Role of Sterols in the Functional Reconstitution of Water-Soluble Mitochondrial Porins from Different Organisms†

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ABSTRACT: Experiments were performed on lipid bilayer membranes with water-soluble mitochondrial porins from different eukaryotic organisms, such as *Dictyostelium discoideum*, *Paramecium*, and rat liver, to study the requirements of functional reconstitution of the porins. The water-soluble porins lost their associated lipids and sterols and are unable to form channels in lipid bilayer membranes. We demonstrate that the water-soluble porins regain their channel-forming ability after preincubation of the polypeptides with sterols in the presence of detergents. Mitochondrial porin from *Dictyostelium discoideum* maintained after this procedure its original properties, in particular the voltage dependence. Water-soluble mitochondrial porins from *Paramecium tetraurelia* and from rat liver were also activated upon preincubation with different sterols in detergent but showed voltage-dependences that were different from those of detergent-purified porins. Furthermore, the voltage dependence depended on the sterol used for preincubation. Interestingly, the preincubation with sterols can likewise be used to activate detergent-purified mitochondrial porins that may have lost associated sterol during isolation and purification procedures.

While the inner mitochondrial membrane contains a variety of very specific transport systems, the outer mitochondrial membrane is permeable to most metabolites (O'Brien & Brierly, 1965; Pfaff et al., 1968). Responsible for this permeability is the mitochondrial porin (Zalman et al., 1980; Roos et al., 1982), also called VDAC[1] (voltage-dependent anion-selective channel) (Schein et al., 1976; Colombini, 1979). The mitochondrial porin acts as a general diffusion pathway with a defined exclusion limit for hydrophilic mitochondrial substrates (Benz, 1985; Mannella et al., 1992) and shows slight anion selectivity in the open state (Schein et al., 1976; Roos et al., 1982; Colombini, 1983). The mitochondrial porin is voltage-gated and switches for voltages exceeding about 20−30 mV into different substates in an asymmetric fashion (Schein et al., 1976; Colombini, 1979; Freitag et al., 1982a). The closed states are cation selective and seem to be, in contrast to the open state, impermeable to ADP and ATP (Benz et al., 1990; Benz & Brdiczka, 1992; Liu & Colombini, 1992). In this way the mitochondrial porin may play an important role in the control of mitochondrial metabolism.

Mitochondrial porins have been isolated and characterized from many different organisms (Roos et al., 1982; Freitag et al., 1982a; Colombini, 1983; De Pinto et al., 1987a,b). The primary sequence of mitochondrial porins from *Saccharomyces cerevisiae* (Mihara & Sato, 1985), *Neurospora crassa* (Kleene et al., 1987), a human B-lymphocyte cell line (Kayser et al., 1989), and *Dictyostelium discoideum* (Troll et al., 1992) have been sequenced by determination of either the cDNA or the protein sequence. The biosynthesis of mitochondrial porins takes place on free cytoplasmic ribosomes (Mihara et al., 1982; Freitag et al., 1982b; Gasser & Schatz, 1983). The precursor is posttranslationally imported into the mitochondria without cleavage of any signal sequence (Mihara et al., 1982; Freitag et al., 1982b; Gasser & Schatz, 1983) and without requirement for a membrane potential. However, metabolic energy in form of ATP (Pfanner et al., 1988) or a detergent such as Triton X-100, probably acting as a chaperon, is needed for the insertion process (Pfaller et al., 1985). The mechanisms of the folding and insertion processes have not been elucidated to date.

The mitochondrial porin channels are supposed to be formed by one (Thomas et al., 1991; Peng et al., 1992) or two polypeptide chain(s) (Lindén & Gellerfors, 1983; Krause et al., 1986), associated with sterol and lipid (Freitag et al., 1982a; Pfaller et al., 1985; De Pinto et al., 1989). According to secondary structure predictions the polypeptide chain(s) are arranged in a cylinder containing either 16 antiparallel, amphiphilic β-barrels similar to certain bacterial porins (De Pinto et al., 1991) or 12 β-barrels and the amphiphilic N-terminal α-helix (Blachly-Dyson et al., 1990). So far it is not clear whether the folding process of mitochondrial porin occurs in the aqueous phase or inside the membrane since the protein has an unusual high polarity for a membrane protein and it is difficult to imagine that the protein folds in a membrane (Vogel & Jähnig, 1986; De Cock et al., 1990; Sen & Nikaido, 1990). By precipitation of the isolated mitochondrial porin of *N. crassa* with trichloracetic acid

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[1] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; DiphPC, diphytanoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin layer chromatography; VDAC, voltage-dependent anion-selective channel.

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followed by treatment at high pH, a water-soluble porin with qualities very similar to the precursor form can be obtained (Pfaller et al., 1985). This water-soluble porin requires a cholesterol containing membrane for successful reconstitution as a channel in the planar lipid bilayer (Pfaller et al., 1985). Sterol is present in the outer mitochondrial membrane while the mitochondrial inner membrane contains probably little or no sterol (Lévy & Sauner, 1968; Daum, 1985).

Sterols are major membrane components in eukaryotic cells: cholesterol is the major sterol in mammalian organisms (Asworth & Green, 1966), although a few closely related sterols such as 7-dehydrocholesterol, cholestanol, lathosterol, and desmosterol are also detected (Glover & Green, 1957; Lasser & Clayton, 1966; Smith et al, 1967). The sterols found in plants are typified by stigmasterol and β-sitosterol; the predominant sterol in fungi and yeast is ergosterol (Demel & De Kruijff, 1976). The successful reconstitution of unfolded membrane proteins is a major problem in the study of transport processes. In the present study we investigated the influence of different sterols on the reconstitution of mitochondrial porin in detail. In particular, we studied the influence of different sterols on the function of the mitochondrial porins as a voltage-gated channel and on the single-channel conductance. The results give an interesting insight in the folding of a membrane protein that contains according to secondary structure predictions mostly β-sheet structure.

MATERIALS AND METHODS

Materials. Cholesterol (cholest-5-en-3β-ol) was obtained from Serva. Androstanol (5α-androstan-3β-ol), cholesten (cholest-5-en), cholestanol (dihydrocholesterol, 5α-cholestan-3β-ol), 7-dehydrocholesterol (cholest-5,7-dien-3β-ol), desmosterol (cholest-5,24-dien-3β-ol), ergosterol (cholest-5,7,22-trien-24-methyl-3β-ol), β-sitosterol (cholest-5-en-24-ethyl-3β-ol), stigmasterol (cholest-5,22-dien-24-ethyl-3β-ol), and thiocholesterol (cholest-5-en-3β-thiol) were all bought from Sigma, and epicholesterol (cholest-5-en-3α-ol) was from Ferak. (Figure 1). Genapol X-80 was obtained from Fluka.

Biological Preparations. Cells of *D. discoideum* were grown and their mitochondria prepared as described elsewhere (Troll et al., 1992). Mitochondria from rat liver were isolated by differential centrifugation (Benz et al., 1990). Mitochondria from *Paramecium tetraurelia* were obtained as described previously in detail (Ludwig et al., 1989).

Purification of Porin. The mitochondrial porin was isolated from whole mitochondria and purified by application to a hydroxyapatite column as published previously (De Pinto et al., 1987a,b). This purification procedure yielded the pure mitochondrial porin as controlled by SDS−PAGE (Lämmli, 1970).

Preparation of Water-Soluble Porin. The protein was precipitated according to the method of Wessel and Flügge (1984) to remove detergents, lipid, and sterol associated with the protein. To ensure that the associated sterol was completely removed, the resulting pellet was mixed a second time with 100 μL of chloroform, and 300 μL of methanol was added. The probe was vortexed again and the protein was pelleted by centrifugation at 9000*g* for 5 min. The pellet was dried at room temperature. It was resolubilized by mixing with 1 volume of 0.1 M NaOH for 60 s and immediately neutralized by an equal volume of 0.2 M NaH_2PO_4 as described by Pfaller et al. (1985). This procedure yielded water-soluble porin in 100 mM NaP_i, pH 6.8.

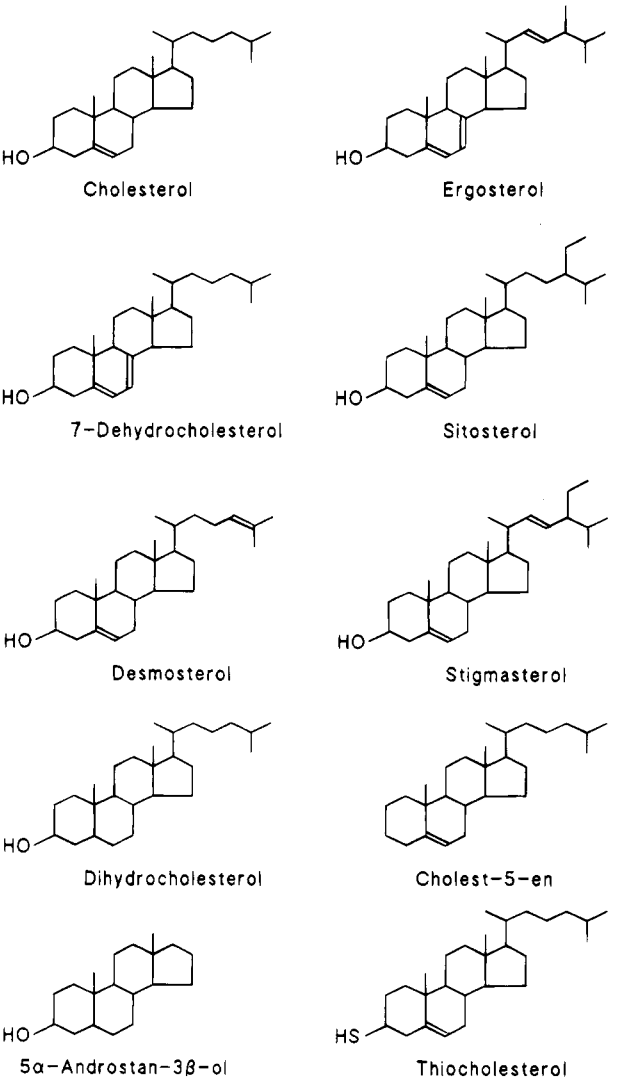

FIGURE 1: (A, top) Structures of sterols from mammalian, plant, or fungi origin. (B, bottom) Structures of some steroids which are related to cholesterol but show structural differences in specific positions.

For quantitation of residual sterol, the protein samples were extracted, and lipids and sterol separated on TLC-plates as described by De Pinto et al. (1989). Sterol was scraped from TLC, extracted by solvents, and quantified by gas chromatography.

Preincubation with Sterols. Suspensions of sterols were made by taking small volumes from solutions of the sterol in chloroform, evaporating the chloroform, and then suspending the sterol in a concentration of 1% (w/v) in 1% Genapol, 0.5 mM EDTA, and 5 mM Hepes, pH 7. For preincubation one volume of porin (10−20 μL), either water-soluble porin in 100 mM NaP_i buffer or isolated porin in 2% Genapol, 10 mM Hepes, and 1 mM EDTA as obtained from the last isolation step, was mixed with one volume of a 1% (w/v) sterol suspension and vortexed for 10s. The obtained samples were stored on ice until use.

Lipid Bilayer Experiments. The methods used for the "black" lipid bilayer experiments have been described previously (Benz et al., 1978). If not mentioned otherwise, membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (DiphPC, Avanti Polar Lipids, Alabaster, AL) in *n*-decane across circular holes (surface area about 0.1 mm^2) in the thin wall of a Teflon

cell separating the two aqueous compartments. For some experiments, phosphatidylserine, phosphatidylethanolamine, asolectin, or asolectin/cholesterol in the molar ratios of 1:1 or 2:1 were used instead of DiphPC. The temperature was kept at 25 °C throughout. All salts and buffers were of analytical grade and obtained from Merck (Darmstadt, F.R.G.). The aqueous solutions were either unbuffered and had a pH around 6 or buffered with 10 mM Hepes at a pH of 7. Porin was added from the stock solutions (either from the detergent solution as obtained from the isolation and purification procedure or in the water-soluble form) to the aqueous phase of the cis compartment (the compartment to which the voltage was applied) after the membranes had turned optically black in reflected light. The current through the membranes was measured with two calomel electrodes switched in series with a voltage source and a model 427 current amplifier (Keithley, Cleveland, OH). The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder. For macroscopic conductance measurements the current amplifier was replaced by a Keithley electrometer (model 602). Zero-current membrane potentials were measured with the same instrument 5−10 min after the application of a salt gradient across the membrane.

Other Methods. The concentration of water-soluble mitochondrial porin was measured according to Sandermann and Strohminger (1972). Epicholesterol was controlled for purity by thin layer chromatography (TLC) on 0.2 mm silica gel plates (Riedel-de-Häen) with 9:1 chloroform/ether as running solvent. Staining was performed with the Carr-and-Price-reaction (Carr & Price, 1926); a solution of antimony trichloride in chloroform (20% w/v) was obtained from Sigma.

RESULTS

Water-Soluble Mitochondrial Porin Does Not Form Ion-Permeable Channels in Lipid Bilayer Membranes. In a first set of experimental conditions we studied the channel formation by water-soluble mitochondrial porin from *D. discoideum*. This form of the porin was not able to increase the conductance of lipid bilayer membrane made of diphytanoylphosphatidylcholine/*n*-decane to any appreciable extent even at very high protein concentration (1 μg/mL). Similarly, we did not observe any membrane activity when we used a variety of pure lipids for membrane formation such as phosphatidylserine or phosphatidylethanolamine and asolectin, a lipid mixture from soy beans. In single-channel experiments we did not resolve current fluctuations similar to those observed with detergent-solubilized mitochondrial porin. Figure 2 shows a single-channel recording in the presence of 1 μg/mL water-soluble porin from *D. discoideum*. Even at this high protein concentration we observed only a minor influence on the membrane current. Similar results were obtained with water-soluble mitochondrial porins from *P. tetraurelia* and from rat liver. It is noteworthy that we observed after addition of detergent some membrane activity with porin samples, which had only been precipitated by the method of Wessel and Flügge (1984) before solubilization. It is possible that residual sterol molecules caused the membrane activity in these cases, although the sterol and detergent content of the sample was reduced to less than 1% of the original one. This membrane activity was absent

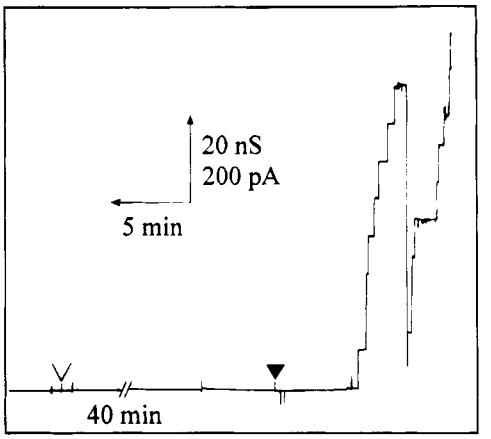

FIGURE 2: Single-channel recording of a diphytanoylcholine/*n*-decane membrane after addition of 1 μg/mL water-soluble *Dictyostelium* porin to the cis compartment. The open symbol (V) indicates the addition of 1 μg/mL water-soluble *Dictyostelium* porin to the cis compartment. The closed symbol (▼) indicates the addition of 100 ng/mL water-soluble *Dictyostelium* porin, preincubated in a buffer containing 1% ergosterol and 1% Genapol, to the same compartment. The aqueous phase contained 1 M KCl (pH 6). The applied membrane potential was 10 mV; $T = 20$ °C.

when the samples had been washed a second time with chloroform as described in Materials and Methods.

Requirements for the Reconstitution of the Water-Soluble Porin from D. discoideum. Obviously, water-soluble mitochondrial porins from *D. discoideum*, *P. tetraurelia*, and rat liver have lost their ability either to insert into artificial bilayers made from different lipids or they have lost their channel-forming ability since the channel-forming complex was destroyed. We consider the latter possibility as more likely since the channel-forming ability of mitochondrial porin was also completely abolished by treatment of the isolated porin by ionic detergents such as sodium dodecyl sulfate, cholate, and desoxycholate. They only formed channels in the lipid bilayer assay when the membranes contained a sterol, for example cholesterol as has previously been described for mitochondrial porin from *N. crassa* (Pfaller et al., 1985). However, the rate of successful reconstitution per amount of protein added (i.e., the channel-forming activity) was still considerably smaller than that of porin isolated in detergent (which we will call native porin further on).

Figure 3 shows a comparison of the channel-forming activities of mitochondrial porin from *D. discoideum* under different conditions. Membranes were formed from diphytanoylphosphatidylcholine/*n*-decane in solutions containing 1 M KCl. Ten minutes after the membrane was in the black state mitochondrial porin in different concentrations and in the different configurations was added to the aqueous phase while stirring to allow equilibration. Then we followed the membrane conductance during approximately 30 min since this is the time needed to obtain most of the conductance increase (Freitag et al., 1982a). Only a small further increase (as compared with the initial one) is normally observed after that time. The same effect was observed irrespective of whether the different forms of the porin were added to one or both sides of the membranes. Lowest activity was obtained for water-soluble porin without any sterol and the specific membrane conductance did not increase above that of membranes without any mitochondrial porin (see above). Furthermore, we did not observe any

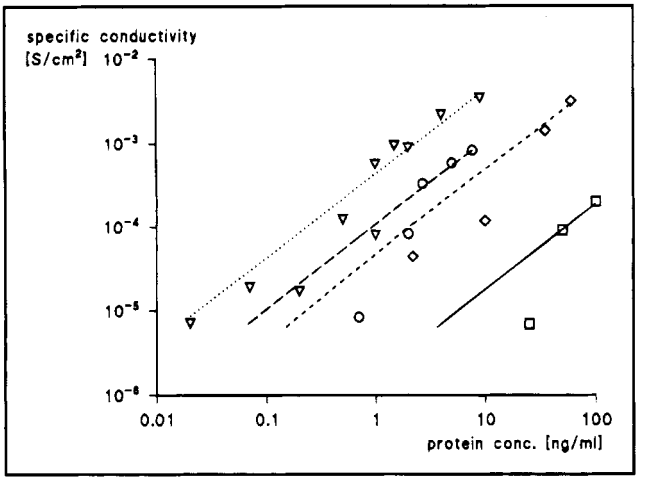

FIGURE 3: Degree of activation of water-soluble mitochondrial porin from *Dictyostelium discoideum* upon preincubation with different sterols and detergent. Membranes were formed from DiphPC. The aqueous phase contained 1 M KCl; the applied voltage was +10 mV. The water-soluble porin was preincubated either in 1% Genapol alone (squares) or in 1% Genapol and 0.5% sterol. The experiments with the different sterols are marked as follows: preincubation with cholesterol by open circles (line: long dashes), with ergosterol by open triangles (line: dotted) and with β-sitosterol by open rhombes (line: short dashes). The lines were fitted under the assumption that there is linear correlation between protein concentration and conductance increase. The aqueous phase contained 1 M KCl (pH 6). The applied membrane potential was 10 mV; T = 20 °C.

nonlinear concentration dependence. The channel-forming activity for membranes containing cholesterol was at least 3−5 times larger but still 30 times below that of isolated porin (data not shown). Interestingly, the preincubation of the water-soluble porin with cholesterol and other sterols and detergent (see Materials and Methods) and its subsequent addition to the aqueous salt solution bathing the bilayer led to an even stronger increase of the membrane conductance although the preincubation with the detergent Genapol alone led to only minor activation (see Figure 3). This was presumably caused by traces of sterol since we did not use the additional "wash" step with chloroform in these experiments. The membrane conductance was a linear function of the protein concentration. It is noteworthy that the membrane activity was dependent on the type of sterol used for preincubation of the water-soluble porin.

This result probably means that the channel-forming complex is already formed under these conditions in the detergent solution. Furthermore, the conductance increase was similar for isolated and for reconstituted porin. It was not necessary for successful reconstitution in these experiments that cholesterol was present in the membrane-forming lipid. Membrane activity after preincubation of the water-soluble porin with detergent and cholesterol was observed irrespective of whether the membrane contained cholesterol or not.

In separate experiments we investigated the concentration of the sterol needed for successful reconstitution. We found out that the concentration of sterol in our preincubation buffer (0.5% w/v final concentration) was in excess. However, the concentration of about 0.5% Genapol in the preincubation buffer resulted in a final concentration of about 0.001% Genapol (and sterol) in the aqueous phase on one side of the membrane, which was necessary for activation of porin.

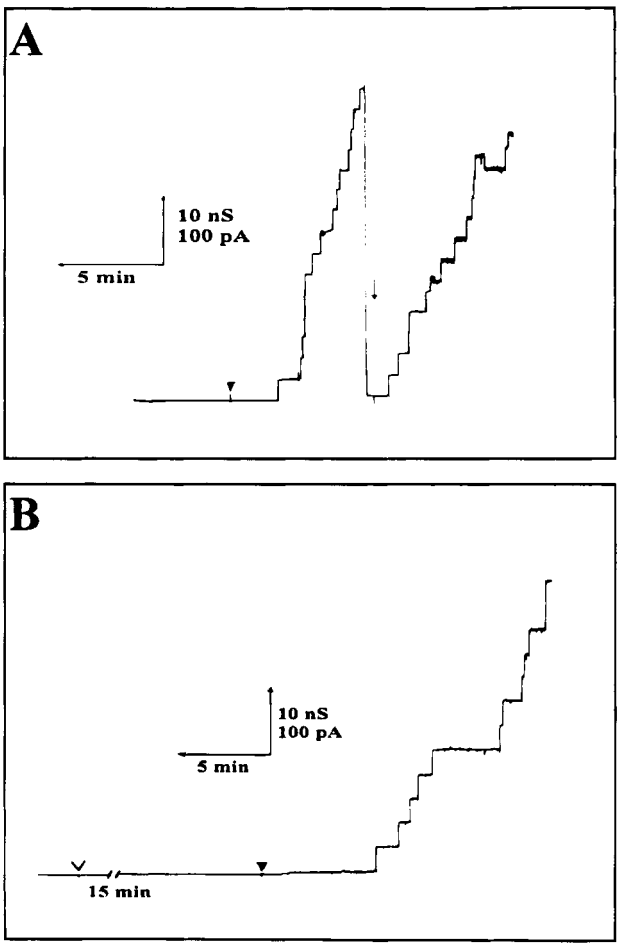

FIGURE 4: (A) Single-channel recording of a diphytanoyl phosphatidylcholine/*n*-decane membrane after addition (▼) of 100 ng/mL native *Dictyostelium* porin to the cis compartment. The downward pointing arrow symbolizes an offset of the baseline. (B) Single-channel recording of a diphytanoylphosphatidylcholine/*n*-decane membrane after addition (▼) of 50 ng/mL water-soluble *Dictyostelium* porin preincubated in a buffer containing 1% ergosterol and 1% Genapol. The V indicates the addition of 100 ng/mL water-soluble *Dictyostelium* porin preincubated in a buffer containing only 1% Genapol. The aqueous phase contained in both cases 1 M KCl (pH 6.0). The applied membrane potential was +10 mV; T = 20 °C.

Figure 4 shows single-channel records of native and reconstituted mitochondrial porin (i.e., water-soluble porin with detergent and sterol) from *D. discoideum*. It is noteworthy that the channels formed by both porin preparations were almost indistinguishable from one another as a comparison of the channel distributions (the histograms) in both cases clearly indicated. Water-soluble porin from *D. discoideum* could be activated with all the sterols shown in Figure 1A. The single-channel conductance was about 3.8 nS in 1 M KCl (with a somewhat broader distribution for the plant sterols). Only the extent of activation differed in dependence of the sterol used for preincubation. The weakest activity was obtained with the plant sterols sitosterol and stigmasterol, the strongest with ergosterol a typical sterol from fungi. The activities after preincubation in cholesterol, desmosterol, and 7-dehydrocholesterol were in between these two. The results of these measurements are shown in Table 1.

Structural Requirements for the Reconstitution of D. discoideum Porin with Sterol. In our reconstitution experiments we tested not only sterols from natural sources (i.e., from different kinds of eukaryotic organisms) for their ability

Table 1: Single-Channel Conductances, Voltage Dependence, and Channel-Forming Activity of Native Mitochondrial Porin of *D. discoideum* and Its Water-Soluble Form Preincubated in Different Sterols and Detergent[a]

D. discoideum	single-channel conductance (nS)	% closure $(1 - G_{50mV}/G_0)100$	activity (μS mL cm^{-2} ng^{-1})
native porin	3.5	51 ± 6	188 ± 90
water-soluble porin, preincubated in cholesterol	3.5	46 ± 1	109 ± 2
water-soluble porin, preincubated in epicholesterol	4	34 ± 11	nd
water-soluble porin, preincubated in ergosterol	3.5	43 ± 6	434 ± 27
water-soluble porin, preincubated in 7-dehydrocholesterol	3.5	44 ± 14	140 ± 25
water-soluble porin, preincubated in desmosterol	3.5	50	157 ± 19
water-soluble porin, preincubated in sitosterol	3	43 ± 7	49 ± 4
water-soluble porin, preincubated in stigmasterol	3.3	nd	6 ± 1

[a] The single-channel conductances represent the peaks of the single-channel histograms. The channel-forming activity is characterized by the specific conductivity (μS/cm^2) per protein in the membrane cell (ng/mL). The voltage dependence was derived from experiments similar to those shown in Figures 9 and 10 and is characterized by percent closure at a membrane potential of +50 mV [= $(1 - G_{50mV}/G_0)100$]. G_{50mV} is the stationary membrane conductance at a voltage of 50mV. G_0 is the stationary conductance at 10 mV. nd means not determined. The aqueous phase contained 1 M KCl (pH 6.0) for all measurements. The applied membrane potential for the determination of the single-channel conductances was +10 mV; T = 20 °C.

to activate the mitochondrial porin. We investigated also several steroids that are structurally related to the sterols but lack one or several groups that are typical for sterols from eukaryotic organisms. Interestingly, the addition of all the naturally occurring sterols such as cholesterol, 7-dehydrocholesterol, desmosterol, ergosterol, β-sitosterol, and stigmasterol to water-soluble porin resulted in the recovery of at least some channel-forming activity and a similar single-channel conductance as the native porin (see Table 1). On the other hand, most of the other compounds tested did not activate the water-soluble porin. For example no channel-forming activity could be detected after preincubation with thiocholesterol or with cholest-5-ene, which both lack the hydroxyl-group at C3 (see Figure 1). This means that this group is essential but not its configuration since the addition of epicholesterol, which has an α-OH group instead an β-OH group as all the other sterols tested, led to full channel-forming activity. No insertion of single channels but only a slight, continuous conductance increase was found upon preincubation with androstanol that lacks the side chain at C17 and the double bond at C5 (see Figure 5). This argues for an important role of the side chain since cholestanol, in which only the double bond is missing, led to very high activity. The double bond at C5, however, is probably not important.

Reconstitution of Other Water-Soluble Porins with Different Sterols. The reconstitution of mitochondrial porins from rat liver and from *Paramecium* yielded similar results. In both cases the water-soluble porins could be activated with all sterols of Figure 1, provided they had an OH group at C3 position and a side chain at C17. Only preincubation with ergosterol lead in both cases to very low channel-forming activity. Nevertheless we observed some differences. For example, the single-channel conductance of water-soluble porin from *Paramecium* had a broader distribution of single-channel conductances as compared with the narrow peak of native porin at 2.5 nS (in 1 M KCl) (Ludwig et al., 1989). Furthermore, the distribution of single-channel conductances depended on the sterol used for reconstitution. After preincubation with cholesterol, epicholesterol, and 7-dehydrocholesterol (Figure 6A), most channels had single-channel conductances between 0.5 and 2 nS in 1 M KCl; with desmosterol the distribution was very broad (between 0.5 and 3.5), with stigmasterol a distinct maximum of the single channel conductances was at 2.5 nS, and with sitosterol there was a clear-cut peak at 3.5 nS (see Figure 6B). This

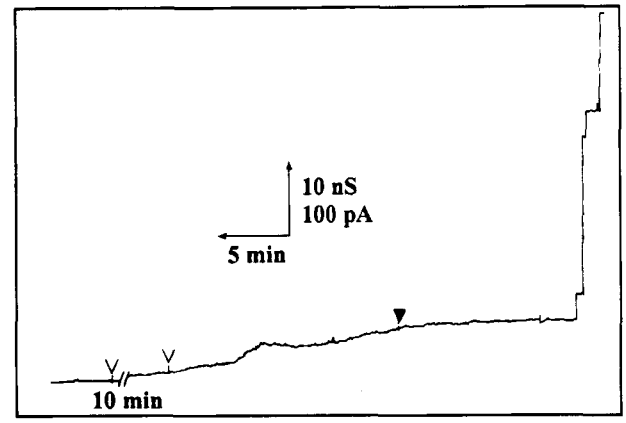

FIGURE 5: Single-channel recording of a diphytanoylphosphatidylcholine/*n*-decane membrane in the presence of water-soluble porin either preincubated with androstanol or with ergosterol. The open symbols (V) indicate the addition of 50 ng/mL water-soluble *Dictyostelium* porin (preincubated in a buffer containing 1% 5α-androstan-3β-ol and 1% Genapol) to the cis compartment. The closed symbols (▼) indicate the addition of 50 ng/mL water-soluble *Dictyostelium* porin (preincubated in a buffer containing 1% ergosterol and 1% Genapol) to the same compartment. The aqueous phase contained 1 M KCl (pH 6.0) The applied membrane potential was 10 mV; T = 20 °C.

can also be seen from the histograms of the single-channel distributions (Figure 7). The results of the single-channel experiments with water-soluble porin from *Paramecium* are summarized in Table 2.

Very similar results were obtained for water-soluble mitochondrial porin from rat liver. Again the single-channel conductance depended somewhat on the sterol used for preincubation (see Table 3). With cholesterol, epicholesterol, 7-dehydrocholesterol, and desmosterol used for preincubation, we found significant proportions of channels smaller than 2.5 nS together with a peak at 3.5−4 nS (1 M KCl), but in this case our original nonsoluble porin probes already had shown two peaks around 3.8 and 1.5 nS, respectively. However, the conductance increments during channel insertion may not reflect the distribution of single-channel sizes. It was pointed out in 1980 (Colombini, 1980) that small insertions were the result of normal channels inserting in a low conducting state and the channel later achieves the normal conductance. With sitosterol only one peak at a single-channel conductance around 3.2 nS appeared, with stigmasterol one peak was observed at 2.2 nS only. These

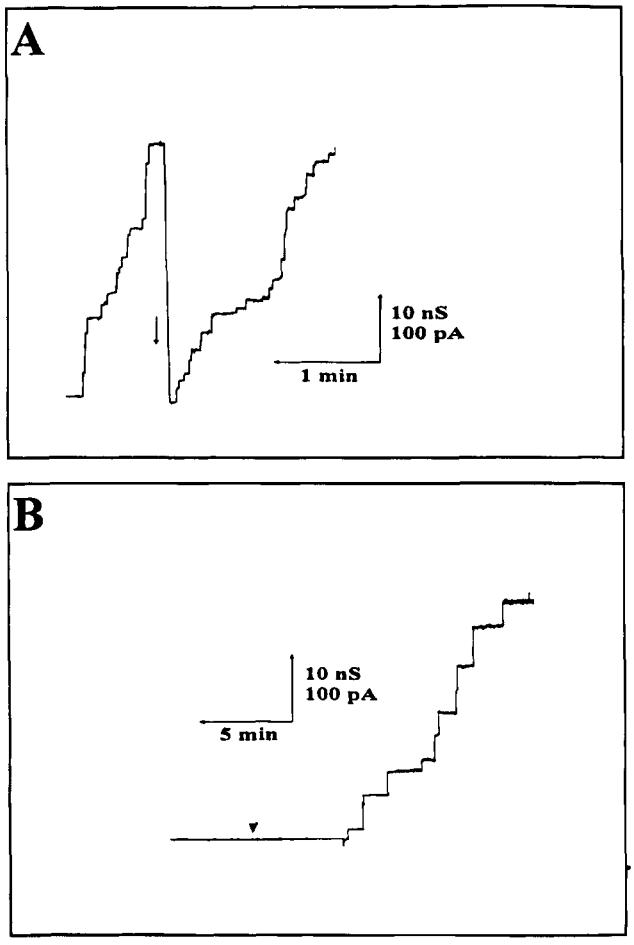

FIGURE 6: (A) Single-channel recording of a diphytanoylphosphatidylcholine/*n*-decane membrane after addition of 60 ng/mL water-soluble mitochondrial porin from *Paramecium* preincubated in 0.5% 7-dehydrocholesterol and 1% Genapol to the cis compartment. (B) Single-channel recording of a diphytanoyl phosphatidylcholine/*n*-decane membrane after addition (▼) of 90 ng/mL water-soluble mitochondrial porin from *Paramecium* preincubated in 0.5% β-sitosterol to the cis compartment. The aqueous phase contained 1 M KCl (pH 6.0) The applied membrane potential was +10 mV; T = 20 °C.

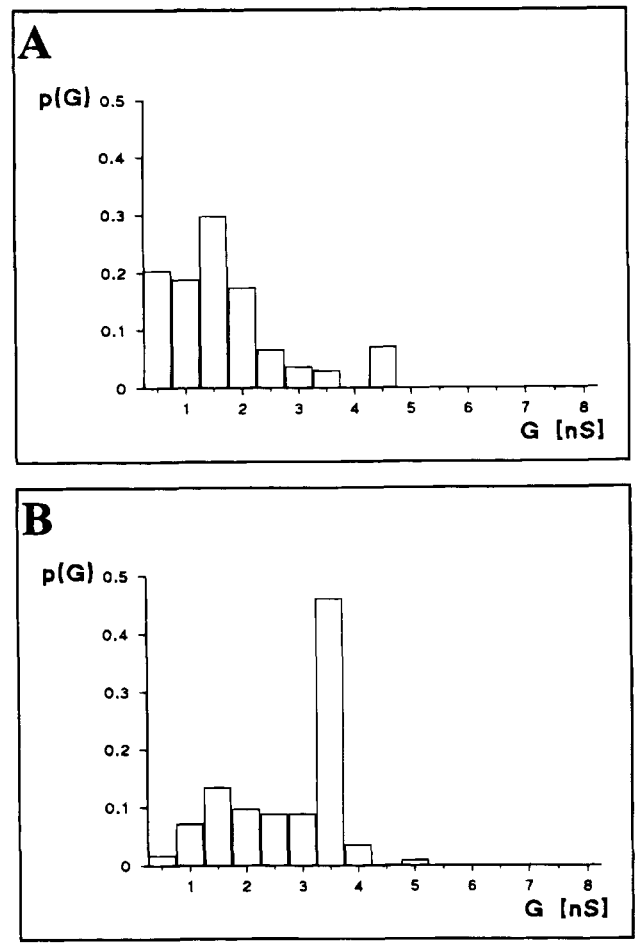

FIGURE 7: (A) Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit observed with membranes formed of diphytanoylphosphatidylcholine/*n*-decane in the presence of water-soluble porin from *Paramecium* preincubated in 7-dehydrocholesterol. (B) Histogram of the probability $P(G)$ for the occurrence of a given conductivity unit observed with membranes formed of diphytanoy phosphatidylcholine/*n*-decane in the presence of water-soluble porin from *Paramecium* preincubated in β-sitosterol. $P(G)$ is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The applied membrane potential was 10 mV; T = 20 °C. The average single-channel conductance was 1.5 nS for 138 single-channel events (A) and 2.8 nS for 113 single-channel events (B).

results mean that we were able to reconstitute some channel function with rat liver porin but only a smaller fraction of the channel had the same single-channel conductance as the native porin.

Voltage Dependence of the Reconstituted D. discoideum Porin. The voltage dependence is a characteristic of mitochondrial porins in contrast to most bacterial porins. Starting with about 20−30 mV, they switch to ion-permeable closed states, which have a completely different selectivity than the open state (Ludwig et al., 1989; Benz et al., 1990). We investigated the voltage dependence of the reconstituted porin from *D. discoideum* and compared it with that of the native form. Figure 8 shows the voltage dependence of the native form derived from four different membranes (closed circles). The bell-shaped curve of Figure 8 was obtained in the following way: after the incorporation of 100−1000 channels into a diphytanoylphosphatidylcholine/*n*-decane membrane, increasing voltages of both polarities were applied to the membrane. The membrane conductance (G) was measured when the opening and closing of channels reached an equilibrium, i.e., after the exponential decay of the membrane current. G was divided by the initial value of the conductance (G_0, which was a linear function of the voltage) obtained immediately after the onset of the voltage. The closed triangles in Figure 8 correspond to the voltage dependence of the porin reconstituted in the presence of ergosterol (also mean of four membranes). It is noteworthy that the voltage dependence for native and reconstituted porin was indistinguishable from one another within the limits of the experimental conditions. Similar results were obtained for reconstitution experiments with water-soluble porin from *D. discoideum* and all the other naturally occurring sterols of Figure 1A. In all cases the channels were irrespective of the sterol likewise voltage dependent.

Voltage Dependence of the Other Reconstituted Porins. We investigated also the voltage dependence of the other porins reconstituted from water-soluble proteins in the presence of detergent and sterol. In both cases (namely, rat liver and *Paramecium*) the original voltage dependence had disappeared. In particular the low conductance state (G) had a higher amplitude, which means that either the closed state-

Table 2: Single-Channel Conductances, Voltage Dependence, and Channel-Forming Activity of Native Mitochondrial Porin from *P. tetraurelia* and Its Water-Soluble Form Preincubated in Different Sterols and Detergent[a]

P. tetraurelia	single-channel conductance (nS)	% closure $(1 - G_{50mV}/G_0)100$	activity
native porin	2.4	61 ± 1	+++
water-soluble porin, preincubated in cholesterol	0.5−2.5	38	+++
water-soluble porin, preincubated in epicholesterol	1.5; 2.5	20 ± 15	+++
water-soluble porin, preincubated in ergosterol	nd	nd	(+)
water-soluble porin, preincubated in 7-dehydrocholesterol	0.5−2	20 ± 12	+++
water-soluble porin, preincubated in desmosterol	0.5−3.5	7	+++
water-soluble porin, preincubated in sitosterol	3.5	1 ± 1	+++
water-soluble porin, preincubated in stigmasterol	2.3; 4	5 ± 5	+++

[a] The single-channel conductances represent the peaks of the single-channel histograms. The channel-forming activity was estimated from single channel measurements and characterized by (+), ++, and +++ from (+) (very weak) to +++ (very strong). The voltage-dependence was derived from experiments similar to those shown in Figures 9 and 10 and is characterized by percent closure at a membrane potential of +50 mV [= $(1 - G_{50mV}/G_0)100$]. G_{50mV} is the stationary membrane conductance at a voltage of 50 mV. G_0 is the stationary conductance at 10mV. nd means not determined. The aqueous phase contained 1 M KCl (pH 6.0) for all measurements. The applied membrane potential for the determination of the single-channel conductances was +10 mV; T = 20 °C.

Table 3: Single-Channel Conductances, Voltage Dependence, and Channel-Forming Activity of Native Mitochondrial Porin from Rat Liver and Its Water-Soluble Form Preincubated in Different Sterols and Detergent[a]

rat liver	single-channel conductance (nS)	% closure $(1 - G_{50mV}/G_0)100$	activity (μS mL cm^{-2} ng^{-1})
native porin	1.5; 4	53 ± 4	++
water-soluble porin, preincubated in cholesterol	1−1.5; 4	14 ± 3	++ (54 ● 4)
water-soluble porin, preincubated in epicholesterol	1.5; 4	7 ± 5	++
water-soluble porin, preincubated in ergosterol	nd	nd	(+) (1 ± 0.3)
water-soluble porin, preincubated in 7-dehydrocholesterol	1−2.5; 3.5	27	++
water-soluble porin, preincubated in desmosterol	3.5	17	++
water-soluble porin, preincubated in sitosterol	3.2	0	++
water-soluble porin, preincubated in stigmasterol	2.2	0	++

[a] The single-channel conductances represent the peaks of the single-channel histograms. The channel-forming activity is estimated from single-channel measurements and characterized by (+), ++, and +++ from (+) (very weak) to +++ (very strong). The voltage dependence was derived from experiments similar to those shown in Figures 9 and 10 and is characterized by percent closure at a membrane potential of +50 mV [= $(1 - G_{50mV}/G_0)100$). G_{50mV} is the stationary membrane conductance at a voltage of 50 mV. G_0 is the stationary conductance at 10 mV. nd means not determined. The aqueous phase contained 1 M KCl (pH 6.0) for all measurements. The applied membrane potential for the determination of the single-channel conductances was +10 mV; T = 20 °C.

(s) had a higher conductance or that part of the channels did not close at all. Otherwise the kinetics of the voltage dependence looked similar after reconstitution with cholesterol, epicholesterol, 7-dehydrocholesterol, dihydrocholesterol, and desmosterol (see Figure 9). With dihydrocholesterol the voltage dependence was closest to that measured for the native porins, but the lowest conductance G reached was about 55% of the open state conductance G_0. Similar effects were observed for *Paramecium* porin. Native porin had closed states, which were only 30−40% of the open state. After reconstitution of the water-soluble porin the closed state had a conductance of about 70−80% of the open state. After reconstitution of the water-soluble porins from *Paramecium* and rat liver with β-sitosterol and stigmasterol, no voltage dependence up to ±80 mV was observed (see Table 3 for a survey).

Effect of Sterols on Native Porin. During our experiments we noticed that the addition of sterols, in particular of cholesterol and ergosterol, to the native porin preparation resulted in a much higher membrane activity. In the case of *D. discoideum* porin the preincubation with ergosterol or cholesterol resulted in an increase of the membrane conductance by a factor of about 10. Similar effects were observed for native porins from *Paramecium* and rat liver although the increase was less pronounced in these cases and depended on the sample.

DISCUSSION

Sterols Are Essential for the Functional Reconstitution of Water-Soluble Mitochondrial Porin. In this study we investigated the reconstitution of water-soluble mitochondrial porin in lipid bilayer membranes. The porin was unfolded and became water-soluble according to a treatment developed previously for mitochondrial porin of *N. crassa* (Pfaller et al., 1985). In that form the protein could mimic the role of the precursor during insertion into the mitochondrial outer membrane. For the correct insertion of the porin in the outer membrane (i.e., in a proteinase insensitive manner) either ATP or Triton X-100 is required (Pfaller et al., 1985). This means probably that the detergent acts as an unfoldase or chaperon. Sterols have been found in isolated and purified mitochondrial porin such as ergosterol in the case of *N. crassa* (Freitag et al., 1982a) and cholesterol in the case of porin of bovine heart mitochondria (De Pinto et al., 1989). The water-soluble porin has no membrane activity and even at very high concentrations it was inactive in lipid bilayer membranes and did not form channels. Channels were observed when the porin was transferred into detergent solution and the membrane contained sterols. A much higher reconstitution yield was achieved when water-soluble porin was preincubated with sterol in the detergent solution. The detergent presumably has in this process the function of a chaperon. Our results strongly suggest that sterols are essential for the proper folding of mitochondrial porin to form

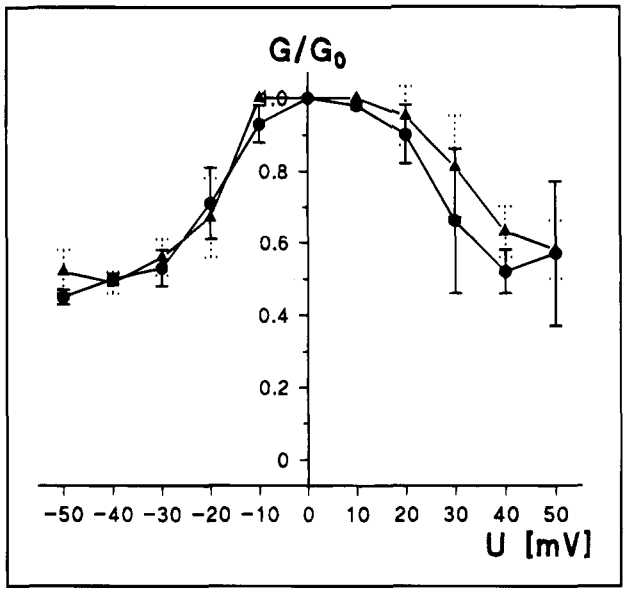

FIGURE 8: Voltage dependence of native mitochondrial porin from *D. discoideum* (closed circles, error bars solid) and of water-soluble porin from *D. discoideum* preincubated in ergosterol suspension in detergent (closed triangles, error bars dotted). The ratio of the conductance G at a given voltage V_m, divided by the conductance G_0 at 10 mV is shown as a function of the voltage. The cis side contained 50 ng/mL *D. discoideum* porin. The sign of the voltage is given with respect to the cis side, the side of the addition of porin. The membranes were formed of DiphPC. The aqueous phase contained 1 M KCl (pH 6.0); $T = 20$ °C.

the channel-forming unit. Probably, they associate with the protein on the outer side of the channel wall, which is oriented toward the lipid phase. This means that we consider the possibility that sterols are only needed for the insertion of the channel into the membranes as rather unlikely.

The Sterols Influence the Channel Properties of Reconstituted Mitochondrial Porin. From the experiments described here it is possible to conclude that the binding of sterols to the mitochondrial porin influences the channel properties and is essential for its activation. Without sterols the water-soluble porin has properties similar to those of the precursor of the mitochondrial porin (Pfaller et al., 1985). The sterols are required for the formation of the functional channel unit itself. According to structural predictions and experiments with antibodies, mitochondrial porin consists mostly of amphiphilic β-barrel sheets forming a hollow cylinder (Kleene et al., 1987; Forte et al., 1987). Our results suggest that at least one sterol molecule is bound to the channel wall. Possibly it contains several sterols since mitochondrial porin from bovine heart contains five molecules of cholesterol per polypeptide chain (De Pinto et al., 1989). The loss of one or several sterols may inactivate the channel. This could be the reason for loss of channel-forming activity observed during the purification process of mitochondrial porin (see also below).

We can only speculate about the function of the sterols in the channel-forming complex since a crystal structure of mitochondrial porin is not known to date. The hydroxyl group of sterols is absolutely essential for formation of the channel-forming unit as our experiments clearly indicated with steroids lacking this group. This could mean that the porin molecule associated with the sterol could get a more hydrophobic outside and proper folding and integration into the lipophilic membrane may be facilitated. Furthermore,

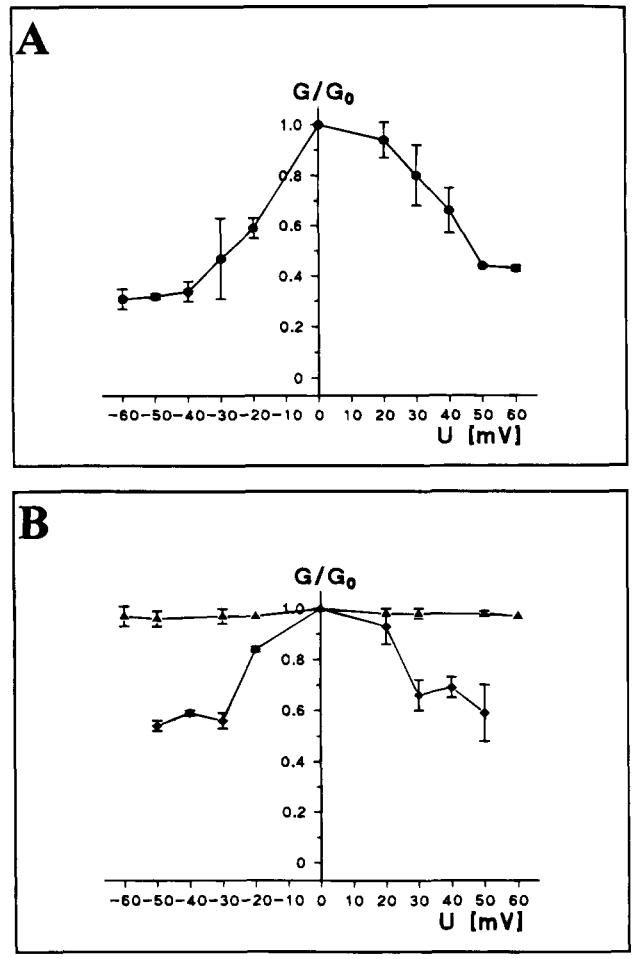

FIGURE 9: Voltage dependence of native mitochondrial porin from *Paramecium* and of water-soluble porin from *Paramecium* preincubated in different sterols. (A) Native mitochondrial porin from *Paramecium*. (B) Water-soluble mitochondrial porin from *Paramecium* preincubated in dihydrocholesterol (closed diamonds) or in β-sitosterol (closed triangles), respectively. The ratio of the conductance G at a given voltage V_m, divided by the conductance G_0 at 10 mV is shown as a function of the voltage. The cis side contained 50 ng/mL *Paramecium* porin. The sign of the voltage is given with respect to the cis side, the side of the addition of porin. The membranes were formed of DiphPC. The aqueous phase contained 1 M KCl (pH 6.0); $T = 20$ °C.

the association with one or more sterol molecules could stabilize the entire protein structure by hydrogen bonds and nonpolar interactions. They could shield relatively polar parts on the protein wall by forming hydrogen bonds between the hydroxyl group and amino acids. It is noteworthy that hydrogen bonds have been found to stabilize the tertiary structure of bacterial porins (Schindler & Rosenbusch, 1984; Markovic-Housley & Garavito, 1986).

The importance of nonpolar interactions along with hydrogen bonding can also be concluded from the fact that a side chain in the sterol molecule is necessary for activation of water-soluble porins. The exact structure of this side chain is not so important as experiments with water-soluble mitochondrial porin from *D. discoideum* indicated. Its preincubation with all naturally occurring sterols that differ from one to the other somewhat in the side chain and also somewhat in the ring system structure lead to activation. Interestingly, the observed channels did not significantly differ from the detergent-purified porin. The same was observed for water-soluble porins from *N. crassa* inserting into sterol-containing membranes (Pfaller et al., 1985). The

quantitative difference in activation may be explained by differences in the association and dissociation constants of the respective sterols with the protein.

In the case of water-soluble mitochondrial porins from *P. tetraurelia* and from rat liver, the structure of the side chain and the ring system of the sterols had a more substantial influence on the properties of the reconstituted channels. The reason for this is probably that these channels did not regain their original conformations. We do not know whether this is caused by nonideal fit of the sterols to the binding region of the protein or also by partially irreversible denaturation of the protein during the procedure to make it water-soluble. It is possible that the fit of the sterol might at least play some crucial role in this context. This can be concluded from the experimental observation that the reconstitution of the porins with sterols allowed the classification of the sterols into groups that argues for an influence of the structure of the sterol on the channel properties. The first group produced at least weak voltage dependence (cholesterol, epicholesterol, 7-dehydrocholesterol) of the reconstituted water-soluble porins from *Paramecium* and rat liver, while the second group did not promote any voltage dependence (sitosterol, stigmasterol). The voltage dependence of mitochondrial porins is presumably caused by loops between β-strands that are folded back inside the channel. The sterols could influence the localization of these loops within the lumen of the channel. Sitosterol and stigmasterol have bulkier side chains than the other sterols. These side chains could interact with the loops and could thus prevent closure of the channel. Another possibility is that certain sterols, which do not fit in the overall channel configuration prevent the closing of the channels since they disturb the channel structure to much. We also observed an influence of the sterol on the selectivity of the reconstituted mitochondrial porin (data not shown). This difference in selectivity could, like the difference in the voltage dependence, be caused by an influence of the sterol on the conformation of the channel protein.

If irreversible denaturation does not play any role for the changed channel properties in the reconstitution experiments with water-soluble porins from *Paramecium* and rat liver, the only reason for these changed properties could be that we did not try the right sterol or that a specific lipid is necessary for proper folding. We consider the first possibility as rather unlikely because we tested most sterols found in mammalian cells. On the other hand, we did not test the influence of 7-dehydrostigmasterol on the channel properties of *Paramecium* porin. Stigmasterol produced pores of about the original pore size of 2.5 nS in 1 M KCl, and some sterols with a second double bond in the ring system promoted some voltage dependence. 7-Dehydrostigmasterol is known to occur in *Paramecium* cells in measurable amounts (Hennessey & Nelson, 1983).

Role of Epicholesterol. Epicholesterol that has the hydroxyl-group in the α-position, while all the other sterols used in our study have this group in the β-position. The isomerization of the hydroxyl group has a strong influence on the overall structure of cholesterol and for example prevents an interaction between the sterol and phospholipids in the membrane (Demel et al., 1972) or with sterol-binding toxins (Alouf & Geoffroy, 1979). Our experimental observation that epicholesterol led to very high activation of water-soluble porin is very interesting. Furthermore, we did not observe any difference in the channel properties between the channels reconstituted with cholesterol and epicholesterol. We cannot completely exclude the possibility that the epicholesterol used in our experiments contained trace amounts of cholesterol although we did not find such impurities by TLC. We also used smaller concentrations of epicholesterol (0.1% instead of 0.5%) for preincubation to suppress the possible activation through trace amounts of cholesterol. Even in these cases we observed no decrease in activation, which means that the activation by epicholesterol was not an artifact. Similarly as epicholesterol, dihydrocholesterol (cholestanol) activated water-soluble porins to a high degree from which it is obvious that the double bond at C5 is not needed.

Effect of Sterols on Detergent-Solubilized, Purified Porin. In other respects our results can be of some interest. The observation that also detergent-purified mitochondrial porins can, at least in some cases, be highly activated by preincubation with sterols can facilitate experiments where high channel-forming activity is needed. Whether this preincubation leads to an activation of the respective pores may depend on the isolation procedure and its number of steps, strictly speaking on the extent to which sterols have been removed from the channel-forming units during purification. The number of sterols that are removed is probably small and may be only one or two out of the five that are tightly attached to mammalian porins (De Pinto et al., 1989). Subsequently, the addition of sterol to purified porin led to a 10-fold increase of the channel-forming activity for porin from *D. discoideum* and to a smaller extent of that of *Paramecium* and rat liver. This result indicates that the stability of the channel-forming complex is much smaller for mitochondrial porins as compared to that of bacterial porins, which easily withstand even treatment with SDS (Schindler & Rosenbusch, 1978; Eisele & Rosenbusch, 1990).

Where Does the Mitochondrial Outer Membrane Channel Form? The secondary structure of mitochondrial porin does not contain any indication for the existence of hydrophobic α-helices. Instead it is similar to bacterial porins (Cowan et al., 1992) preferentially controlled by amphipathic β-structure. This is widely accepted although the exact number of β-strands is still a matter of debate. One model assumes 16 β-strands (De Pinto et al, 1991) as it has been found from the crystal structure of bacterial porins (Weiss et al., 1991) while the other assumes 12 β-strands, which form together with the amphipathic α-helix at the N-terminal end the channel (Blachly-Dyson et al., 1990). The exact mechanism and the location of the folding of neither bacterial nor mitochondrial porins are known to date, but there exist some indications that bacterial porins may first form monomers in the periplasmic space followed by the formation of trimers (De Cock et al., 1990; Sen & Nikaido, 1990). Detergents play a crucial role in this process using an *in vitro* system, which may indicate that it is chaperon-promoted. The folding of bacterial porins has been studied with CD spectroscopy and SDS−PAGE, but the functional integrity of the folded trimers has not been shown. This means that it is not known whether the reconstituted trimers form channels and whether the channel properties are identical to native trimers. Similar questions may be addressed to the folding of the mitochondrial outer membrane channel. Our results suggest that it occurs in the aqueous phase because preincubation of the water-soluble porin with sterols and

detergent led to a higher channel-forming activity than addition of the water-soluble porin together with detergent to membranes containing sterol. Furthermore, the sterol in the membranes had no influence on the membrane activity when the water-soluble porin was preincubated with sterol. We believe that the formation of the porin channels in the aqueous phase is a general feature that occurs also *in vivo* in a similar way as we have observed it in this study since it is difficult to imagine how a polar protein [polarity about 0.5 (Forte et al., 1987; Kayser et al., 1989)] can fold inside a membrane. For the *in vivo* process, however, an ATP-dependent chaperon is needed. Detergents, such as Triton X-100 or Genapol X-80 can mimic the role of the chaperons in the folding and insertion of both, mitochondrial (Pfaller et al., 1985) and bacterial porins (De Cock et al., 1990; Sen & Nikaido, 1990).

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REFERENCES

Alouf, J. E., & Geoffroy, C. (1979) *FEMS Microbiol. Lett. 6*, 413−416.
Asworth, L. A. E., & Green, C. (1966) *Science 151*, 210−211.
Benz, R. (1985) *CRC Crit. Rev. Biochem. 19*, 145−190.
Benz, R., & Brdiczka, D. (1992) *J. Bioenerg. Biomembr. 24*, 33−29.
Benz, R., Janko, K., Boos, W., & Läuger, P. (1978) *Biochim. Biophys. Acta 511*, 305−319.
Benz, R., Kottke, M., & Brdiczka, D. (1990) *Biochim. Biophys. Acta 1022*, 311−318.
Blachly-Dyson, E., Peng, S., Colombini, M., & Forte, M. (1990) *Science 247*, 1233−1236.
Carr, F. M., & Price, E. A. (1926) *Biochem. J. 20*, 497.
Colombini, M. (1979) *Nature 279*, 643−645.
Colombini, M. (1980) *J. Membr. Biol. 53*, 79−84.
Colombini, M. (1983) *J. Membr. Biol. 74*, 115−121.
Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Gosh, R., Pauptit, R. A., Jansonius, J. N., & Rosenbusch, J. P. (1992) *Nature 356*, 727−733.
Daum, G. (1985) *Biochim. Biophys. Acta 822*, 1−42.
De Cock, H., Hendriks, R., De Vrije, T., & Tommassen, J. (1990) *J. Biol. Chem. 265*, 4646−4651.
Demel, R. A., & De Kruijff, B. (1976) *Biochim. Biophys. Acta 457*, 109−132.
Demel, R. A., Bruckdorfer, K. R., & Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 255*, 321−330.
De Pinto, V., Ludwig, O., Krause, J., Benz, R., & Palmieri, F. (1987a) *Biochim. Biophys. Acta 894*, 109−119.
De Pinto, V., Prezioso, G., & Palmieri, F. (1987b) *Biochim. Biophys. Acta 905*, 499−502.
De Pinto, V., Benz, R., & Palmieri, F. (1989) *Eur. J. Biochem. 183*, 179−187.
De Pinto, V., Prezioso, G., Thinnes, F., Link, T. A., & Palmieri, F. (1991) *Biochemistry 30*, 10191−10200.
Eisele, J.-L., & Rosenbusch, J. P. (1990) *J. Biol. Chem. 265*, 10217−10220.
Forte, M., Guy, H. R., & Mannella, C. (1987) *J. Bioenerg. Biomembr. 19*, 341−350.
Freitag, H., Neupert, W., & Benz, R. (1982a) *Eur. J. Biochem. 123*, 629−636.
Freitag, H., Janes, M., & Neupert, W. (1982b) *Eur. J. Biochem. 126*, 197−202.
Gasser, M., & Schatz, G. (1983) *J. Biol. Chem. 258*, 3427−3430.
Glover, J., & Green, C. (1957) *Biochem. J. 67*, 308−316.
Hennessey, T. M., & Nelson, D. L. (1983) *Biochim. Biophys. Acta 728*, 145−158.
Kayser, H., Kratzin, H. D., Thinnes, F. P., Götz, H., Schmidt, W. E., Eckart, K., & Hilschmann, N. (1989) *Biol. Chem. Hoppe-Seyler 370*, 1265−1278.
Kleene, R., Pfanner, N., Pfaller, R., Link, T. A., Sebald, W., Neupert, W., & Tropschung, M. (1987) *EMBO J. 6*, 2627−2633.
Lämmli, U. K. (1970) *Nature 227*, 680−685.
Lasser, N. L., & Clayton, R. B. (1966) *J. Lipid Res. 7*, 413−421.
Lévy, M., & Sauner, M. T. (1968) *Chem. Phys. Lipids 2*, 291−295.
Lindén, M., & Gellerfors, P. (1983) *Biochim. Biophys. Acta 763*, 125−129.
Liu, M. Y., & Colombini, M. (1992) *Biochim. Biophys. Acta 1098*, 255−260.
Ludwig, O., Benz, R., & Schultz, I. E. (1989) *Biochim. Biophys. Acta 978*, 319−327.
Krause, J., Hay, R., Kowollik, C., & Brdiczka, D. (1986) *Biochim. Biophys. Acta 860*, 690−698.
Mannella, C. A., Forte, M., & Colombini, M. (1992) *J. Bioenerg. Biomembr. 24*, 7−19.
Marcovic-Housley, Z., & Garavito, R. M. (1986) *Biochim. Biophys. Acta 869*, 158−170.
Mihara, K., & Sato, R. (1985) *EMBO J. 4*, 769−774.
Mihara, K., Blobel, G., & Sato, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7102−7106.
O'Brien, R. L., & Brierly, G. (1965) *J. Biol. Chem. 240*, 4527−4539.
Peng, S., Blachly-Dyson, E., Colombini, M., & Forte, M. (1992) *J. Bioenerg. Biomembr. 24*, 27−32.
Pfaff, E., Klingenberg, M., Ritt, E., & Vogel, W. (1968) *Eur. J. Biochem. 5*, 222−232.
Pfaller, R., Freitag, H., Harmey, M. A., Benz, R., & Neupert, W. (1985) *J. Biol. Chem. 260*, 8188−8193.
Pfanner, N., Pfaller, R. Kleene, R., Ito, M., Tropschug, M., & Neupert, W. (1988) *J. Biol. Chem. 263*, 4049−4051.
Roos, N., Benz, R., & Brdiczka, D. (1982) *Biochim. Biophys. Acta 686*, 204−214.
Sandermann, H., & Strohminger, J. (1972) *J. Biol. Chem. 247*, 5123−5131.
Schein, S. J., Colombini, M., & Finkelstein, A. (1976) *J. Membr. Biol. 30*, 99−120.
Schindler, H., & Rosenbusch, J. P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3751−3755.
Schindler, M., & Rosenbusch, J. P. (1984) *FEBS Lett. 173*, 85−89.
Sen, K., & Nikaido, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 743−747.
Smith, M. E., Fumagallio, R., & Paoletti, R. (1967) *Life Sci. 6*, 1085−1092.
Thomas, L., Kocsis, E., Colombini, M., Erbe, E., Trus, B. L., & Steven, A. C. (1991) *J. Struct. Biol. 106*, 161−171.
Troll, H., Malchow, D., Müller-Taubenberger, A., Humbel, B., Lottspeich, F., Eck, M., Gerisch, G., Schmid, A., & Benz, R. (1992) *J. Biol. Chem. 267*, 21072−21079.
Vogel, H., & Jähnig, F. (1986) *J. Mol. Biol. 190*, 191−199.
Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., & Schultz, G. E. (1991) *Science 254*, 1627−1630.
Wessel, D., & Flügge, U. I. (1984) *Anal. Biochem. 138*, 141−143.
Zalman, L. S.,Nikaido, H., & Kagawa, Y. (1980) *J. Biol. Chem. 255*, 1771−1774.

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