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Pore formation by the mitochondrial porin of rat brain in lipid bilayer membranes

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The porin of the outer membrane of rat-brain mitochondria was isolated and purified. The protein showed a single band of apparent M_r 35500 on dodecyl sulfate-containing polyacrylamide gels. The incorporation of rat-brain porin into artificial lipid bilayer membranes showed that it is able to form pores with an average single-channel conductance of 400 pS in 0.1 M KCl. The pores were found to be voltage-dependent and switched to substates at higher transmembrane potentials. The voltage-dependence of the rat brain pore was considerably smaller than that of the other known eukaryotic porins. The possible role of the rat-brain porin in the regulation of transport processes across the outer mitochondrial membrane is discussed.

Introduction

The outer mitochondrial membrane seems to be freely permeable to a variety of different solutes [1,2]. It is, on the other hand, impermeable to molecules with higher molecular weights [3]. These observations indicated that the outer mitochondrial membrane contains a defined pathway for the mostly anionic substrates of mitochondria. In fact, a pore-forming protein was identified in the outer mitochondrial membrane of a variety of eukaryotic cells (Refs. 4–7, see also Refs. 8 and 9 for recent reviews). This protein (also known as the voltage-dependent anion-selective channel – VDAC [4,5]) was named mitochondrial porin in analogy to the bacterial porins and in agreement with the endosymbiontic theory [6–10]. Mitochondrial porins have molecular weights between 30000 and 35000 [6–12]. They are encoded in the nucleus and are synthesized at free cytoplasmic ribosomes without leader sequence [13–15]. The mature protein binds dicyclohexylcarbodiimide (DCCD) at very low concentrations, which indicated a negatively charged group in a hydrophobic environment [12]. Experiments with water-soluble porin from *Neurospora crassa* suggested that the channel-forming unit is a complex composed of polypeptide(s), phospholipids and sterols [16].

The role of the mitochondrial pore in the metabolism and physiology of mitochondria is unresolved at present. Its 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 higher than 10–20 mV [4–9]. The substates have a smaller permeability and a different ionic selectivity than the open pore [4,8,9,17]. Another control of the channel permeability could be generated by the binding of kinases

Abbreviations: DCCD, dicyclohexylcarbodiimide; HTP, hydroxyapatite; SDS, sodium dodecyl sulfate.

to the pore. The binding of hexokinase and glycerokinase could have the advantage that these enzymes utilize directly the mitochondrial ATP-pool [18–20]. On the other hand, the binding of these enzymes could control the channel permeability as was suggested for rat brain mitochondria [21]. These mitochondria contain more than 80% of hexokinase tightly but reversibly bound [22].

In this publication we describe the isolation and purification of porin from rat-brain mitochondria. The porin was reconstituted in lipid bilayer membranes and the properties of the pores were studied in detail. The rat-brain porin formed general diffusion pores with a small preference for Cl^- over K^+. The voltage-dependence was considerably less than those of the other eukaryotic porins studied so far. The possible role of the smaller voltage dependence in the physiology of mitochondria is discussed.

Materials and Methods

Purification of rat-brain porin

Mitochondria from rat brain were isolated as described elsewhere [23]. The protein was determined according to the Biuret method [24]. The methods of isolation and purification of the porin were essentially the same as described previously for the pig heart porin (DCCD-binding protein [12]). Rat-brain mitochondria were solubilized for 20 min at 0°C in 3% Triton X-100/20 mM KH_2PO_4 (pH 6.5)/20 mM KCl/1 mM EDTA in such a way that the total protein concentration was 10 mg/ml. The unsolubilized material was removed by centrifugation at $147000 \times g$ for 30 min. The Triton X-100 extract (6 ml of the supernatant) was applied to a hydroxyapatite column (6 g of dry Bio-Gel HTP from Bio-Rad, Richmond, CA) and the elution of the column was performed with the solubilization buffer. The first eluted 7 ml were applied to a Affi-Gel 501 (Bio-Rad) column which was preequilibrated with the solubilization buffer. The unretarded proteins from the Affi-Gel column were subsequently applied to a dry HTP/Celite column (ratio 1 : 1 (w/w); total 6 g; Celite 535 was obtained from Roth, Karlsruhe, G.F.R.). The HTP/Celite column was eluated with the solubilization buffer and the first eluted 7 ml contained the pure protein.

The purity of the protein was tested by electrophoresis on a polyacrylamide-sodium dodecyl sulfate (SDS) gel [25]. The separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 150. The porin was precipitated in 90% cold acetone. The pellet obtained by centrifugation was dissolved in SDS sample buffer [25] before application to the gel. The porin concentration was determined by the Lowry method modified for the presence of Triton X-100 [26].

Membrane experiments

The methods used for black lipid bilayer experiments have been described previously in detail [27]. 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 and phosphatidylserine (Avanti Biochemicals, Birmingham, AL) in *n*-decane.

The temperature was kept at 25°C throughout.

All salts were obtained from Merck (Darmstadt, F.R.G. analytical grade). The aqueous solutions were unbuffered and had a pH around 6. To prevent protein inactivation, the protein was added to the aqueous phase from the 10-fold diluted stock solutions (containing 0.1% Triton X-100) either prior to membrane formation or after the membrane had turned completely black.

The membrane current was measured at given voltages using a pair of calomel electrodes with salt bridges, which were inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a current amplifier (Keithley, Cleveland OH, model 427) monitored on a storage oscilloscope (Tektronix, Beaverton OR, model 5115) and recorded on a strip chart or 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, [28].

Results

Purification of rat brain porin

The same purification procedure as established for pig-heart porin resulted also in a pure preparation of rat-brain porin (DCCD-binding protein [12]). The electrophoretic mobility of this porin was very similar but not identical to that of pig heart porin, since it showed a somewhat lower mobility on a SDS-polyacrylamide gel corresponding to an apparent M_r of 35 500. The yield of the purification procedure for rat-brain porin was approx. 60% of that for pig-heart porin [12].

Membrane experiments

Macroscopic conductance

When the rat brain porin was added in small quantities (10 to 100 ng/ml) to the aqueous solutions bathing a lipid bilayer membrane, the specific conductance of the membranes increased by several orders of magnitude. The time-course of the conductance increase was similar to that described previously for mitochondrial porin from rat liver [6] and from *Neurospora crassa* [7]. After an initial rapid increase for 15–20 min, the membrane conductance increased at a much lower rate. The slow increase continued usually until membrane breakage. The conductance increase was observed regardless of whether the protein was added to only one side or to both sides of the membrane. The addition of detergent alone at the same concentration as present in the protein solutions did not lead to any appreciable increase in the specific membrane conductance above that in the absence of protein and detergent (10^{-8} to 10^{-7} S/cm^2).

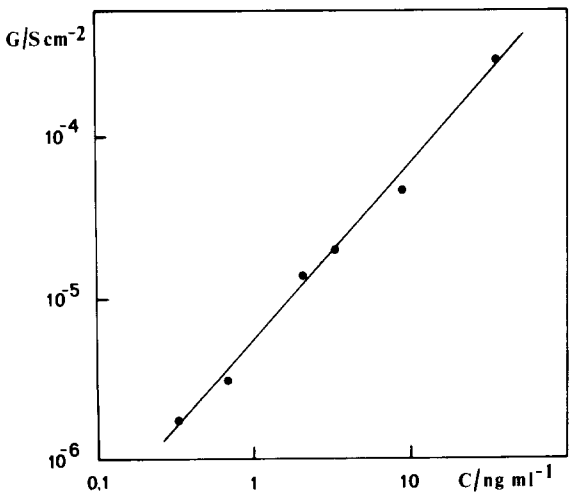

Fig. 1. Dependence of the specific membrane conductance, G, on the rat-brain porin concentration c in the aqueous phase, G was measured 20 min after the addition of the protein to the black lipid membrane. The membranes were formed from diphytanoylphosphatidylcholine in *n*-decane. The aqueous phase contained 1 M KCl and less than 0.1 μg/ml Triton X-100; $T = 25$ °C; $V_m = 10$ mV.

Because of the time-dependence of the membrane conductance in the presence of brain porin, it was somewhat difficult to determine the dependence of the specific membrane conductance on the protein concentration in the aqueous phase. However, a meaningful comparison was possible when we used the conductance value at a fixed time after the addition of the protein. Fig. 1 shows the dependence of the membrane conductance on the rat-brain porin concentration in the aqueous phase measured 20 min after the addition of the protein to the black lipid membranes. As can be seen from Fig. 1, there exists a linear relationship between membrane conductance and protein concentration in the aqueous phase for at least a 100-fold range of protein concentration. Similar linear relationships were also observed for mitochondrial porins from rat liver and *Neurospora crassa* [6,7].

It has to be noted that the process of the insertion of the mitochondrial porin into lipid bilayer membranes was highly dependent on the type of lipid used for membrane formation. Whereas the kinetics of the conductance increase were approximately the same for different lipids, the pore formation probability was at least 100-fold higher in membranes prepared from oxidized cholesterol than from diphytanoylphosphatidylcholine. The reason for this different behavior remains unclear so far. The similar 'lipid specificity' of bacterial porins was discussed on the basis of different lateral pressures in lipid bilayer membranes made of different lipids [29,30].

The mitochondrial pores from rat liver [6,31] and *Neurosopora crassa* [7] were reported to be voltage-dependent. This voltage-dependence and its magnitude were observed irrespective of the

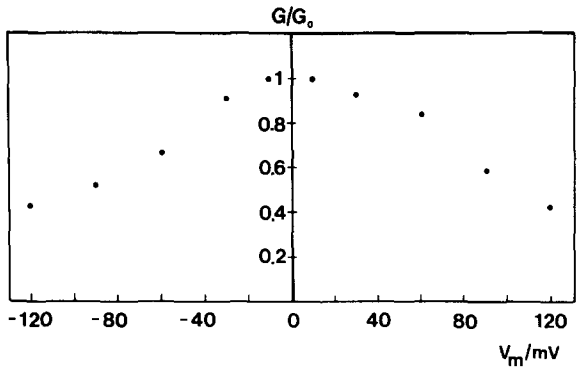

Fig. 2. Ratio of the conductance, G, at a given membrane potential divided by the conductance, G_0, at zero potential as a function of the applied membrane potential, V_m. The membranes were formed from a mixture of diphytanoylphosphatidylcholine/phosphatidylserine (molar ratio 4:1) in *n*-decane. The aqueous phase contained 1 M KCl. The *cis* side contained 9 ng/ml rat brain porin. The sign of V_m is given with respect to the *cis* side; $T = 25°C$.

type of membrane-forming lipid [6,7] and irrespective of the methods used for membrane formation [5,6]. Current-voltage curves were measured with lipid bilayer membranes in presence of rat-brain porin to test whether this porin has a voltage dependence similar to that of the pore from rat liver. Fig. 2 shows the ratio of the conductance, G, at a given membrane potential divided by the conductance, G_0, at zero potential as a function of the applied membrane potential. Surprisingly, the membrane conductance induced by rat-brain porin appeared to be less voltage-dependent than that of rat liver ([6,31], see also Discussion). A similar smaller voltage dependendence was also observed for the time constant of the conductance decrease when the membrane potential was switched from zero to the potential V_m. Fig. 3 shows such relaxation processes. V_m was rapidly taken from 0 to 50 mV (curve 1) and from 0 to 100 mV (curve 2, same membrane). Whereas at 50 mV membrane potential only an insignificant conductance decrease was observed, the membrane conductance decreased by about 40% at 100 mV with a time constant of about 1 s. The initial conductance (i.e., immediately after switching on the membrane potential) was a linear function of the applied potential.

The time constant of the single exponentional relaxation process varied with the membrane

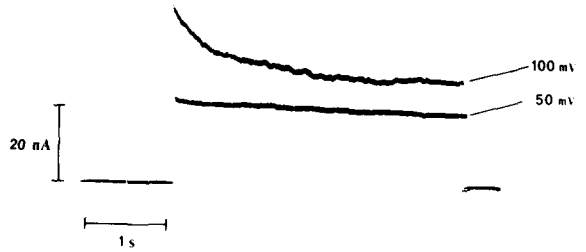

Fig. 3. Relaxation of the membrane current in the presence of the rat-brain porin. The membrane potential was rapidly taken from 0 to 50 mV (curve I) and from 0 to 100 mV (curve II, same membrane). The membrane was formed from diphytanoylphosphatidylcholine/*n*-decane, and had an area of 1 mm^2. The aqueous phase contained 1 M KCl and about 2 ng/ml porin from rat-brain mitochondria; $T = 25°C$.

potential. Fig. 4 shows a semilogarithmic plot of the relaxation time constant as a function of the membrane potential. The experimental results could be fitted to a straight line. This line corresponds to an e-fold decrease for the time constant for about 23 mV, which means that the number of gating charges traversing the entire membrane

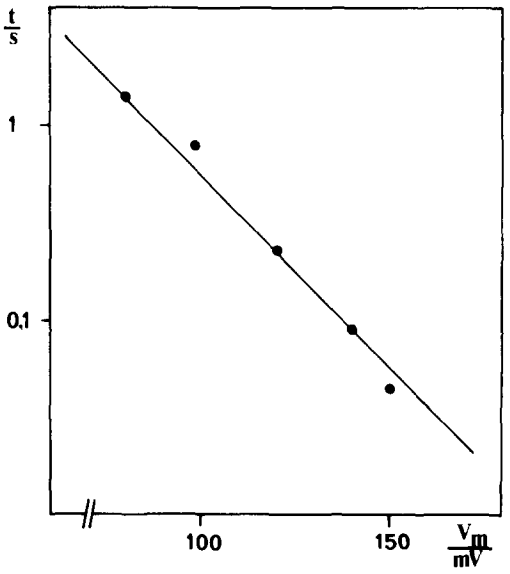

Fig. 4. Semilogarithmic plot of the relaxation time constant, τ, as a function of the membrane potential V_m. The membrane was formed from a mixture of diphytanoylphosphatidylcholine/phosphatidylserine (molar ratio 4:1) in *n*-decane. The aqueous phase contained 1 M KCl, and 1.3 ng/ml porin from rat-brain mitochondria; $T = 25°C$.

potential in channel closing is about one (see Discussion). The time constant of the switching of the pores from the 'closed' to the 'open' state could not be followed within the time resolution of our experimental instrumentation (1 ms). This corresponds to largely different reaction rates for the closing and the opening of the mitochondrial pore at small voltages.

Single-channel analysis

The addition of low concentrations (1–5 ng/ml) of rat brain porin to the aqueous phase on one or both sides of a black lipid bilayer membrane of small surface resulted in a stepwise increase of the membrane current at a fixed voltage. Such an experiment is demonstrated in Fig. 5, in which rat brain porin was added in a final concentration of 1 ng/ml to a black lipid bilayer membrane of diphytanoylphosphatidylcholine/*n*-decane. The current steps shown in Fig. 5 were specific to the presence of rat-brain porin and were not observed when only the detergent Triton X-100 was added

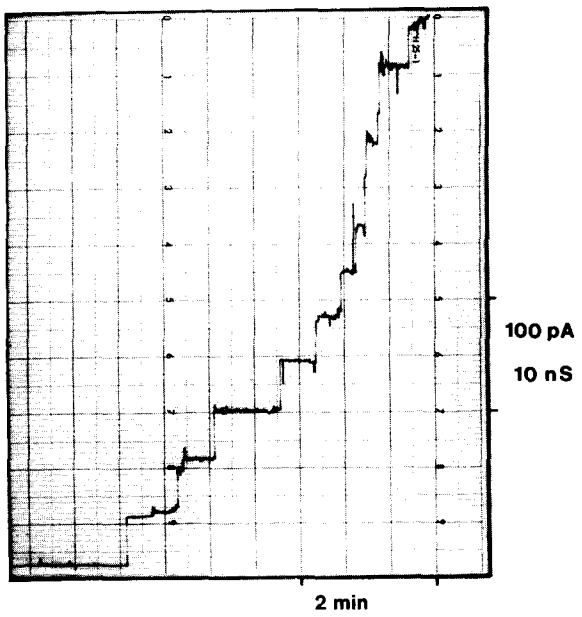

Fig. 5. Stepwise increase in the membrane current after the addition of the porin from rat-brain mitochondria. The aqueous phase contained 1.2 ng/ml porin and 1 M KCl. The membrane was formed from diphytanoylphosphatidylcholine in *n*-decane. The applied voltage was 10 mV; the current prior to the addition of the protein was less than 0.2 pA; $T = 25°C$.

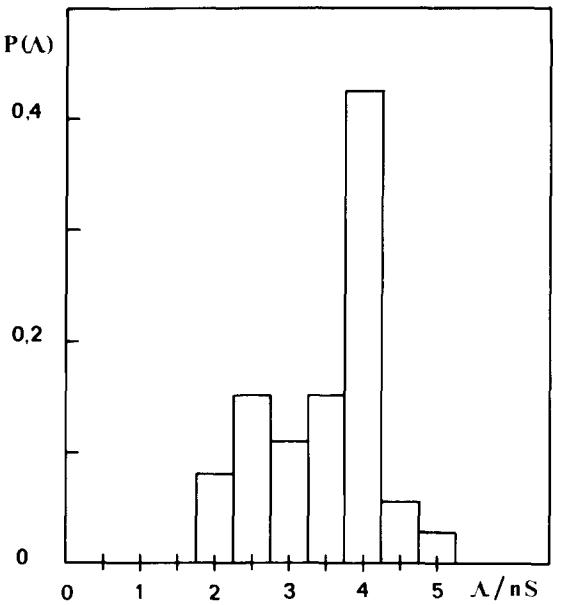

Fig. 6. Histogram of conductance fluctuations observed with membranes from diphytanoyl phosphatidylcholine in *n*-decane in the presence of porin from rat-brain mitochondria. The aqueous phase contained 1 M KCl. The applied voltage was 10 mV. The mean value of all upward-directed steps was 4.0 nS for 73 single events; $T = 25°C$.

to the aqueous phase. Most current steps were directed upward and terminating steps were only rarely observed at transmembrane potentials of 5 and 10 mV, indicating a long lifetime of the pores at small voltages.

The size of rat brain pores was not homogeneous (see Fig. 6). The most frequent value for the single channel conductance is about 3.5 to 4.5 nS. A second peak in the distribution of single-channel conductances is observed between 2 and 3 nS, which contains about 30% of the total number of single events. It has been suggested earlier that the smaller steps are substates of the 4 nS channel [7,9] which eventually may revert to the open state. In fact, the increase in the transmembrane potential above 50–60 mV results in the occurrence of substates of the rat brain porin. The absolute level of the substates of the pores varied with the applied membrane potential, indicating many different substates of the pore.

The channel from the outer mitochondrial membrane of rat brain was permeable to a variety of different ions. Table I shows the average

TABLE I

AVERAGE SINGLE-CHANNEL CONDUCTANCE IN DIFFERENT SALT SOLUTIONS OF CONCENTRATION c

The solution contained 1.2 ng/ml rat-brain porin and less than 0.1 μg/ml Triton X-100; the pH was between 6.0 and 7.0. The membranes were made from diphytanoylphosphatidylcholine in n-decane (1%, w/v) $T = 25°C$; $V_m = 10$ mV. $\overline{\Lambda}$ was determined by recording at least 70 conductance steps and averaging over the distribution of values. σ is the specific conductance of the aqueous salt solutions.

Salt	c (M)	$\overline{\Lambda}$ (nS)	$\overline{\Lambda}/\sigma$ (10^{-8} cm)
KCl	0.01	0.05	4.2
	0.05	0.27	4.5
	0.1	0.4	4.2
	0.3	1.4	4.1
	1.0	4.0	3.6
	3.0	11.5	4.6
NaCl	1.0	4.0	4.7
RbCl	1.0	5.0	4.3
CH_3COOK	1.0	2.5	4.0
LiCl	1.0	3.5	4.9
$MgCl_2$	0.5	3.0	4.7
Na_2SO_4	0.5	2.5	4.6
Tris-HCl	0.5	1.5	5.0

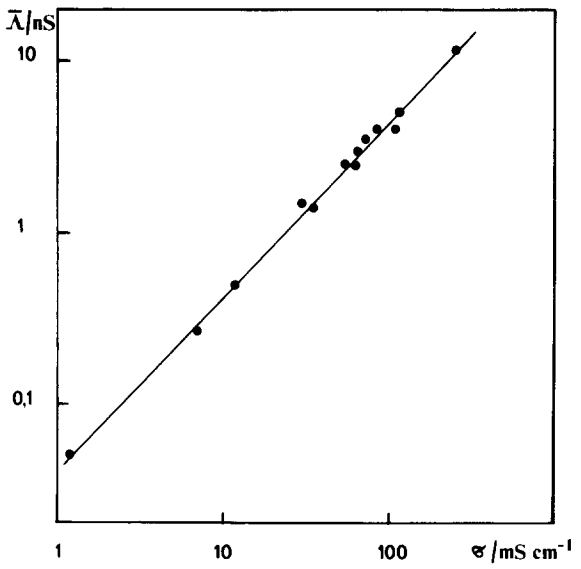

Fig. 7. The average single-channel conductance, $\overline{\Lambda}$, of rat-brain porin from different salts given as a function of the specific conductance of the corresponding aqueous salt solutions. $T = 25°C$. The data were taken from Table I.

single-channel conductance, Λ, of rat-brain porin in different salt solutions. Although there exists a considerable influence of the different salts on $\overline{\Lambda}$, the ratio between single channel conductance and the specific conductance, σ, of the aqueous phase (i.e., the ion mobility) varied only little. This may be explained by the assumption that the ions move inside the pore in a manner similar to that in which they move in an aqueous environment. This can also be seen from Fig. 7, which shows the average single channel conductance, $\overline{\Lambda}$, of rat brain porin as a function of σ. The data points in the double-logarithmic plot could be reasonably well fitted to a straight line, suggesting a 1 : 1 relationship between the movement inside the pore and that in the aqueous phase.

Selectivity of rat-brain porin

Further information about the structure of the pore formed by the rat-brain porin was obtained by zero-current membrane potential measurements in the presence of salt gradients. Table II shows the results of the measurements for the 10-fold gradients of KCl, LiCl and CH_3COOK. The potentials were found to be negative on the dilute side for LiCl and KCl, indicating preferential movement of Cl^-, whereas it was positive for CH_3COOK, which indicated preferential movement of K^+ over acetate through the pore. The zero-current membrane potentials were analyzed using the Goldman-Hodgkin-Katz equation [28]. The ratio of the anion permeability, P_a,

TABLE II

ZERO-CURRENT MEMBRANE POTENTIALS V_m, IN THE PRESENCE OF A 10-FOLD CONCENTRATION GRADIENT OF DIFFERENT SALTS

V_m is the electrical potential on the dilute side minus the potential of the concentrated side. The membranes were formed from diphytanoylphosphatidylcholine in n-decane. The aqueous solutions were unbuffered and had a pH as indicated. The ratio of the permeabilities P_c (cation) and P_a (anion) was calculated according to the Goldman-Hodgkin-Katz equation [28].

Salt	pH	V_m (mV)	P_c/P_a
KCl	6	−11	0.60
LiCl	6	−16	0.46
CH_3COOK	7	42	10.4

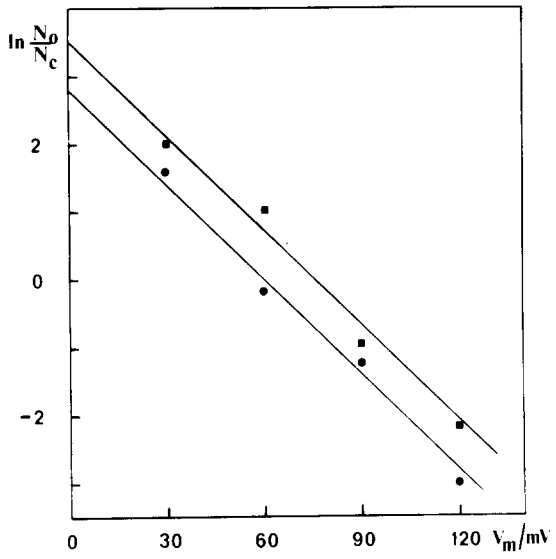

Fig. 8. Semilogarithmic plots of the ratio N_o/N_c as a function of the transmembrane potential V_m. The closed circles (●) indicate the measurements with a positive transmembrane potential, V_m, while the squares (■) shows measurements with the negative potential with respect to the addition of the porin. The data were taken from Fig. 2; $V_o(+) = +60$ mV; $V_o(-) = -67$ mV.

divided by the cation permeability, P_c, suggests that Cl^- has a 1.7-fold higher mobility inside the pore than K^+, despite the same mobility in the aqueous phase [32]. The permeability rates for the other two salts are consistent with the mobility sequences of the cations and the anions in the aqueous phase. The small anion selectivity in the case of KCl may be explained by an excess of positively charged groups in or near the pore [9,31].

Discussion

In this publication we have shown that a polypeptide of M_r 35 500 from rat-brain mitochondria is able to form ion-permeable pores in lipid bilayer membranes. The pores are wide and allow the passage of large organic ions without any detectable interaction with the pore interior. Furthermore, the pore conductance was a linear function of the specific conductance of the aqueous salt solution and did not show saturation with increasing salt concentration which would in principle be expected for an ion-specific pore [33]. The absence of any binding site and the high single-channel conductance, Λ, allows a rough estimate of the effective diameter of the pore from outer membrane of rat brain mitochondria. Assuming that the pores are filled with a solution of the same specific conductivity, σ, as the external solution and assuming a cylindrical pore with a length, l, of 6 nm, the effective pore diameter d ($= 2r$) can be calculated according to

$$\Lambda = \sigma \pi r^2 / l \qquad (1)$$

The single-channel conductance is about 4 nS in 1 M KCl. This means that the effective diameter of the pore is about 1.7 nm. The diameter of most other mitochondrial porins is very similar to this value [8,9]. Only porin from *Paramecium* had a smaller single-channel conductance (2.4 nS in 1 M KCl) and thus a smaller effective diameter (1.3 nm) [8,9].

A diameter of 1.7 nm for the rat-brain porin would be consistent with the electron microscopic studies. Negative staining of isolated outer membranes from mung bean mitochondria have revealed stain-filled pits of 2–3 nm diameter [34]. More recent studies of outer mitochondrial membrane of *Neurospora crassa* showed that the pores are organized in a hexagonal array if the outer membranes are dialyzed against low salt buffer and loose phospholipids [35,36]. Averaged electron microscopic images of these crystalline arrays show that the mitochondrial pore is in fact a cylinder with a diameter of 2 nm and a length of 5 to 6 nm [37,38]. This means that a diameter of the mitochondrial pore of 4 nm which was found in the liposome swelling assay using poly(ethylene glycols) is most likely an overestimate of the channel size [39].

All mitochondrial porins studied so far are voltage-dependent and switch to substates of reduced conductance at higher transmembrane potentials [8,9,40]. Rat-brain porin is in this respect to exception. Exceptional is, on the other hand, the rather low voltage-dependence (see Fig. 3). Whereas a transmembrane potential of more than 100 mV is needed to reduce the initial conductance to about 60% in the case of the rat-brain porin, less than 50 mV is sufficient for the same effect for other mitochondrial porins from

eukaryotic cells [4,6,7]. The data given in Fig. 2 can be analyzed using the equation [4]:

$$N_o/N_c = \exp[nF(V_m - V_o)/RT] \quad (2)$$

where F, R and T have the usual meaning, n is the number of gating charges moving through the entire transmembrane potential gradient for channel gating and V_o is the potential where 50% of the total number of channels are in the closed configuration. The open to closed ratio N_o/N_c may be calculated from the data given in Fig. 2 according to [4]:

$$N_o/N_c = (G - G_{min})/(G_0 - G) \quad (3)$$

G is the conductance at a given membrane potential V_m, G_0 and G_{min} are the conductances at zero voltage and very high potentials, respectively. Fig. 8 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 was about 21 mV for an e-fold change of N_o/N_c, which suggested that the number of charges involved in the gating process was approximately one. This result agreed nicely with the relaxation time constants, where we also found a slope of 23 mV for an e-fold change of τ (see Fig. 4).

This result was somewhat unexpected, because the number of charges involved in the gating process of rat liver porin was approximately three [9,31]. So far it is not known if eukaryotic cells contain the genes of several porins. However, the different number of gating charges for rat-liver porin and rat-brain porin, which were found to be independent of the lipid composition of the membranes, suggested that there could be a difference either in the sequence or in the pore-forming complex of both pores. In this respect it is interesting to note that the hexokinase is tightly bound to brain mitochondria, whereas it is only loosely associated to the surface of rat-liver mitochondria [22]. This could be due to the existence of different hexokinase isoenzymes in both cells [41]. On the other hand, the difference in the hexokinase binding could also be caused by a change of the binding site on the pore-forming complex, which would support the existence of different genes for porins in the nucleus of an eukaryotic cell. These considerations would also apply to cancer cells, where hexokinase is also firmly bound to the surface of mitochondria, whereas in the normal cells the situation could be similar to that in the rat liver [42].

All mitochondrial porins studied so far were found to be voltage-gated [8,9] and the rat brain porin is no exception. This result indicated that the permeability of the mitochondrial pore and of the outer mitochondrial membrane could be voltage-controlled. Increasing transmembrane potentials could reduce the exclusion limit of the outer membrane and thus control the metabolism of mitochondria. However, no evidence was found for such a regulation process. Furthermore, the pore diameter appears too large and the pore selectivity too small for an ionic gradient to be stable for a time larger than a few milliseconds [8,9]. The generation of an intrinsic membrane potential could also control the permeability of the outer mitochondrial membrane. But such a process could be too slow for a rapid regulation of mitochondrial metabolism. Nevertheless, there could exist a metabolic control of outer membrane permeability. In vitro, mitochondria have an ultrastructure somewhat different from that of the in situ mitochondria, where all membranes are closely apposed and form five-layered, 12 nm wide structures [43]. This close apposition of the outer and the inner membranes could result in electrical coupling of both membranes in situ [9,44]. The electrical coupling of both membranes could lead to a change in the permeability of the outer membrane (i.e., the pore) for substrates of the oxidative phosphorylation and its products, if the potential across the inner membrane changed. The mitochondrial pore presumably plays an important role also in the microcompartment formation in mitochondria. Such a microcompartment was described for hexokinase and the adenylate translocator which in energized mitochondria excludes the adenylate kinase from these mitochondrial ATP pool [17].

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