hepes	0.5	0.03	2.06
Sodium-Hepes	0.5	0.27	1.20
Potassium-Mes	0.5	0.23	1.10
Sodium acetate	1	0.72	1.65

TABLE II

Zero-current membrane potentials, V_m, of membranes from diphytanoylphosphatidylcholine/n-decane in the presence of Paramecium porin measured for a 10-fold gradient of different salts

V_m is defined as the potential of the dilute side (10 mM) relative to that of the concentrated side (100 mM); the temperature was 25 °C. P_a/P_c was calculated from the Goldman-Hodgkin-Katz equation [37] from at least four individual experiments.

Salt	V_m (mV)	P_a/P_c
KCl (pH 6)	−11	1.7
LiCl (pH 6)	−24	3.4
Potassium acetate (pH 7)	+14	0.50

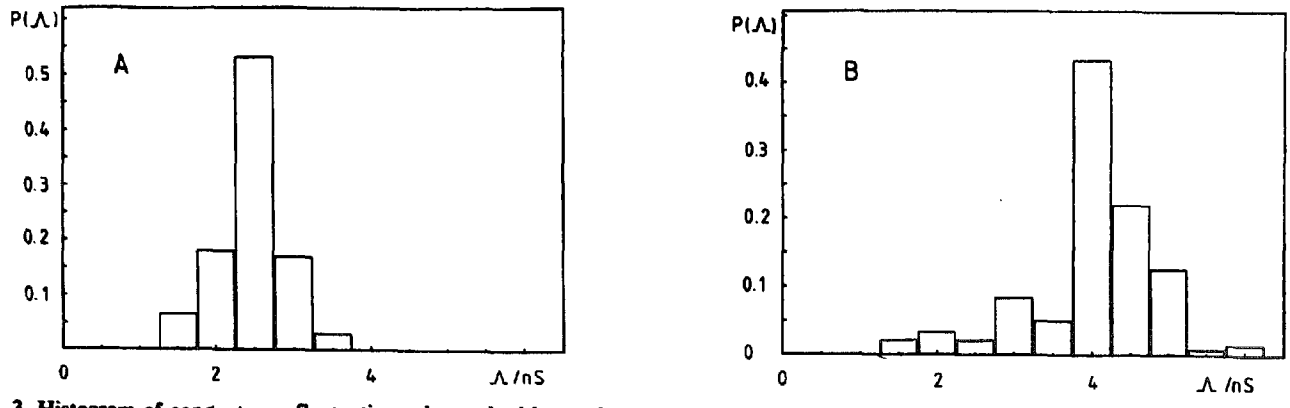

Fig. 3. Histogram of conductance fluctuations observed with membranes of diphytanoylphosphatidylcholine/*n*-decane in the presence of porins from *Paramecium* (A) and yeast (B) mitochondria. The aqueous phase contained 1 M KCl. The applied voltage was 10 mV. The mean value of all upward directed steps was 2.5 nS for 156 single events in the case of *Paramecium* porin and 4.0 nS in the case of yeast porin (181 single events [13]); *T*, 25 °C.

and Cl^- which have equal mobilities in bulk water [38]. Use of LiCl in similar experiments resulted in an even higher anion selectivity and P_a/P_c was calculated to be 3.3 from an asymmetry potential of −24 mV on the more dilute side of a 10-fold LiCl gradient (see Table II). This result indicated that the selectivity of the *Paramecium* porin is not absolute and changed with the mobility of the ions in the aqueous phase. To test whether this was correct we also performed zero-current membrane potential measurements in the presence of potassium acetate. In these experiments the asymmetry potential became positive on the more dilute side. We calculated a permeability ratio, P_a/P_c, of 0.5 from an asymmetry potential of +14 mV on the more dilute side of a 10-fold potassium acetate gradient (see Table II).

Voltage-dependence

At 10 mV transmembrane potential, the terminating events represented only a minor fraction of the total number of current fluctuations. At higher voltages, the terminating events became more and more frequent. This finding indicated that the *Paramecium* mitochondrial porin has a voltage dependency similar to those of the mitochondrial porins from rat liver [6,7], rat brain [9], *N. crassa* [11,12] and yeast [13]. The voltage dependence of the *Paramecium* porin was studied as follows. The voltage across a membrane bathed in 1 M KCl and containing a large number of pores was switched from zero to a given voltage, V_m. The initial current, i.e., the current immediately after application of the voltage, was a linear function of the membrane potential. Subsequently, the membrane current showed an exponential decay with a defined time contant, τ. This effect was first noticeable at about 20 mV and the maximal conductance decreased reached was about 40 to 50% of the initial conductance, G_0, at a transmembrane potential of about 80 mV. Table III shows the results of an experiment of this type. G_0 is the initial conductance, G the conductance after the decay was complete and G/G_0 is the ratio of both values at the corresponding membrane potential.

It is interesting that the time constants, τ, of the exponential decay were also found to be voltage-dependent and decreased strongly with increasing membrane potential (as shown in Table III). τ decreased by about a factor of 150 when the applied voltage was increased from 30 to 100 mV. Although the time constants of the relaxation from the open to the closed state of the pore could easily be measured, the inverse relaxation (i.e., back to the open state of the pore) could not be resolved within the time resolution of our experimental instrumentation (1 ms). This means, that the reaction rates for the closing and the opening of the mitochondrial pore were very different.

TABLE III

Voltage-dependence of the Paramecium porin-mediated membrane conductance

The membrane was formed from diphytanoylphosphatidylcholine/*n*-decane. The aqueous phase contained 1 M KCl and 50 ng/ml mitochondrial porin from *P. tetraurelia*; *T*, 25 °C. The experiments were performed 30 min after membrane formation (after the membrane conductance became stationary). n.d., not detectable.

V_m (mV)	G_0 (μS/cm^2)	G (μS/cm^2)	G/G_0	τ (s)
20	108	92	0.85	n.d.
30	108	80	0.74	0.75
40	110	70	0.64	0.52
50	112	62	0.55	0.25
60	112	56	0.50	0.085
70	115	55	0.48	0.035
80	117	54	0.46	0.022
90	120	55	0.46	0.007
100	120	54	0.45	0.005

Ionic selectivity of the closed state

As described above, the mitochondrial pores of *Paramecium* switched to the closed state if the transmembrane voltages exceeded 15–20 mV. This state definitely has a reduced permeability towards ions. Its ionic selectivity, however, is not known because it is impossible to perform zero-current membrane potential measurements under the conditions in which the pores are in the closed state. To gain some insight into the ionic selectivity of the closed state, we performed the following measurements. The decay of the membrane current was studied as a function of time after the application of a voltage in salts other than KCl. For these experiments a highly mobile cation was combined with a less mobile anion, and vice versa. Fig. 4 shows an experiment in which a voltage of ±50 mV was applied to a membrane bathed in 0.5 M Tris-HCl (pH 7.2) contain-

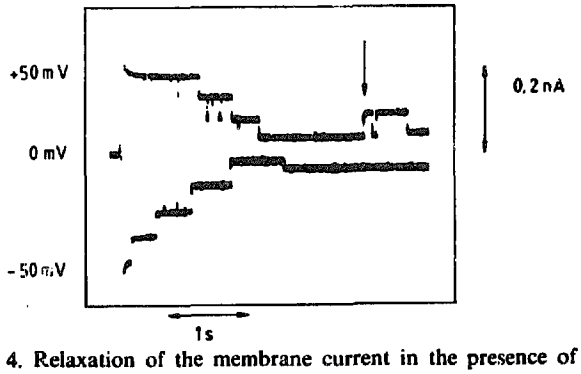

Fig. 4. Relaxation of the membrane current in the presence of the *Paramecium* porin. The membrane potential was first switched to +50 mV and then to −50 mV and cotnained first three (upper trace) and then four pores (lower trace). The arrow indicates the insertion of the fourth pore. The membrane was formed of diphytanoylphosphatidylcholine/*n*-decane. The aqueous phase contained 0.5 M Tris-HCl (pH 7.2); *T*, 25 °C.

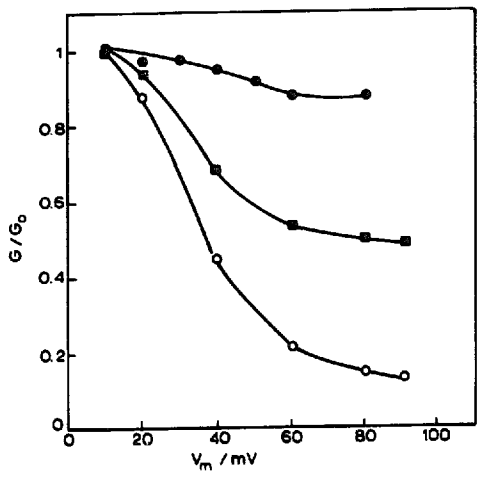

Fig. 5. Ratio of the conductance G at a given membrane potential (V_m) divided by the conductance G_0 at 5 mV as a function of the voltage V_m. The membranes were formed from diphytanoylphosphatidylcholine/*n*-decane. The aqueous phase contained either 0.5 M KCl (pH 7.2) (squares), 0.5 M potassium Mes (pH 7.2) (closed circles), or 0.5 M Tris-HCl (pH 7.2) (open circles). The *cis* side contained 50 ng/ml *Paramecium* porin. The sign of V_m is given with respect to the *cis* side. The experiments were performed 30 min after membrane formation when the conductance was stationary; T, 25 °C.

ing three (upper trace) and four pores (lower trace). Surprisingly, the current through the pores decayed to less than 20% of the initial value (as compared to about 55% in KCl, see Table III). In the case of 0.5 M potassium Mes (pH 7.2; data not shown) the membrane conductance after decay remained higher than 90% of its initial value (i.e., immediately after the application of the 50 mV).

Fig. 5 shows G/G_0 as a function of the transmembrane voltage for 0.5 M KCl (pH 7.2), 0.5 M Tris-HCl (pH 7.2), and 0.5 M potassium Mes (pH 7.2). It is obvious that the voltage dependence was in all similar cases (see Discussion). However, G/G_0 was found to be dependent on the type of the cation and anion present in the aqueous solution. G/G_0 was considerably larger (at high voltages) in potassium Mes than in KCl or in Tris-HCl. This result could only be explained by the assumption that the substate of the *Paramecium* porin has a much lower permeability towards anions than to cations. Otherwise, the results of Fig. 5 could not be explained. The exact value for the permeability ratio of potassium and chloride was difficult to obtain because the mobility of Tris and Mes inside the pore is not known. However, because of the comparably small mobility of Tris and Mes in the aqueous phase, we are sure that P_K/P_{Cl} of the closed state is at least 10. The value may be even larger if the closed state is impermeable to anions. On the other hand, it is also evident from Fig. 5 that potassium is almost equally mobile through the open and the closed state which also suggests that the latter state has completely different properties.

Discussion

In this publication we have described the isolation and purification of a major protein of the mitochondrial outer membrane from *P. tetraurelia*. This protein has an apparent molecular weight of 37000 on a SDS-polyacrylamide electrophoretogram (see Fig. 1). Reconstituted into lipid bilayer membranes the protein formed ion-permeable pores, which is consistent with its role as the permeability channel of the outer membrane. The properties of the mitochondrial porin of *Paramecium* were very similar to those of the porins of other eukaryotic cells, although the single-channel conductance was smaller. The pore conductance was a linear function of the specific conductance of the bulk aqueous phase and did not show the saturation with increasing salt concentration which is expected for a pore which is highly ion-specific [39]. Thus, the pore does not contain any binding site for anions or cations inside when in the open state. There is no doubt that, in the open state, it exists as a water-filled general diffusion pore similar to most bacterial porins [15] and other mitochondrial porins [40]. Furthermore, the selectivity of the *Paramecium* porin was a function of the mobility of the ions in the aqueous phase, which has also been found for other weakly anion-selective mitochondrial and Gram-negative bacterial porins [9,13,15]. No similar shift of the selectivity was observed for the highly anion-selective protein P channel of *Pseudomonas aeruginosa* outer membrane [39].

The absence of any binding site inside the pore and the fact that ions move inside the channel similar to the way in which they move in the bulk aqueous phase allows a rough estimate of the effective diameter of the pore [15]. Assuming that the porin pores are filled with a solution of the same specific conductivity as the external bulk aqueous solution, and assuming that the pore is cylindrical with a length, l, of 6 nm, the average pore radius can be estimated as proposed previously [15,40]. The single-channel conductance of *Paramecium* porin is 0.26 nS in 0.1 M KCl, i.e., the effective diameter ($d = 2r$) of the pore is about 1.3 nm ($\sigma = 14$ mS/cm). The other mitochondrial porins have single-channel conductances of about 0.40 nS under identical conditions (0.1 M KCl); see Table IV for a comparison. Their effective diameter is about 1.7 nm [10,15,40], which agrees well with the results of electron microscopic studies [41,42], but not with the diffusion of poly(ethylene glycols) of various molecular weights [11]. It must be noted that a similar estimate cannot be used for the calculation of the pore size of a specific pore such as the anion-selective channel of *Ps. aeruginosa* outer membrane [39]. The basic differences between a selective (or specific) channel and a general diffusion pore are that the latter must have not only a small electrostatic barrier but also very good shielding of the pore interior

from the low permittivity of the membrane hydrocarbon.

All mitochondrial porins studied to date are voltage dependent [5,6,10,15,40]. *Paramecium* porin is no exception, as Table III and Fig. 4 clearly show. Transmembrane voltages of 20 mV or higher are sufficient to close the pore. The data given in Table III can be analyzed using the equation [5]:

$$N_o/N_c = \exp(nF(V_m - V_0)/RT) \quad (1)$$

where F, R and T have their usual meanings, n is the number of channel gating charges that are assumed to move through the entire transmembrane potential gradient, and V_0 is the potential where 50% of the channels are closed. The open-to-closed ratio N_o/N_c may be calculated from the data given in Table III according to:

$$N_o/N_c = (G - G_{min})/(G_0 - G) \quad (2)$$

where G is the conductance at a given membrane potential V_m, G_0 and G_{min} are the conductances at zero voltage and at very high potentials, respectively. Fig. 6 shows semilogarithmic plots of the ratio N_o/N_c as a function of the transmembrane potential, V_m. The slope of the straight lines gave an e-fold change in N_o/N_c for 13 mV change in V_m, which suggested that the number of charges involved in the gating process was approx. 2.50% of the channels were closed at a voltage of 32 mV. The number of gating charges derived from the conductance voltage data is in good agreement with that calculated from a semilogarithmic plot of the relaxation time constant against the membrane voltage. This plot exhibited a slope of 12 mV for an e-fold change of τ (see Fig. 6), which is also consistent with approx. two gating charges. It is interesting to note that this number of gating charges can be considered typical of other mitochondrial porins, which show gating charge

TABLE IV

Single-channel conductance of mitochondrial porins from different eukaryotic cells in 1 M KCl

The effective diameter, d, of the pores was calcualted assuming the length of the pore to be 6 nm and a specific conductance of the pore interior of 110 mS/cm. The membranes were made of diphytanoylphosphatidylcholine/n-decane. The voltage was 10 mV; T, 25°C.

Porin	Λ (nS)	d (nm)	Ref.
Rat liver	4.3	1.7	7
Rat heart	4.0	1.7	10
Rabbit liver	4.0	1.7	10
Rat brain	4.0	1.7	9
N. crassa	4.5	1.8	11, 12
Yeast	4.2	1.7	13
Paramecium	2.4	1.3	this work

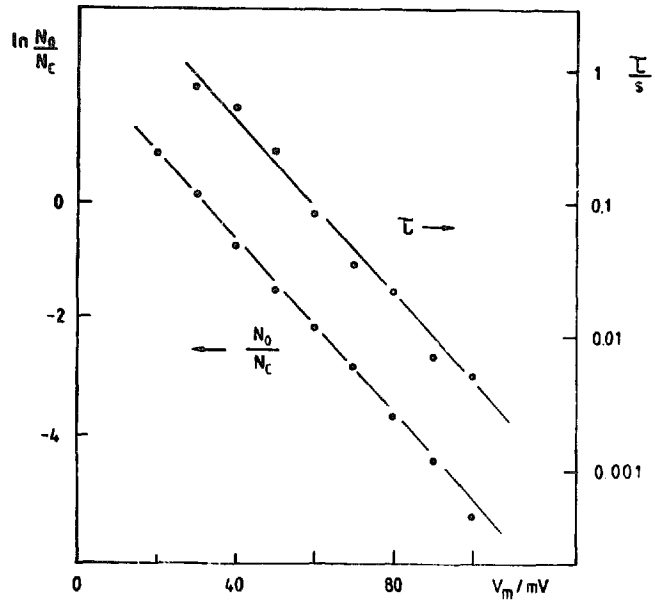

Fig. 6. Semilogarithmic plot of the ratio, N_o/N_c (closed circles), and of the relaxation time, τ (full circles), as a function of the transmembrane potential V_m. The data were taken from Table III. The slope of the straight lines is such that an e-fold change of N_o/N_c or of τ is produced by a change in V_m of 12 mV. The midpoint potential of the N_oN_c distribution (i.e., $N_o = N_c$) was at 32 mV.

numbers between one and three under the same conditions [10].

It has been suggested [6] that the properties of *Paramecium* porin have already been reported in a paper by Schein et al. [5]. In that study, the pore formation of a crude hexane extract of *Paramecium* mitochondria was investigated in lipid bilayer membranes. However, the properties of the purified *Paramecium* porin described here differ considerably from the results of Schein et al. [5]. Examples of these differences are: the single-channel conductance in 0.1 M KCl (0.26 nS compared to 0.5 nS); the number of gating charges ($n = 2$ compared to $n = 4$–6); and the ionic selectivity of the open state ($P_a/P_c = 1.7$, compared to $P_a/P_c = 7$). Furthermore, Schein et al. found that acetate had a higher permeability than potassium through the *Paramecium* porin, which we definitely did not observe. Thus, we have to conclude that either Schein et al. studied a different pore or that the integrity of their porin was influenced by the use of hexane during the isolation process. The difference was definitely not caused by the purification of the porin, because we received the same results with a crude Triton X-100 extract of mitochondria and we found that the addition of hexane to our porin preparations completely destroyed their pore-forming activity (Ludwig, O. and Benz, R., unpublished observations), presumably because the sterol and other lipid essential for pore-formation was removed from the pore-forming complex [22,43]. On the other hand, it cannot be excluded that the discrepancy between our data and those of Schein et

al. is caused by the subtraction of the voltage-independent part of the pore conductance (Colombini, M., personal communication).

Voltage dependence is a general property of the mitochondrial pore: mitochondrial porins from rat liver and other mammalian tissue [7,10] and *N. crassa* [11,12] are also voltage-dependent. The pore conductance in a variety of salts is reduced at higher voltages. The reduction of the conductance of the pore was dependent on the type of the salt used for the experiments. The combination of a highly mobile cation with a less mobile anion (potassium MES) resulted in only a small decrease in membrane conductance. The opposite combination, i.e., that of a mobile anion with a less mobile cation (Tris-HCl) resulted in a more substantial decrease of the pore conductance. This clearly indicated that the pore favors the permeation of cations in the closed state. The permeability ratio, P_K/P_{Cl}, of the closed state is at least 10 (see Fig. 5), if not even larger. This means that the permeation of anionic solutes through the closed state of the pore is either very limited or impossible.

In previous publications [7,10,12,15,28] we have suggested that the closure of the mitochondrial porin may be part of the control of mitochondrial metabolism. This would be possible only if this state had permeability and selectivity different from those of the open state. In fact, our data provided the first evidence that the closed state of the mitochondrial pore is impermeable to anionic solutes. This means that a voltage-dependent closure of the pore could stop mitochondrial metabolism [15,28]. In fact, we have shown recently [24], that ATP and ADP cannot penetrate the mitochondrial outer membrane if the pores are in the closed state. These experiments were performed with a synthetic polyanion of molecular weight 10000, which is the copolymer of methacrylate, maleate, and styrene (in a molar ratio 1:2:3). This polyanion has been reported to inhibit oxidative phosphorylation [44] and the carriers located in the mitochondrial inner membrane [45]. Its direct action is, however, to shift the voltage-dependence of the porin so that the closed state occurs at very small voltages [24].

So far, it is not clear how such a transmembrane potential could be created across the outer mitochondrial membrane. This is because the existence of an ionic gradient across the outer mitochondrial membrane seems to be rather unlikely, as judged by the large permeability of the pore and its very limited ion selectivity. Another possibility would be that the membranes were asymmetric enough to possess a significant intrinsic potential. However, the change of an intrinsic membrane potential would probably be too slow to allow rapid control of the mitochondrial metabolism. A further possibility is based on the observation that the mitochondrial outer and inner membranes form a five-layered structure with a width of 12 nm, as seen in electron micrographs of in situ mitochondria [46]. This means that the space between the two membranes is very small, which could result in electric coupling between the two membranes. The membrane potential or the outer surface potential of the inner mitochondrial membrane could thereby influence the permeability of the outer mitochondrial membrane and thus in turn control the metabolism of mitochondria. This proposed electrical coupling is critically dependent on the actual distance between both membranes and the Debye length in the intermembrane space. A large distance and a short Debye length would both result in loose coupling, whereas distances of the order of 2–3 nm and a large Debye length (caused by a high dielectric constant or by low ionic strength of the intermembrane space) could result in tight coupling between the two membranes. This interpretation would be consistent with the small number of contact sites between inner and outer membranes found in cancer mitochondria while energized mitochondria have a large number of contact sites and a much better metabolic control [47,48].

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