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Purification and characterization of the pore forming protein of yeast mitochondrial outer membrane

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Abstract. One of the major outer membrane proteins of yeast mitochondria was isolated and purified. It migrated as a single band with an apparent molecular weight of 30 kDa on a SDS-electrophoretogram. When reconstituted in lipid bilayer membranes the protein formed pores with a single channel conductance of 0.45 nS in 0.1 *M* KCl. The pores had the characteristics of general diffusion pores with an estimated diameter of 1.7 nm. The pore of mitochondrial outer membranes of yeast shared some similarities with the pores formed by mitochondrial and bacterial porins. The pores switched to substates at voltages higher than 20 mV. The possible role of this voltage-dependence in the metabolism of mitochondria is discussed.

Key words: Ion-channel, porin, mitochondrial outer membrane, lipid bilayer, yeast mitochondria, voltage-dependence

Introduction

Mitochondria are presumably descendents of advanced, strictly aerobic Gram-negative bacteria (John and Whatley 1975; Schwartz and Dayhoff 1978). The mitochondrial outer membrane of a variety of eukaryotic cells contains, in agreement with the endosymbiontic theory, a pore-forming protein termed mitochondrial porin (Roos et al. 1982; Freitag et al. 1982a, b; Benz 1985; Benz et al. 1985a; Zalman et al. 1980). This channel forming protein (called also VDAC) was first reconstituted by Schein et al. (1976) from crude extracts of *Paramecium* mitochondria into planar lipid bilayer membranes. Some of these mitochondrial porins (or VDAC) were studied in detail (Colombini 1979, 1980a; Roos et al. 1982; Freitag et al. 1982b; Ludwig et al. 1986). The outer membrane of yeast mitochondria contains a major protein with a molecular weight of 30 kDa, which constitutes of at least one fifth of the protein mass of the membrane (Gasser and Schatz 1983; Mihara et al. 1982). Based on vesicle experiments it was proposed that this protein is the yeast porin (Mihara et al. 1982). Its gene is located in the nucleus of the yeast cell and it is synthesized at cytoplasmic ribosomes without leader sequence (Freitag et al. 1982a; Mihara et al. 1982; Gasser and Schatz 1983). The primary structure of the 30 kDa protein was derived from the sequence of the gene (Mihara and Sato 1985). The sequence is not particularly hydrophobic, which may indicate that arrangement of the polypeptide chain in secondary, tertiary, and quaternary structure is presumably responsible for the role of the protein as an intrinsic membrane protein (Migara and Sato 1985; Capaldi and Vanderkoi 1972).

In this paper we describe an elegant way for the isolation and purification of the 30 kDa protein from yeast mitochondria. The protein spontaneously inserted into lipid bilayer membranes. Single channel analysis indicated that this protein formed pores and is the mitochondrial porin of yeast. The single channel conductance was found to depend on the membrane potential. In many respects the yeast mitochondrial porin resembles the properties of other mitochondrial porins and of the porins isolated from the outer membrane of Gram-negative bacteria.

Materials and methods

Isolation of yeast mitochondria

Wild-type cells of *Saccharomyces cerevisiae* strain D273-10B (ATCC 25657) were grown on medium containing 2% lactate, converted to spheroplasts, homogenized, and the mitochondria isolated therefrom as described previously (Daum et al. 1982; Hay and Oppliger 1984).

Purification of porin from yeast mitochondria

The mitochondrial pellet (about 15 mg protein) was resuspended to 3 mg/ml in buffer A (20 m*M* HEPES pH 6.8 containing 20 m*M* Na_2SO_4, 1 m*M* EDTA and 0.05% Triton X-100 (SIGMA, Deisenhofen, FRG)) and incubated on ice for 30 min. Following a 30 min centrifugation at 100,000 × *g*, the pellet was resuspended in buffer A and 2.4 mg Genapol X-80 (FLUKA, Neu-Ulm, FRG) per mg of starting protein were added. After another 30 min on ice and a second centrifugation at 100,000 × *g* for 30 min, the resulting supernatant was applied to a hydroxyapatite column (1 ml Bio-Gel HTP (Bio Rad, München, FRG) per 2 mg of starting protein). The column was equilibrated and eluted with buffer A. 1 ml fractions were collected and their protein content was monitored by the absorbance at 280 nm. The protein-containing fractions were pooled and stored at − 25 °C.

SDS-*gel electrophoresis*

The protein was precipitated from the detergent-containing solution according to the method of Wessel and Flügge (1984). For SDS-gel electrophoresis the pellet was dissolved in 50 µl SDS solution containing 5% SDS and 2% mercaptoethanol and kept for 10 min at 97 °C. Gel electrophoresis was carried out on 12.5% acrylamide gels according to Douglas and Butow (1976). Staining was performed with coomassie blue.

Incorporation of yeast porin into planar lipid bilayer membranes and conductance measurements

Artificial lipid bilayer membranes were obtained as described previously (Benz et al. 1978) from a solution of 1% diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in *n*-decane. The cell used for bilayer formation was made of Teflon. The circular hole in the wall separating the two aqueous compartments had an area of either 2 mm^2 (for macroscopic conductance measurements) or 0.1 mm^2 (for single-channel experiments). All salts were obtained from Merck (Darmstadt, FRG, analytical grade). The aqueous solutions were used unbuffered with a pH of 6. The protein was added from a stock solution either immediately before membrane formation or after the membrane had completely turned black in order to prevent protein inactivation. Calomel electrodes with salt bridges were inserted into the aqueous compartments on both sides of the membrane. The macroscopic conductance measurements were performed with a Keithly 610C electrometer (Keithley, Cleveland OH). A Keithley 427 current amplifier was used for the single-channel experiments. The amplified signal was monitored with a Tektronix 7834 storage oscilloscope (Tektronix, Beaverton OR) and recorded with a strip-chart recorder. Zero-current membrane potentials were measured with a Keithly 610C electrometer 5–10 min after the application of a salt gradient across the membranes, as described earlier (Benz et al. 1979).

Results

Purification of the pore protein

The mitochondrial outer membrane of *Saccharomyces cerevisiae* contains a much smaller number of major proteins than that of mammalian tissue (Gasser and Schatz 1983; Benz 1985). Thus, the isolation procedure of the mitochondrial porin was relatively simple. Mitochondria from yeast were washed with Triton buffer A (see Materials and methods) followed by centrifugation. The pellet was extracted with Genapol X-80 (a detergent which is very similar to Triton but which contains isotridecyl groups instead of aromatic rings). The mitochondrial inner membrane was only solubilized by the detergent Genapol X-80 to a smaller extent. Thus, the protein solution contained only small amounts of the carriers located in the mitochondrial inner membrane (Benz 1985; Palmieri et al. 1985). The supernatant of the subsequent centrifugation was applied to a hydroxyapatite column. The eluate of this column contained the porin. Gel electrophoretic analysis showed a single band with an apparent molecular weight of 30 kDa (see Fig. 1). Bands with similar molecular weights have also been observed in mitochondrial outer membrane from other sources, such as *Neurospora crassa* (Freitag et al. 1982b) and rat liver (Roos et al. 1982; Linden et al. 1982).

Incorporation of the yeast porin into lipid bilayer membranes

When the 30 kDa protein was added in small quantities (10 to 100 ng/ml) to the aqueous solutions 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 described previously for other mitochondrial porins (Roos et al. 1982; Freitag et al. 1982b). After an initial rapid increase for 15 to 20 min, the membrane conductance increased at a much slower rate. This slow increase continued usually until membrane breakage. When the rate of conductance increase was relatively slow (about 20–30 min after the addition of the protein) it could be shown for differ-

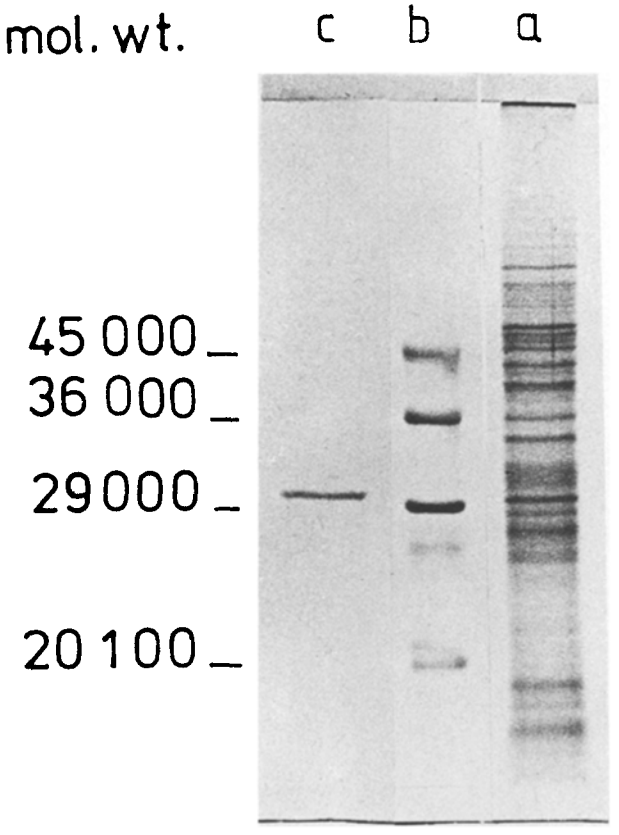

Fig. 1. SDS – polyacrylamide gel electrophoretogramm of yeast porin with coomassie blue staining. 10 μg of the purified porin (*line c*) were applied to the gel and migrated on the gel with an apparent molecular weight of about 30,000 Dalton; *line b*: molecular standards; *line a*: Genapol X-80 eluate before application to the HTP-column

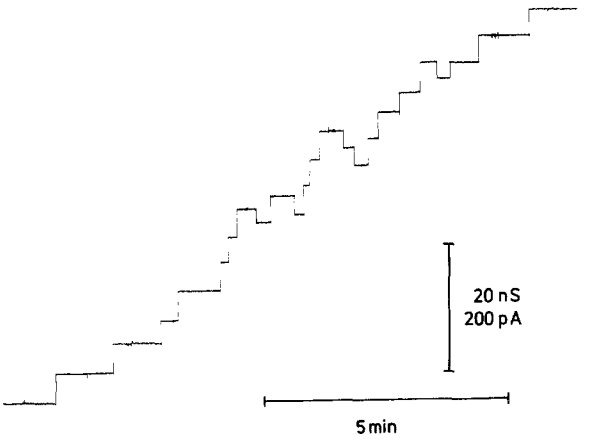

Fig. 2. Stepwise increase of the membrane current after the addition of the yeast porin to the aqueous phase bathing a black membrane. The aqueous phase contained 2 ng/ml porin, and 1 *M* KCl, pH 6. The membrane was formed from 1% diphytanoyl phosphatidylcholine/*n*-decane. The applied voltage was 10 mV; the current prior to the addition of the protein was less than 0.5 pA; T = 25 °C

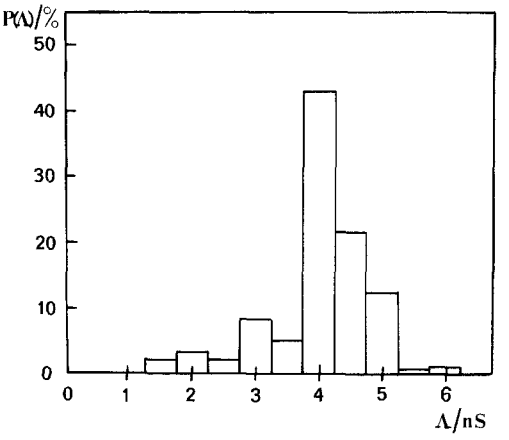

Fig. 3. Histogram of conductance fluctuations observed with membranes from diphytanoyl phosphatidylcholine/*n*-decane in the presence of yeast porin. The aqueous phase contained 1 *M* KCl. The applied voltage was 10 mV. The mean value of all upward directed steps was 4.0 nS for 181 single events; T = 25 °C

ent protein concentrations that the membrane conductance was a linear function of the protein concentration in the aqueous phase. The conductance increase occurred regardless of whether the protein was added to only one side or to both sides of the membrane. The addition of the detergent Genapol X-80 alone in a similar concentration to that used in the presence of the protein did not lead to any appreciable increase in the membrane conductance above the specific conductance in the absence of the protein (10^{-7} to 10^{-8} S/cm^2).

Single-channel analysis

The addition of smaller amounts of the porin from yeast to lipid bilayer membranes having small surface areas (0.1 mm^2) allowed the resolution of step increases in conductance (Fig. 2). These conductance steps were not observed when only the detergent Genapol X-80 was added. Most of the conductance steps were directed upwards and closing steps were only rarely observed at transmembrane potentials of 5 or 10 mV. Figure 3 shows a histogramm of 181 conductance steps in 1 *M* KCl at a membrane potential of 10 mV. The most frequent value for the single-channel conductance of the yeast porin was 4 nS. At 10 mV transmembrane potential, the closing events represented only a minor fraction of the total number of conductance fluctuations. However, at higher potentials the closing events became more and more frequent. Furthermore, the closing events had in many cases a smaller amplitude than the initial on-steps. This result indicated that the yeast porin pore switched to substates at high voltages. The pore may have different conductance states depending on the magnitude of the applied potential (see below).

Voltage-dependence

The mitochondrial pores from *Neurospora crassa* (Freitag et al. 1982b), rat liver (Roos et al. 1982; Doring and Colombini 1985), pig heart (De Pinto et al. 1985) and rat brain (Ludwig et al. 1986) were reported to be voltage-dependent. The results presented above indicated that the mitochondrial porin from yeast was also voltage-dependent. This could be demonstrated in experiments in which only a few channels were reconstituted into the membrane and in multi-channel experiments. An experiment of the first type is shown in Fig. 4. Four channels were incorporated in a membrane from diphytanoyl phosphatidylcholine/*n*-decane. The voltage across the membrane was switched to 40 mV (with respect to the cis-side, the side of the addition of the protein) and then to − 40 mV. The four channels switched to substates of the open state.

In the multi-channel system the decay of the membrane current following a voltage step could be described by a single exponential decay. The time constant of this exponential decay was found to decrease with increasing membrane potential as was shown for the porin from rat brain (Ludwig et al. 1986). The steady-state conductance showed a bell-shaped curve as a function of the applied voltage. Figure 5 shows the ratio of the conductance, G, at a given membrane potential divided by the conductance G_0, at the zero potential as a function of the applied membrane potential. The data given in Fig. 5 could be analysed as proposed by Schein et al. 1976:

$$N_o/N_c = \exp\left[nF(V_m - V_0)/RT\right], \quad (1)$$

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_0 is the potential where 50% of the total number of channels are in the closed configuration. The open to closed ratio of the channels, N_o/N_c may be calculated from the data given in Fig. 5 according to:

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

G is in this equation 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. A semilogarithmic plot of the data given in Fig. 5 showed that they could be fitted to a straight line with a slope of 12 mV for an e-fold change of N_o/N_c.This result suggested that the number of charges involved in the gating process was approximately two (see Fig. 6).

The time constant τ of the single exponential relaxation process varied with the membrane potential. The experimental results for τ could also be fitted to a straight line in a semilogarithmic plot (data not

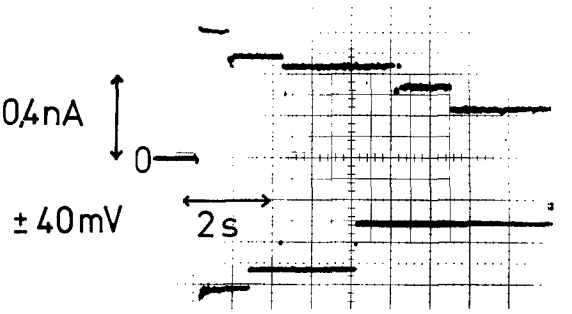

Fig. 4. Voltage-dependence of yeast porin. Four channels were incorporated in a membrane from 1% diphytanoyl phosphatidylcholine/*n*-decane. The voltage across the membrane was switched to 40 mV (with respect to the cis-side, the side of the addition of the protein) and then to − 40 mV. The four channels switched to substates of the open state. Yeast porin was added in a concentration of 2 ng/ml to the aqueous phase containing 1 *M* KCl; T = 25 °C

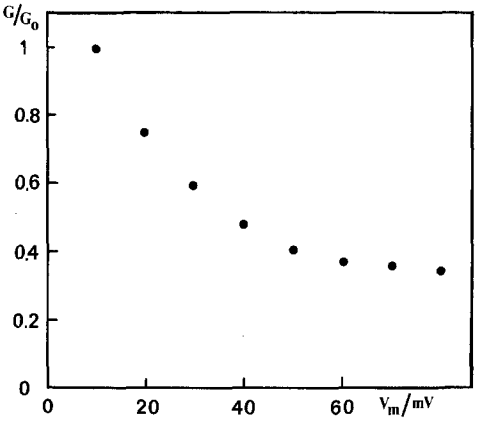

Fig. 5. Ratio of the conductance G at a given membrane potential divided by the conductance G_0 at zero potential as a function of the applied membrane potential V_m. The membranes were formed from diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 *M* KCl. The cis-side contained 12 ng/ml yeast porin. Only the positive branch (with respect to the cis side) of the bell-shaped curve is shown; T = 25 °C.

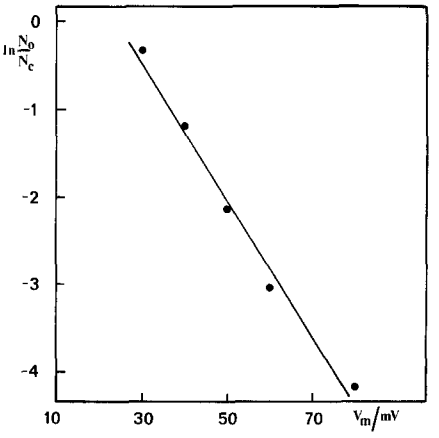

Fig. 6. Semilogarithmic plot of the ratio N_o/N_c as a function of the transmembrane potential V_m. The data were calculated from the results of Fig. 5 using Eq. (1). The slope of the line was about 13 mV for an e-fold change of N_o/N_c; $V_0 = 16$ mV.

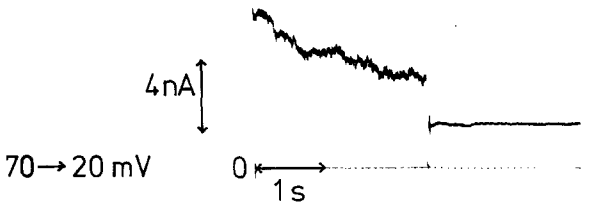

Fig. 7. Voltage-dependence of the open and closed state of yeast porin. The voltage across the membrane was switched from 0 to 70 mV (with respect to the cis-side, the side of the addition of the protein) and then to 20 mV. The current through the pores decreased with a time constant of approximately 1.5 s. After switching to 20 mV, the increase of the current through the pores could not be followed at the same time scale. Yeast porin was added in a concentration of 20 ng/ml. 33 channels were incorporated in a membrane from 1% diphytanoyl phosphatidylcholine/*n*-decane. The aqueous phase contained 1 *M* KCl; T = 25 °C

shown). This line corresponded to an e-fold decrease of the time constant τ for an increase of the voltage by about 13 mV, which means that the number of gating charges traversing the entire membrane potential in channel closing is about two. This result agreed nicely with the number of gating charges derived from the plot of G/G_0 (see above). 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 is also demonstrated in Fig. 7. The transmembrane potential across a diphytanoyl phosphatidylcholine /*n*-decane membrane in which about 33 porin pores were incorporated was switched from 0 to 70 mV. The current through the pores decreased with a time constant of approximately 1.5 s. Then the membrane potential was decreased to 20 mV, but the increase of the current through the pores could not be followed at the same time scale. This result indicated largely different reaction rates for the closing and the opening process of the yeast porin at small voltages.

Zero-current membrane potentials

Further information about the structure of the pore formed by yeast porin may be obtained from zero-current membrane potential measurements in the presence of salt gradients. A ten fold KCl gradient, across a lipid bilayer membrane in which porin was incorporated, resulted in an asymmetry potential of about −7 mV (negative at the more diluted side). This result indicated some preferential movement of anions over cations through the pore at neutral pH. The zero-current membrane potentials were analysed using the Goldman-Hodgkin-Katz equation (Benz et al. 1979). The ratio of the anion permeability P_a divided by the cation permeability P_c was about 1.4, suggesting a small anion selectivity of the mitochondrial pore from yeast in the case of the equally mobile potassium and chloride ions. Using LiCl in similar experiments resulted in an even higher anion selectivity and P_a/P_c was calculated to be 2.6 from an asymmetry potential of − 20 mV at the dilute side of a ten fold LiCl gradient. This result indicated that the selectivity of the yeast porin is not an absolute one 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 at the more dilute side. We calculated a permeability ratio $P_a/P_c = 0.5$ from an asymmetry potential of 14 mV at the more dilute side of a ten fold potassium acetate gradient. It is interesting to note that weakly anion selective bacterial porins also change their selectivity as a result of the change of the mobility of the ions in the aqueous phase (Benz et al. 1985b), whereas the highly anion selective protein *P* channel of *Pseudomonas aeruginosa* outer membrane does not (Benz and Hancock 1987). The selectivity of the yeast porin may be explained by the presence of positively charged groups, possibly lysines (Doring and Colombini 1985; Benz 1985), in or near the pore.

Estimation of the pore diameter

The channel in the mitochondrial outer membrane was permeable for a variety of different ions. Table 1 shows the single channel conductances of yeast porin in the presence of different salt solutions. Although there existed a considerable influence of the salt on the pore conductance, the ratio Λ/σ varied less than a factor of two, i.e. the ions seemed to move inside the pore in a manner similar to the way they move in an aqueous environment. This finding together with the results obtained above from the selectivity measurements allowed a rough estimate of the effective diameter of the pores. Assuming that the porin pores are filled with a solution of the same specific conductivity σ as the external solution and assuming a cylindric pore with a length, l, of 6 nm [which is very likely according to electron microscopic studies (Mannella 1981; Mannella and Frank 1984a, b)], the average pore diameter d (= 2r) can be calculated according to the equation:

$$\Lambda = \sigma \pi r^2 / l \qquad (3)$$

The effective diameter of the yeast porin pores was calculated from the average single channel-conductance of the pores in 0.1 *M* KCl ($\Lambda = 0.45$ nS; $\sigma = 13$ mS/cm) to be about 1.7 nm. This was very similar to the diameter of most mitochondrial pores (ex-

Table 1. Average single-channel conductance of yeast porin in different salt solutions of concentration c. The solution contained 5 ng/ml yeast 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. $\bar{A}$ was determined by recording at least 70 conductance steps and averaging over the distribution of the values. σ is the specific conductance of the aqueous salt solutions

Salt	c [M]	$\bar{A}$ [nS]	$\bar{A}/\sigma$ (10^{-8}) cm
KCl	0.1	0.4	3.7
	0.3	1.24	3.6
	1	1.24	4.0
	3	9.7	3.8
NaCl	1	3.0	4.5
LiCl	1	3.0	4.8
KBr	1	4.2	3.6
KF	1	2.2	2.9
$NaCH_3COO$	1	2.5	5.6
$MgCl_2$	0.5	0.94	4.2
K_2SO_4	0.5	3.0	3.2

cept for *Paramecium* porin; Benz et al. 1985 a). Such a diameter would allow the permeation of hydrophilic solutes up to molecular weights of 2,500–3,000 Da through the pores.

Discussion

We have isolated and purified the major protein component of the mitochondrial outer membrane of yeast. This protein is firmly integrated into the outer membrane. It was isolated employing nonionic detergents. The isolated protein has an apparent molecular weight of 30 kDa on a SDS-electrophoretogram (see Fig. 1) which is very close to the molecular weight derived from the sequence (Mihara and Sato 1985).

The studies on the incorporation of the purified protein into artificial lipid bilayer membranes define its function as a pore or channel forming component. Reconstitution at very low protein concentrations in the aqueous phase shows an increase of the membrane conductance in distinct steps; this single conductance unit most probably corresponds to a reconstituted channel. The single channel conductance is about 0.45 nS in 0.1 *M* KCl. Assuming a pore length, l, of 6 nm (corresponding to a similar thickness of the outer mitochondrial membrane and to the length of the pore according to electron microscopic studies (Mannella and Frank 1984 a; Mannella et al. 1984), and assuming, that the pore is filled with a solution of the same specific conductance σ as the external solution, the pore diameter is 1.7 nm according to Eq. (3). Other mitochondrial porins have a similar diameter as judged from their single channel conductance in 0.1 *M* KCl (Benz 1984, 1985; Benz et al. 1985 a). Only mitochondrial porin from *Paramecium* had with 1.3 nm a somewhat smaller diameter (Benz et al. 1985 a). Such a diameter of the pore in the outer mitochondrial membrane is consistent with the results of research into the permeability of isolated mitochondria (Pfaff et al. 1968) and with those of research into the electron microscopy of outer mitochondrial membranes (Mannella and Frank 1984 b; Mannella et al. 1984). X-ray diffraction patterns of plant outer mitochondrial membranes have suggested that a protein of about 30 kDa forms a predominant subunit structure with an inner low-electron-density core of approximately 2 nm (Mannella 1981; Mannella and Bonner 1975).

It has to be noted that the diameter of the yeast porin pore and other mitochondrial pores as obtained from electron microscopy and the single-channel analysis is about half of the diameter obtained by Colombini (1980 b) for the diffusion of polyethylene glycols through the porin pore. We do not know the reason for this discrepancy but we would like to stress the point that the purity of the polydisperse polyalcohols is very critical for Colombini's approach (Colombini 1980 b). There exists in the literature excellent agreement between the pore sizes of general diffusion pores (e.g. porins) calculated from swelling experiments using different hydrophilic solutes and those estimated from the single-channel data (Nikaido and Rosenberg 1983; Yoshimura et al. 1983; Benz et al. 1985 b). We are thus very confident that a pore diameter of 4 nm (Colombini 1980 b) represents an overestimate of the pore size of the mitochondrial porin.

On the other hand, it is obvious that a similar estimate as given in Eq. (3) cannot be used for the calculation of the pore size of a specific pore such as gramicidin or the anion selective channel of *Pseudomonas aeruginosa* outer membrane (Benz and Hancock 1987). The basic difference between a selective (or specific) channel and a general diffusion pore is obviously the small electrostatic barrier of the latter and a perfect shielding of the pore interior against the low dielectric of the membrane.

The mitochondrial porin from yeast forms voltage-dependent pores in lipid bilayer membrane. This result is in agreement with what was observed earlier for mitochondrial porins from rat liver (Roos et al. 1982) and *Neurospora crassa* (Colombini 1980 b; Freitag et al. 1982 b). The pore conductance is reduced at higher voltages. This could also result in the restriction of the permeability of the pore for larger solutes. So far, it is not clear how such a transmembrane potential could be created across the outer mitochondrial membrane, because the existence of an ionic gradient across the outer mitochondrial membrane seems to be rather unlikely. Another possibility would be the assumption of a membrane asymmetry. However, the generation

and the change of an intrinsic membrane potential would probably be too slow to account for a rapid control of the mitochondrial metabolism.

There exists only one indication in the literature, that the outer mitochondrial membrane may be involved in control of mitochondrial metabolism. Recently it was shown that a polyanion, which is the copolymer of methacrylate, maleate, and styrene (in a molar ratio 1 : 2 : 3) inhibits oxidative phosphorylation (König et al. 1977) and may act by closing the mitochondrial porin pore (Yeung et al. 1986). For the discussion of the role of the voltage dependence of the pore in the control of mitochondrial metabolism, it has to be borne in mind that in situ mitochondria have a different structure from that of in vitro mitochondria. Mitochondrial outer and inner membranes form a 5-layered 12 nm wide structure in electron microscopic analysis of in situ mitochondria (Malhotra 1966), which means that the space between both membranes could be very small. This situation could result in electric coupling between both membranes. The membrane potential or the surface potential of the inner mitochondrial membrane could influence the permeability of the outer mitochondrial membrane and thus in turn control the metabolism of mitochondria. Critical for this proposed electrical coupling is the actual distance between both membranes and the Debye-length of the intermembrane space. A long distance and a short Debye-length would both result in loose coupling, whereas distances on the order of 2–3 nm and a long Debye-length (caused by a high dielectric constant or by low ionic strength of the intermembrane space) could result in a tight coupling between both membranes.

The mitochondrial pore presumably also plays an important role in the microcompartment formation of the mitochondria. Such compartments were described for hexokinase and creatine kinase (Inui and Ishibashi 1979; Jacobus et al. 1983). In fact, it has been shown that hexokinase bound to the surface of mitochondria has a higher activity with ATP coming from oxidative phosphorylation as compared with ATP added to the cytoplasmic side of mitochondria (Gots and Bessman 1974; Inui and Ishibashi 1979). This finding is now understood because both, hexokinase and glycerokinase bind to the pore, i.e. to the hexokinase binding protein (Fiek et al. 1982; Lindén et al. 1982; Östlund et al. 1983). Energized mitochondria showed the formation of a microcompartment between hexokinase and adenylate translocator, thus excluding adenylate kinase (located between mitochondrial inner and outer membranes) from the ATP-pool in energized mitochondria (Brdiczka et al. 1986).

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