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PROPERTIES OF THE LARGE ION-PERMEABLE PORES FORMED FROM PROTEIN F OF *PSEUDOMONAS AERUGINOSA* IN LIPID BILAYER MEMBRANES

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The incorporation of porin protein F from the outer membrane of *Pseudomonas aeruginosa* into artificial lipid bilayers results in an increase of the membrane conductance by many orders of magnitude. The membrane conductance is caused by the formation of large ion-permeable channels with a single-channel conductance in the order of 5 nS for 1 M alkali chlorides. The conductance has an ohmic current vs. voltage relationship. Further information on the structure of the pore formed by protein F was obtained by determining the single-channel conductance for various species differing in charge and size, and from zero-current potential measurements. The channel was found to be permeable for large organic ions ($Tris^+$, $N(C_2H_5)_4^+$, $Hepes^-$) and a channel diameter of 2.2 nm could be estimated from the conductance data (pore length of 7.5 nm). At neutral pH the pore is about two times more permeable for cations than for anions, possibly caused by negative charges in the pore. The consistent observation of large water filled pores formed by porin protein F in model membrane systems is discussed in the light of the known low permeability of the *Ps. aeruginosa* outer membrane towards antibiotics. It is suggested that this results from a relatively low proportion of open functional porin protein F pores in vivo.

Introduction

The cell envelopes of Gram-negative bacteria consist of a cytoplasmic (inner) membrane, a layer of peptidoglycan and an outer membrane. The outer membrane acts as a molecular filter with a defined exclusion limit for hydrophilic substances [1–3]. This property results largely from the presence in outer membranes of a class of proteins called porins, which form large water filled pores through the hydrophobic core of the outer membrane [1,4–6].

Experiments with reconstituted outer membrane vesicles have suggested that the maximum molecular weight for permeable substances in the case of enteric bacteria like *Escherichia coli* and *Salmonella typhimurium* [1,4,5] is about 500–700, whereas molecules with molecular weights up to 6000 can penetrate the outer membrane of *Pseudomonas aeruginosa* [3,6]. Such exclusion limits can be obtained whether whole outer membranes [3] or purified porin proteins [6] are used to reconstitute the pores. While reconstituted vesicles are able to provide limited information about the presence and size of the pores formed by porins, a much more detailed analysis of porins can be obtained from black lipid bilayer studies. The incorporation of *E. coli* and *S. typhimurium* porins into lipid bilayers results in the formation of large ion-permeable pores with a high electrical conductance (in the order of 0.2 nS for an 0.1 M alkali chloride solution [7–10]). These experiments suggest that the porins form large water-filled pores in lipid bilayer membranes with a diameter in the order of 1.4 nm.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Ps. aeruginosa is an opportunistic pathogen of major importance in hospital-acquired infections, largely due to its high natural resistance to antibiotics. One of the important factors contributing to this antibiotic resistance is the general low permeability of *Ps. aeruginosa* to antibiotics [11]. For the β lactam class of antibiotics which have targets on the periplasmic side of the cytoplasmic membrane, the only potential permeability barrier is the outer membrane [1,11]. Thus there is the apparent anomaly of a bacterium with an outer membrane which constitutes a significant permeability barrier towards hydrophilic antibiotics of around 400 daltons, but which contains large amounts of a porin which can reconstitute pores with an exclusion limit of around 6 000 daltons [3,6]. In order to gain insight into this apparent dilemma, we performed lipid bilayer experiments in the presence of *Ps. aeruginosa* outer membranes or isolated porin protein F. The results presented here are consistent with the assumption that porin protein F forms large water-filled pores in lipid bilayer membranes with a diameter in the order of 2 nm. The large size and weak selectivity of porin protein F suggest that the low permeability of outer membranes to antibiotics is most likely explained by a relatively small proportion of functional open pores in the outer membrane of *Ps. aeruginosa* compared to enteric bacteria. Presumptive evidence is presented to support this conclusion.

Materials and Methods

Isolation of outer membranes and purification of porin protein F. This was performed as described previously [6]. The total outer membranes were dissolved in a solution containing 1% sodium deoxycholate, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl (pH 8.0) and 0.02% sodium azide in a concentration of about 5 mg/ml. The purified protein F was dissolved in 0.1% (v/v) Triton X-100 and 3 mM sodium azide in concentrations between 0.1 and 1 mg/ml. About 2–3 molecules lipopolysaccharide were associated with each molecule of purified porin. The stock solutions were kept in the refrigerator or in the freezer and remained active under these conditions for at least 3 months.

Membrane experiments. Black lipid bilayers were obtained [7,8] from a 1–2% (w/v) solution of oxidized cholesterol or egg lecithin in *n*-decane (Fluka, Buchs, Switzerland, purum). Oxidized cholesterol was prepared as described earlier [7] and egg lecithin was isolated and purified according to standard methods [13,14]. The chamber used for bilayer formation was made from Teflon. The circular holes in the wall between the two aqueous compartments has an area of either 2 mm^2 (in the case of the macroscopic conductance measurements) or 0.1 mm^2 (for the single channel measurements). The temperature was kept at 25°C throughout.

All salts, besides Hepes (Sigma, analytical grade), tetramethylammoniumhydroxide and tetraethylammoniumhydroxide (Fluka, Buchs, Switzerland, purum) were obtained from Merck (Darmstadt, F.R.G., analytical grade). The pH of the aqueous salt solution was adjusted to the values given in the text by adding the corresponding hydroxide or acid. Thus, in experiments with large organic ions, no small ions were present. The aqueous solutions were used without buffering. The protein was added to the aqueous phase prior to membrane formation or after the membrane had turned optically black (thus signifying bilayer formation). All protein containing solutions were prepared immediately before use in order to prevent protein inactivation.

For the electrical measurements, Ag/AgCl or platinized platinum electrodes were inserted into the aqueous solutions on both sides of the membranes. The current-fluctuation experiments were performed using a Keithley 427 preamplifier. The amplified signal was monitored with a Tektronix 5115 storage oscilloscope (plug-in amplifier 5A22) and recorded with a stripchart recorder or a tape recorder. The time resolution of the measurements were in the order of 1 kHz. Zero-current membrane potentials were measured by a slight modification of an earlier described method [8]. Bilayer membranes were formed in a 10^{-2} M salt solution in the presence of 10^{-9} M porin protein F. After the membranes had turned completely black, a voltage of 10 mV was applied and the membrane conductance was observed to increase, within 10 to 20 min, to a value of about 1 $\mu S \cdot cm^{-2}$ in the presence of alkali chlorides. For other salts the conductance was lower, corresponding to a lower single-channel conductance. The applied voltage was then removed and the salt concentration

on one side of the membrane raised by the addition of small amounts of concentrated salt solution. After about 10 min the zero-current membrane potential (mV) reached a steady-state value and was measured with a Keithley 610 C electrometer, using calomel electrodes with salt bridges (Methrohm, Herisau, Switzerland).

Results

Macroscopic conductance measurements

The conductance of lipid bilayer membranes increased many orders of magnitude when isolated protein F from *Ps. aeruginosa* was added to the aqueous phase prior to membrane formation or after the membranes had turned black. The conductance increase in the presence of the porin was not instantaneous as it has been described in experiments with macrocyclic carriers [15]. A typical experiment is given in Fig. 1. A membrane from oxidized cholesterol/*n*-decane was formed in a solution of 1 M NaCl and 32.5 ng/ml (approx. 10^{-9} M) protein F. After blackening of the membrane (arrow in Fig. 1) the membrane conductance increased rapidly for about 30 min. After this time the rate of increase was slower but no stationary conductance level was reached and the membrane conductance increased continuously until membrane breakage. This behavior did not change whether the porins were added to one or to both sides of the membranes. There was also no basic difference if isolated protein F or the total outer membrane of *Ps. aeruginosa* (dissolved in sodium deoxycholate) was added to the aqueous phase. The absolute conductance level in the latter case when corrected for the content of protein F (about 15% of outer membrane proteins) was about a factor of 10 lower than the conductance observed when isolated protein F was present in the aqueous phase. This difference might have been caused by the shorter lifetime of the pores created by the total outer membrane in the bilayer membranes (see below) or by a partial inactivation of protein F by deoxycholate. The relation between macroscopic conductance and protein concentration in the aqueous phase was found to be linear for at least a 100-fold range of protein concentration. Fig. 2 shows the concentration dependence of the macroscopic conductance measured in the presence of 1 M KCl and different concentrations of isolated porin F in the aqueous phase at a given time (usually 30 min) after blackening of the membrane. A linear relationship was observed when either of two different lipids was used for membrane formation (Fig. 2). However, in agreement with results obtained for porins from *E. coli* [7] and from *S. typhimurium* [10], a considerable difference in the macroscopic

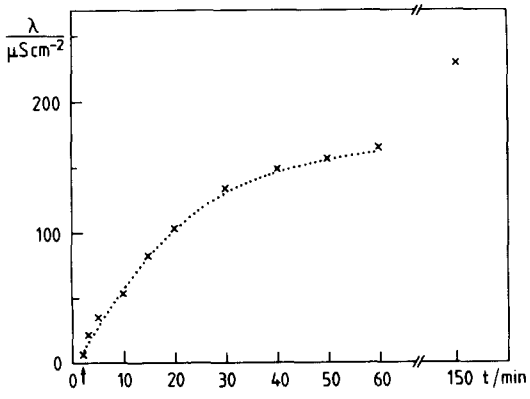

Fig. 1. Specific membrane conductance λ as a function of time t after the formation of the membrane from oxidized cholesterol/*n*-decane. The aqueous phase contained 1 M NaCl, 32.5 ng/ml ($9 \cdot 10^{-10}$ M) protein F and 0.5 μg/ml Triton, pH 6.5; T = 25°C; V_m = 10 mV. The arrow indicates when the membrane was completely black.

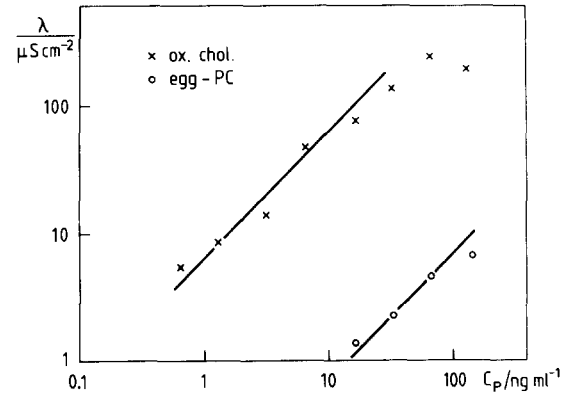

Fig. 2. Specific membrane conductance as a function of the protein concentration c_p of protein F in the aqueous phase. The membranes were formed either from oxidized cholesterol/*n*-decane (ox. chol.) or from egg-lecithin/*n*-decane (egg-PC). The aqueous phase contained 1 M NaCl and less than 1 μg/ml Triton X-100. The results were obtained from at least three membranes 30 min after blackening of the membranes.

conductance was measured for membranes formed from different lipids. With membranes made from oxidized cholesterol the conductance at a given protein concentration was about a factor of 100 larger than with membranes made from phospholipids like egg lecithin. The reason for this difference remains unclear to date, but did not seem to be caused by a different pore size (see below). Thus, the kinetic process of the insertion of the protein into the membrane may be responsible for the variable activity of porin in membranes of various composition.

As described above, a steady conductance level could not be reached (Fig. 1) and therefore the dependence of the conductance on various parameters was difficult to obtain. A meaningful comparison was possible, however, on the basis of $\lambda(t)$ curves which were reproducible to ±30%. Fig. 3 shows the effect of the salt concentration on the membrane conductance in the presence of a constant concentration of porin F at a given time after the formation of the bilayer. This time was chosen to be 30 min in all cases. As can be seen from Fig. 3 the conductance λ was a linear function of the salt concentration in the aqueous phase within the limits of experimental uncertainty. This result suggests that the rate of protein incorporation was independent

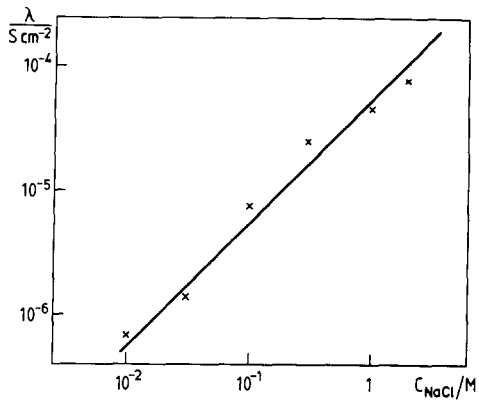

Fig. 3. Specific membrane conductance λ as a function of the NaCl-concentration in water. The aqueous phase contained 10 ng/ml protein F and 0.5 μg/ml Triton, pH 6.5; T = 25°C. The membranes were formed from a 1% (w/v) solution of oxidized cholesterol in *n*-decane and the conductance was measured 30 min after membrane formation (applied voltage V_m = 10 mV). Each point represents at least three membranes.

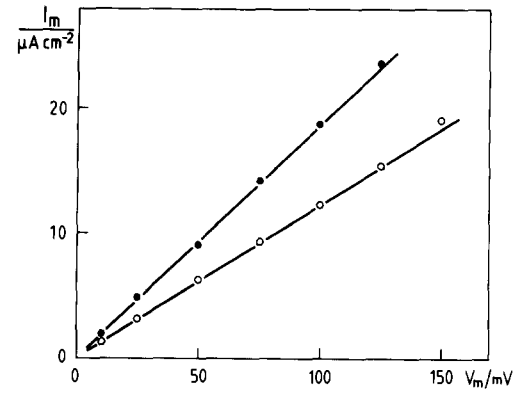

Fig. 4. Current vs. voltage characteristic of a membrane from oxidized cholesterol/*n*-decane doped with protein F. The aqueous phase contained 20 ng/ml protein, 0.5 μg/ml Triton X-100 and 1 M NaCl, pH 6.5; T = 25°C. The results from two different membranes are shown.

of the salt concentration. Thus, porin protein F probably inserts into the membrane through hydrophobic interactions.

Fig. 4 shows the current-voltage behavior of two membranes in the presence of porin protein F. The membrane current observed was a linear function of the applied voltage up to at least 150 mV for the application of the voltage for about 5 s. For a larger application of the voltage, only a slight increase of the current was observed presumably caused by facilitated incorporation of protein F into the membranes. The observed linear current-voltage relationship indicated that the properties of the single-conductive unit were virtually independent of voltage. Extrapolation of the lines for the current-voltage relationship to zero current (at zero voltage) strongly suggested that there was no fixed minimal voltage required to initiate the passage of ions i.e. the pores were not voltage gated.

Single-channel experiments

When porin protein F from a stock solution in Triton X-100 was added in small quantities to the aqueous solutions bathing the membrane, the membrane conductance started to increase in a stepwise fashion. An example of such an experiment is given in Fig. 5. The occurrence of these steps was specific for the presence of protein F and was not observed when only the detergent was present

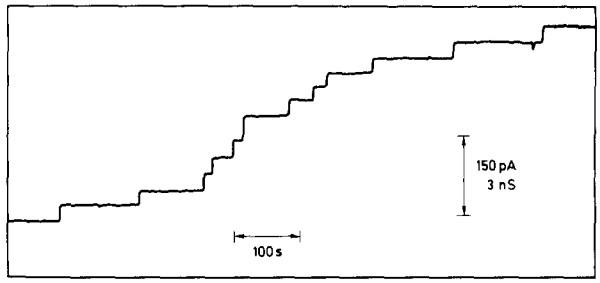

Fig. 5. Stepwise increase of the membrane current after the addition of 5 ng/ml protein F to the aqueous phase contained 0.1 M NaCl, pH 6.5; $T = 25°C$. The membrane was formed from a 1% (w/v) solution of egg-lecithin in *n*-decane. The applied voltage was 50 mV.

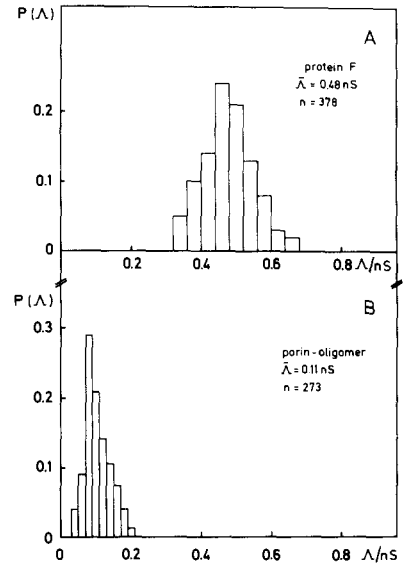

Fig. 6. Histogram of the conductance fluctuations observed with membranes from egg-lecithin dissolved in *n*-decane in the presence of protein F (A) or porin from *E. coli* K-12 (B). The aqueous phase contained 0.1 M NaCl, pH 6.5; $T = 25°C$. The applied voltage was 50 mV. A: Protein F, $n = 378$; $\bar{\Lambda} = 4.8 \cdot 10^{-10}$ S. B: porin from *E. coli*, K-12 $n = 273$; $\bar{\Lambda} = 9.3 \cdot 10^{-11}$ S. n is the number of single steps.

at a concentration equivalent to or 100-times higher than that in the single-channel experiments. Channels caused by Trition, as described in the literature [16], were only observed if the detergent was present in the aqueous phase at a concentration of 0.1 mg/ml, a concentration never reached in all experiments described here. In addition, at the concentrations of Triton used in the experiments described here, the background conductance of the membranes in the order of 10^{-7} S · cm^{-2} was not changed. This data was consistent with results showing the lack of effects of Triton X-100 on vesicle reconstitution experiments. The conductance steps were observed irrespective of whether the protein F was added to the aqueous solution on one side of the membrane only or to both solutions. Most of the steps in the current records were directed upward, whereas terminating events were rarely observed. The lifetime of the F-porin pores was at least 5 min as derived from records extending over long times. Similar stability was previously found for purified porins from *E. coli* and *S. typhimurium* [7,10]. However, in single channel experiments using whole outer membrane from *Ps. aeruginosa*, the lifetime of the porin pores was found to be much shorter. In this case the lifetime was in the order of 50 to 100 ms, although the absolute level of the current fluctuation remained the same as in the experiments with the isolated porin. Changes in the salt concentration and in the lipid composition had no influence on the lifetime of the single-conductance unit, irrespective of whether isolated porin or total outer membrane was used in the experiments. Both preparations showed much lower activity in single-channel experiments on phospholipid membranes than with membranes from oxidized cholesterol, and about 100 times more porin had to be added in order to obtain the same number of pores. The risetime of the single-current fluctuations was always faster than the bandwidth of the preamplifier and no indication of smaller intermediate steps could be observed.

In contrast to findings with *E. coli* and *S. typhimurium* porin [8,10], the single-conductance increments were almost uniform in magnitude and only small variations were observed. Fig. 6 shows a histogram observed for porin protein F in egg-lecithin membranes. For comparison, the results for porin from *E. coli* K-12 are also shown in Fig. 6. As can be seen from Fig. 6 the variation of the conductance fluctuation is much smaller in the case of the porin from *Ps. aeruginosa*. In single-channel experiments with porin protein F larger steps were occasionally observed (not shown in Fig. 6). This might have been caused by the incorporation

of more than one channel forming unit at the same time, presumably caused by the aggregation of protein F in the aqueous phase at the membrane interphase.

Single-channel experiments were performed with a variety of different electrolytes and concentrations. From similar records to those given in Fig. 5, the average conductance increase $\bar{\Lambda}$ over long periods of time was obtained by measuring a large number (73–418) of individual events. In addition to $\bar{\Lambda}$, the specific conductance σ of the given aqueous solution of the salt as well as the ratio $\bar{\Lambda}/\sigma$ are presented in Table I. Despite a variation of $\bar{\Lambda}$ and σ by a factor of about 70, the ratio $\bar{\Lambda}/\sigma$ varied less than 2-fold. Thus the average conductance of a single channel ($\bar{\Lambda}$) followed more or less the mobility sequence of salts in the aqueous phase. In contrast to the results presented in Table I, for *S. typhimurium* and *E. coli*

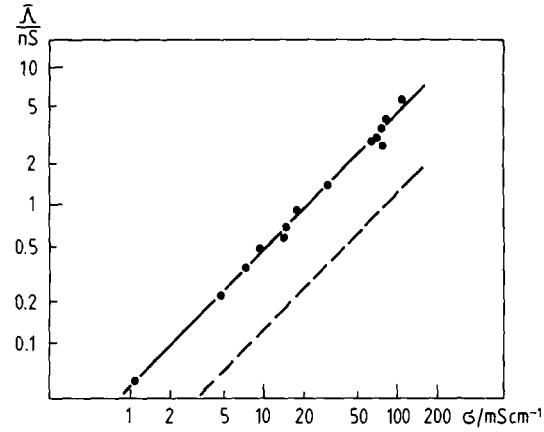

Fig. 7. Average pore conductance $\bar{\Lambda}$ of protein F given as a function of the specific conductance of the corresponding aqueous salt solution; $T = 25°C$. The data were taken from Table I. The broken line corresponds to the result obtained with *E. coli* or *Salmonella* porins [8,10].

TABLE I

AVERAGE CONDUCTANCE INCREMENT $\bar{\Lambda}$ MEASURED ON MEMBRANES FROM OXIDIZED CHOLESTEROL/*n*-DECANE OR EGG LECITHIN/*n*-DECANE IN THE PRESENCE OF PROTEIN F

The pH of the salt solutions with the concentration c was between 6 and 7 if not otherwise indicated; $T = 25°C$; $U = 50$ mV. $\bar{\Lambda}$ was determined by recording a large number n of conductance steps and averaging. σ is the specific conductance of the aqueous salt solution.

Salt	c (M)	$\bar{\Lambda}$ (nS)	σ (mS · cm^{-1})	$\bar{\Lambda}/\sigma$ (10^{-8} cm)	n
Egg lecithin/*n*-decane					
NaCl	0.1	0.48	9.4	5.1	378
	1	4.5	84	5.4	149
KCl	1	5.6	110	5.1	166
Oxidized cholesterol/*n*-decane					
NaCl	0.01	0.052	1.1	4.7	328
	0.1	0.45	9.4	4.8	185
	1	4.3	84	5.1	295
KCl	0.1	0.57	14	4.1	628
	1	5.9	110	5.4	398
$MgCl_2$	0.5	2.9	64	4.5	245
$CaCl_2$	0.5	2.7	78	3.5	357
K_2SO_4	0.5	3.6	76	4.7	418
Na^+ $Hepes^-$ (pH 9)	0.5	0.91	18	5.1	75
$Tris^+$ Cl^-	0.5	1.4	30	4.7	241
$Tris^+$ $Hepes^-$ (pH 8)	0.5	0.35	7.2	4.9	65
$(CH_3)_4N^+$ Cl^-	0.5	3.1	71	4.4	285
$(CH_3)_4N^+$ $Hepes^-$ (pH 8.5)	0.5	0.68	15	4.5	73
$(C_2H_5)_4N^+$ $Hepes^-$	0.5	0.22	4.8	4.6	284

porins [8,10] there was a 3-fold decrease in the ratio $\bar{\Lambda}/\sigma$ for large salts like $(CH_3)_4N^+Hepes^-$ compared to smaller salts like KCl suggesting some restriction of the movement of these larger ions for the porins of bacteria. For *Ps. aeruginosa* porin protein F, the data points for the different salts could be fitted by a highly significant straight line with a correlation coefficient for linear regression of 0.985 (Fig. 7). This indicated that even large ions such as $(C_2H_5)_4N^+$ or $Hepes^-$ were able to pass through the porin channel with little or no interaction with the pore interior.

The ratio $\bar{\Lambda}/\sigma$ was about two (for KCl) to six (for $(CH_3)_4N^+Hepes^-$) times larger for porin protein F from *Ps. aeruginosa* when compared to results obtained for the porins from *E. coli* and *S. typhimurium* [8,10], a finding which can be explained by a larger pore diameter. This would be consistent with the results of vesicle permeability assays using carbohydrates of different sizes [2–6].

Zero-current membrane potentials

Information about the structure of the pore may be obtained from zero-current potential measurements. Fig. 8 shows the results of such experiments

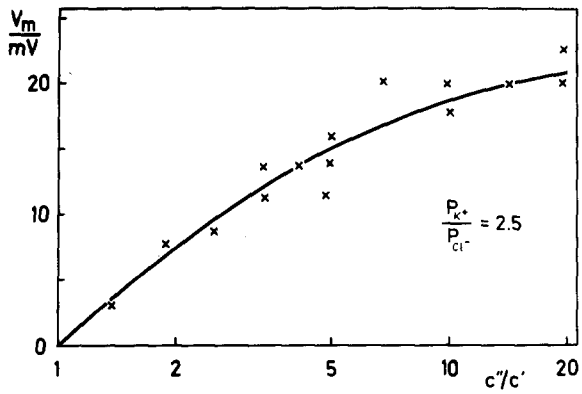

Fig. 8. Zero-current membrane potentials $V_m = \psi' - \psi''$ as a function of the ratio c''/c' of the salt concentrations on both sides of the membrane. c' was fixed to 10^{-2} M KCl and c'' was varied between 10^{-2} M and 0.2 M KCl. Besides the salt, the aqueous phase contained 32.5 ng/ml protein F and 0.5 μg/ml Triton X-100; T = 25°C. The membranes were made from oxidized cholesterol/*n*-decane. V_m was positive at the more dilute side (10^{-2} M KCl). The lines were drawn according to the Goldman-Hodgkin-Katz equation [8] with $P_{K^+}/P_{Cl^-} = 2.5$. The results were obtained from 4 different membranes.

TABLE II

ZERO-CURRENT MEMBRANE POTENTIALS V_m IN THE PRESENCE OF A 10-FOLD SALT CONCENTRATION GRADIENT

V_m is the electrical potential of the dilute side (10^{-2} M) minus the potential of the concentrated side (10^{-1} M). The membranes were formed either from egg lecithin or from oxidized cholesterol dissolved in *n*-decane. The aqueous solutions were unbuffered and had a pH between 6 and 7 if not otherwise indicated; T = 25°C. P_c/P_a (the ratio of the permeability of the cationic species to the permeability of the anionic species) was calculated as described previously [8,10]. V_m was derived from at least three membranes.

Salt	V_m (mV)	P_c/P_a
Egg lecithin/*n*-decane		
NaCl	18 ± 3	2.4 ± 0.4
KCl	20 ± 4	2.7 ± 0.6
Oxidized cholesterol/*n*-decane		
NaCl	16 ± 3	2.2 ± 0.3
KCl	19 ± 3	2.5 ± 0.5
$Tris^+ Cl^-$	15 ± 2	2.1 ± 0.2
$Tris^+ Hepes^-$ (pH 8)	18 ± 3	2.4 ± 0.4

obtained on four different membranes made from oxidized cholesterol/*n*-decane with NaCl as the electrolyte. The zero-current potential (V_m) was found to be positive on the more dilute side of the black lipid bilayer membrane. This indicated that porin protein F forms cation selective pores. From the measured V_m and the concentration gradient c''/c' across the membrane, the ratio of the permeabilities P_c for cations and P_a for anions was calculated according to the Goldman-Hodgkin-Katz-equation as previously described [8]. Table II contains the permeability ratio P_c/P_a for a variety of different salts and for membranes from egg-lecithin and oxidized cholesterol. The observed slight cationic selectivity for all salts may be explained by the presence of negative charges in or near the pore.

Discussion

The experiments in this communication show that protein F isolated from *Ps. aeruginosa* and the total outer membrane dissolved in deoxycholate are

able to form large ion-permeable pores in lipid bilayer membranes. Both the formation and the properties of the pore seem to have some analogy to the pores obtained with porins from *E. coli* and from *S. typhimurium*. However, *Ps. aeruginosa* porin shows two significant differences. The pore conductance was considerably higher (up to 4-fold) than other porins indicating a larger pore size and the activity per unit weight of purified porin was about 100-times lower. There are a number of possible trivial explanations for these results. For example, a molecular rearrangement of the porin might be considered to have occurred during purification, such that the putative trimer structure (by analogy to *E. coli* [17] and *S. typhimurium* [18] porins) became a hexamer. However, recently it was demonstrated that *Ps. aeruginosa* porin is tightly associated with the peptidoglycan and cannot be released by the procedures used for purification [19]. It seems likely, therefore, that the purified porin protein F must have retained a small fragment of peptidoglycan to which it is attached. The firmness of this attachment would seem to preclude the possibility of molecular rearrangement during purification. Furthermore, since there is no evidence of membrane damage upon insertion of *Ps. aeruginosa* porin (in fact there is less variation in pore size that seen for other porins), or of a hydrophilic interaction between porin and the membrane bilayer (see Fig. 3), it would seem unlikely that this attached peptidoglycan is influencing the conductance measurements. The mild procedure used for porin protein F purification, i.e. the use of a non-ionic detergent Triton X-100 as opposed to the powerful ionic detergent sodium dodecyl sulfate used in other porin preparations, again argues that the possibility of either molecular rearrangement or denaturation during the purification was minimized. In addition, the individual conductance increase varied over only a 2-fold range and pores of one half or one third of the average conductance were never observed in thousands of individual events. Thus, we believe the data strongly argue against subunit rearrangement as a mechanism explaining the large conductance increases. In agreement with this, as described below, the conductance increase measured in the black lipid bilayer system using both whole outer membranes and purified porin are in substantial agreement with the results obtained for the pore sizes as estimated by saccharide leakage from reconstituted outer membrane vesicles [3,6] or whole cells with expanded periplasmic spaces (see Refs. 1 and 6 for discussion).

The magnitudes of the conductance increases for *Ps. aeruginosa* were considerably higher than has been observed for other bacterial porins. The largest of these, the *E. coli* outer membrane maltose pore (the λ receptor) still had a conductance 2-fold lower than porin protein F (Table III). The conductance of all of the others has been found to be approximately 3- to 4-fold smaller. This indicates that the diameter of the F-porin pore is larger than the diameter of the other porin pores. If it is assumed that the pore is a cylinder with a spherical cross-section and is filled with an aqueous solution of the same conductance as the external bulk phase, according to the equation: $\bar{\Lambda} = \sigma\pi r^2/l$, the average pore diameter d (=2r) may be calculated for a certain length of the

TABLE III

COMPARISON OF THE PORES FORMED BY DIFFERENCE PORE- FORMING PROTEINS FROM THE OUTER MEMBRANES OF GRAM-NEGATIVE BACTERIA

The diameter d was calculated from the results obtained with 1 M KCl according to the equation: $\bar{\Lambda} = \sigma\pi r^2/l$, using the assumption that the pore is a cylinder of 7.5 nm length (thickness of the outer membrane [25]) and that the pore interior has the same specific conductance as the bulk aqueous phase (for 1 M KCl, 110 mS · cm^{-1}).

Pore	$\bar{\Lambda}$ (nS)	d (nm)	Area (nm^2)
Escherichia coli			
Ia [a]	2.3	1.4	1.6
Ib [a]	2.2	1.3	1.3
Ic [a]	1.7	1.2	1.2
λ-receptor [b]	2.7	1.5	1.8
Salmonella typhimurium			
38 K [c]	2.4	1.4	1.6
39 K [c]	2.2	1.4	1.5
40 K [c]	2.4	1.4	1.5
Pseduomonas aeruginosa			
F [d]	5.6	2.2	3.8

[a] Benz, R. and Henning, U., unpublished results.
[b] From Ref. 9.
[c] From Ref. 10.
[d] This study.

pore *l*. Assuming a pore length of 7.5 nm (corresponding to the thickness of the outer membrane), which seems to be likely, the pore diameter d can be calculated to be about 2.2 nm. Table III shows the diameter of the different porin pores of *E. coli* and *Salmonella* calculated by the above equation from the conductance of the pores in 1 M KCl. As can be seen from Table III the diameter and cross-sectional area of the protein F pore is considerably larger than the diameter of the other pores and it may well account for the permeation of hydrophilic solutes of molecular weights up to 6000, as demonstrated in reconstituted vesicle experiments [3,6]. This indicates that the rough estimates in Table I have some relevance, as has also been shown for the other porin pores.

Both single channel experiments and macroscopic conductance measurements supported the assumption of a large water-filled pore formed by protein F from *Ps. aeruginosa*. For instance, the current vs. voltage relationship of a wide channel should be, and was, ohmic (Fig. 4). In addition, the single-channel conductance varied only little in the series LiCl to CsCl (Table I) and the salt concentration dependence (Fig. 3) showed no saturation. The F-pore is also freely permeable to ions as large as $(C_2H_5)_4N^+$ and $Hepes^-$ and the single channel conductance $\overline{\Lambda}$ was proportional to the conductance in the aqueous phase for all salts studied here.

From the zero-current potential measurements it can be derived that the pore has a slight preference for cations over anions. For a 2.2 nm wide pore, specific interactions with the permeating ions seem rather improbable. A more likely explanation for the observed cationic selectivity would be provided by the assumption that fixed negative charges are present in (or near) the pore. Conceivably lipopolysaccharide, a negatively charged molecule which co-purifies with porin, may contribute to this selectivity. In any case, the selectivity cannot be caused by the neutral detergent Triton or by the negative charges on the membranes from oxidized cholesterol. Porin pores in uncharged egg-lecithin bilayers show the same selectivity and it is therefore most likely that negative charges are located on the pore walls or at the mouth of the pore.

The demonstration of large pores in the outer membrane of *Ps. aeruginosa*, seems superficially to contradict previous results demonstrating the high natural antibiotic resistance, known as permeability-type resistance. However, there are at least two ways by which these results can be reconciled. Either a gated porin (i.e. one that opens or shuts according to specific stimulus) or a large proportion of closed or inactive porin pores would explain the reduced permeability of the outer membrane, given the existence of such large pores. It should be noted that this might limit the growth rate of the organism in dilute media [1], but the maximum growth rate of *Ps. aeruginosa* even in rich media is only half of that of *E. coli*, and thus this might not be a serious problem. We consider it unlikely that the porin is gated and the experiments reported in Fig. 4 would certainly seem to rule out voltage gating (see also Ref. 10 for discussion). Furthermore, given the low selectivity of protein F pores towards cations (Table I) and saccharides [3,6], it seems improbable that specific opening of porin pores in response to a chemical stimulus occurs. Thus, in the absence of specific regulation of pore opening or closing, it would seem likely that the most probable explanation resides in a low proportion of functional pores. As pointed out above, under identical conditions of salt concentrations, membrane composition and protein concentration, about 100- to 300-times less pores per unit area were formed in the membrane by *Ps. aeruginosa* porin protein F than by *E. coli* or *S. typhimurium* porins. This may be due to a number of possible reasons including a large content of inactive proteins, either due to the preparation procedure or due to the presence of inactive porin in the original outer membrane, or due to a lower hydrophobic interaction between the membrane and the porin protein F during black lipid bilayer experiments. The latter possibility would seem to be ruled out since protein F also showed much lower activity in the vesicle permeability assay, an assay in which the bilayers were formed in the presence of the porin protein F and in which vesicle bilayers apparently contained all of the protein (Hancock, R.E.W., preliminary results). We are unable to rule out loss of activity during purification, despite the relatively mild procedures used (see above), but we consider that the in vivo antibiotics data strongly suggest that there are a lower proportion of functional porin proteins in the *Ps. aeruginosa* than in the *E. coli*

or *S. typhimurium* outer membranes. Various lines of evidence are consistent with this, but to date there is little real proof. For example, in our black lipid bilayer experiments, we measured a 10-fold reduction in porin activity (per unit weight of porin protein F) rather than an increase when whole outer membranes in deoxycholate were substituted for purified porin protein F. Furthermore, in the vesicle reconstitution assay [6], 20 μg of purified protein F resulted in the same enhancement of vesicle permeability as 130 μg of unfractionated outer membrane, corresponding closely to the 15% (w/w) content of porin protein F in outer membranes. In addition, it was recently demonstrated that the cationic antibiotics streptomycin and gentamicin which should potentially be taken up by the hydrophilic (porin-mediated) pathway [1,20], use another mechanism for crossing the outer membrane of *Ps. aeruginosa* suggesting a relative paucity of hydrophilic pores in the *Ps. aeruginosa* outer membrane (see Ref. 21 for further discussion). Thus, we postulate that the *Ps. aeruginosa* outer membrane has very large hydrophilic pores, thus allowing a greater size and range of substrates to pass through the pores (e.g. peptides, hydrophobic compounds; see Ref. 1, 3 for discussion), but that relatively few of these pores are open at any given time, allowing slower access of antibiotics into the periplasmic space. This slowing of the rate of antibiotic movement into the periplasm, would be especially effective in combination with inactivating enzymes, such as the periplasmic inducible β-lactamase of *Ps. aeruginosa*.

There is often a tendency amongst microbiologists to consider *E. coli* and enteric organism as a standard and any result differing from this as exceptional. However, as pointed out by Nikaido [1], the large pore size of the *Ps. aeruginosa* porin might provide a better model for porins in general than the smaller pore sizes observed for enteric porins. For example, the porins (voltage-induced anion channels) of the outer membranes of mitochondria from a variety of eukaryotic cells [22,23] show similar conductance increases (approx. 4.5 nS in 1 M KCl) to protein F. In addition, a purified protein from the outer membrane of mung bean mitochondria has a similar exclusion limit for saccharides as protein F [24]. Other non-enteric bacterial outer membranes have also been observed to have similar large exclusion limits (Hancock, R.E.W., Zalman, L. and Nikaido, H., unpublished data). It will be interesting to see if an evolutionary relationship can be established for such porins.

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