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Ion Selectivity of Gram-Negative Bacterial Porins

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Twelve different porins from the gram-negative bacteria *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Yersinia pestis* were reconstituted into lipid bilayer membranes. Most of the porins, except outer membrane protein P, formed large, water-filled, ion-permeable channels with a single-channel conductance between 1.5 and 6 nS in 1 M KCl. The ions used for probing the pore structure had the same relative mobilities while moving through the porin pore as they did while moving in free solution. Thus the single-channel conductances of the individual porins could be used to estimate the effective channel diameters of these porins, yielding values ranging from 1.0 to 2.0 nm. Zero-current potential measurements in the presence of salt gradients across lipid bilayer membranes containing individual porins gave results that were consistent with the conclusions drawn from the single-channel experiments. For all porins except protein P, the channels exhibited a greater cation selectivity for less mobile anions and a greater anion selectivity for less mobile cations, which again indicated that the ions were moving inside the pores in a fashion similar to their movement in the aqueous phase. Three porins, PhoE and NmpC of *E. coli* and protein P of *P. aeruginosa*, formed anion-selective pores. PhoE and NmpC were only weakly anion selective, and their selectivity was dependent on the mobility of the ions. In contrast, cations were unable to enter the selectivity filter of the protein P channel. This resulted in a high anion selectivity for all salts tested in this study. The other porins examined, including all of the known constitutive porins of the four gram-negative bacteria studied, were cation selective with a 3- to 40-fold preference for K^+ ions over Cl^- ions.

The cell envelope of gram-negative bacteria consists of three different layers, the outer membrane, the peptidoglycan layer, and the inner membrane (32). The inner membrane, which is a phospholipid bilayer, represents a real diffusion barrier. Movement of molecules such as substrates and small ions across the cytoplasmic membrane occurs via transport systems involving specific integral membrane proteins. The outer membrane acts as a molecular filter with a defined exclusion limit for hydrophilic solutes (32). These molecular sieving properties result from integral outer membrane proteins called porins (28), which form large water-filled pores through the outer membrane. Purified porin trimers have been reconstituted in lipid vesicles (18, 27, 28, 33) and artificial lipid bilayer membranes (3, 4, 6). In both systems they form water-filled pores with effective diameters between 1 and 2 nm (3, 4, 6, 18, 28, 33).

The study of the permeability properties of porin trimers in lipid vesicles has presented weak evidence that the pores are not simply water-filled channels but exhibit a certain selectivity for cations or anions (33). The lipid bilayer technique provides a much better tool for the study of the single conductive unit (i.e., porin trimer) and its properties under various conditions. Furthermore, this technique allows access to both sides of a membrane, and the pore properties can be studied as the function of changes of pH and salt concentration (4). The measurement of zero-current membrane potentials yields precise information on the selectivity of the pores under all conditions. Furthermore, the lipid bilayer technique allows the study of a single unit, which is not possible by other methods.

In this paper we studied the ionic selectivity of a variety of different porins of *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Yersinia pestis* in the presence of three different salts. Most of the pores appear to be cation selective. Only 3 of 13 porins studied here showed anion selectivity. Two of them (PhoE and protein P) are induced in phosphate-starved cells (19, 39), whereas the third (NmpC) has been found in phenotypic revertants of *E. coli* K-12 mutants lacking the OmpC and OmpF porins in the outer membrane (24). The results presented here, with one exception, demonstrate that these porins formed large water-filled channels in which the small ions showed the same mobility sequence as they did in the aqueous phase. The exception, *P. aeruginosa* outer membrane protein P, formed a small, highly anion-selective channel. In contrast to the large water-filled channels, the permeability of the protein P pore for anions was related to their size and not to their aqueous mobility.

MATERIALS AND METHODS

Bacterial strains and porin isolation. The OmpC and OmpF (B) porins were purified from strains JF700 (16) (obtained from B. Bachmann, Coli Genetic Stock Center, Yale University, New Haven, Conn.) (*proC24 ompF254 ompA256 his-53 purE41 ilv-277 met-65 lacY29 xyl-14 rpsL97 cycA1 cycB2 tsx-63*) and *E. coli* B, respectively, by Gordon Crockford as described previously (38). The OmpF (K-12) porin was a generous gift of Taiji Nakae and was obtained from *E. coli* K-12 strain KY2209 (30). Purification of the *E. coli* NmpC porin (21) by Gordon Crockford, of the *E. coli* PhoE porin (1) and *Y. pestis* protein E (12) by Richard P. Darveau, of the *E. coli* protein K porin (41) by Chris Whitfield, and of the *P. aeruginosa* protein P porin (19) by Keith Poole have been described previously.

The *S. typhimurium* porins were generously supplied by Taiji Nakae. They were obtained from strains HN407 (OmpF), SH5551 (OmpC), and SH6017 (OmpD), producing single species of porin as described elsewhere (38).

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Lipid bilayer experiments. The methods used for black lipid bilayer experiments have been described in detail in a previous publication (6). The instrumentation consisted of a Teflon chamber with two compartments separated by a thin wall. The circular hole in the wall connecting the two compartments had an area of either 0.1 mm^2 (for the single-channel measurements) or 2 mm^2 (for the selectivity measurements). The membranes were formed across the hole by painting on a 1% (wt/vol) solution of different lipids dissolved in *n*-decane. Bilayer formation was indicated when the membrane turned optically black to reflected light. The temperature was kept at 25°C throughout.

All salts were obtained from Merck, Darmstadt, Federal Republic of Germany (analytical grade). The pH of the aqueous salt solutions was adjusted to pH 6 without buffering by the addition of the corresponding acid or base. The protein was added to the aqueous phase from the stock solutions either before membrane formation or after the membrane had completely turned black. To prevent inactivation, proteins were added to the salt solutions immediately before the start of individual experiments. Two different lipids were used for membrane formation: diphytanoyl phosphatidylcholine was obtained from Avanti Biochemicals, Birmingham, Ala., and oxidized cholesterol was prepared as described earlier (6). The single-channel conductance of the pores was measured after application of a fixed transmembrane potential with a pair of calomel electrodes with salt bridges (Metrohm, Herisau, Switzerland) inserted into the two aqueous salt solutions on both sides of the membrane. The current through the pores was boosted by a current amplifier (Keithley 427) monitored on a storage oscilloscope (Tektronix 5115) and recorded on a strip chart or tape recorder.

The zero-current membrane potential measurements were made as previously described (7). The membranes were formed in a 10 mM salt solution containing a predetermined concentrated protein stock so that the conductance of the membrane increased about 100- to 1,000-fold within 20 to 30 min. The voltage was then switched off and the instrumentation was switched to allow measurement of zero-current membrane potentials. The salt concentration on one side of the membrane was raised by adding small amounts of concentrated solutions while stirring. The zero-current membrane potential reached its final value within 5 to 10 min.

RESULTS

Single-channel experiments. The porins used in this study were able to increase the conductance of artificial lipid bilayer membranes by many orders of magnitude. This increase was found to depend on the type of lipid used for membrane formation. For membranes from oxidized cholesterol in *n*-decane, the membrane conductance at a fixed porin concentration was about 100- to 1,000-fold larger than it was for membranes formed from diphytanoyl phosphatidylcholine in *n*-decane. It should be noted, however, that the properties of the single channels were virtually identical for both lipids. This suggests that the insertion of the preformed pore (most probably a trimer of three identical polypeptide subunits) into the bilayer membranes is governed by a kinetic process, as has been discussed in detail elsewhere (4).

Adding small amounts of the porins to membranes with small surface areas allowed the resolution of step increases in membrane conductance. These steps indicate that the unit of conductance is a defined structure. Furthermore, by analogy with other single-channel experiments (3, 4, 6), they represent the incorporation of trimers into the membranes. The lifetime of the single channels was on the time scale of minutes. Most of the conductance steps were directed upwards, and terminating events were only rarely observed. The single-channel conductance was in most cases distributed over a two- to threefold range as reported elsewhere (4). Only for protein P of *P. aeruginosa* outer membrane was a sharp histogram observed (20).

Single-channel conductances were measured for a variety of different porins of *E. coli* and protein E from *Y. pestis*. The average single-channel conductance $\bar{\Lambda}$ was calculated by counting a sufficient number of single events (at least 100). Values of $\bar{\Lambda}$ for the three different electrolytes KCl, LiCl, and KCH_3COO are given in Table 1 together with some earlier-published data obtained with porins of *S. typhimurium* (5) and *P. aeruginosa* (3). The three different salts were chosen to provide a comparison of the single-channel conductance as a function of the aqueous mobility of the different ions. Potassium ions and chloride ions have about the same mobility in the aqueous phase (limiting molar conductivities of 73.5 and 76.4 S × cm^2 per mol, respectively [10]), whereas the mobilities of Li^+ and CH_3COO^- are considerably smaller (limiting molar conductivities of 38.7 and 40.9 S × cm^2 per mol, respectively). The single-channel conductance of a large water-filled pore which is cation

TABLE 1. Average single-channel conductance in the presence of porins[a]

Porin	$\bar{\Lambda}$ (nS)			Pore diam (nm)
	1 M KCl	1 M LiCl	1 M KCH_3COO	
E. coli				
OmpF (B)	2.1	0.73	1.3	1.2
OmpF (K-12)	1.9	0.62	1.1	1.1
OmpC	1.5	0.68	0.96	1.0
PhoE	1.8[b]	1.2[b]	0.62	1.1
K	1.5	0.52	1.0	1.0
NmpC	1.3	1.0	0.52	1.0
LamB[c]	2.7[d]/0.2	1.3[d]/0.05	—[e]	1.4[d]
S. typhimurium[f]				
OmpF (39K)	2.2	1.1	—	1.2
OmpC (40K)	2.4	0.90	—	1.3
OmpD (38K)	2.5	0.97	—	1.3
P. aeruginosa				
F[g]	5.6	—	—	2.0
P	0.28	0.27	0.033	0.6
Y. pestis				
E	1.7	0.76	0.85	1.1

[a] Average single-channel conductance $\bar{\Lambda}$ measured on membranes from diphytanoyl phosphatidylcholine–*n*-decane or oxidized cholesterol–*n*-decane in the presence of porins of *E. coli*, *S. typhimurium*, *P. aeruginosa*, and *Y. pestis*. Aqueous salt solution pH, 6; temperature, 25°C; V_m, 50 mV. $\bar{\Lambda}$ was determined by recording and averaging at least 100 conductance steps. The specific conductances of the aqueous salt solutions were 110 mS/cm (1 M KCl), 71 mS/cm (1 M LiCl), and 70 mS/cm (1 M KCH_3COO).

[b] Data are from reference 1.

[c] More recent investigations of LamB preparations have indicated that LamB forms two types of pores in lipid bilayer membranes. The small pore occurs about 20 times more frequently than the large pore and can be blocked by maltose and maltodextrins. Equation 1 cannot be used to calculate the size of the small pore (R. Benz, A. Schmid, T. Nakae, and G. Vos-Scheperkeuter, unpublished data).

[d] From reference 9.

[e] —, Not done.

[f] Data for *S. typhimurium* are from reference 5.

[g] Data are from reference 3.

selective will follow the aqueous mobility sequence of the cations. This is in fact the case for most of the porins of *E. coli*, all the porins of *S. typhimurium*, protein F of *P. aeruginosa* (3), and protein E of *Y. pestis*. This suggests that all of these porins formed cation-selective pores. On the other hand, the single-channel conductance of PhoE and NmpC of *E. coli* did not follow the mobility sequence of the cations but instead followed that of the anions, indicating that these pores could be anion selective. The channel formed by protein P had the same single-channel conductance for 1 M KCl and 1 M LiCl, but a drastically reduced single-channel conductance for 1 M KCH_3COO. This result is consistent with the observation that protein P forms a highly anion-selective channel with a small selectivity filter (2).

The finding that small ions move inside the pores in a fashion similar to the way they move through the aqueous phase allowed a rough estimate of the channel size to be made as discussed in detail elsewhere (4). The conductance Λ of a hollow cylinder filled with an aqueous solution of the same specific conductance σ as the bulk aqueous phase is given by the equation

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

where r is the radius and l is the length of the pore. Assuming a length of 6 nm, which is in agreement with the dimensions of the *E. coli* OmpF trimers as derived from X-ray diffraction studies of crystallized OmpF (17), the pore diameters can be calculated (σ = 110 mS/cm) (Table 1). The diameters calculated from equation 1 are in close agreement with those estimated from the results of the liposome swelling assay (33) and from the exclusion limits of pores (28). It has to be noted, however, that the diameter of the protein P pore of *P. aeruginosa* cannot be calculated in a similar way because of the high selectivity of this channel for anions (2).

Zero-current membrane potentials. The conclusions drawn from the single-channel experiments were supported by measurements of zero-current membrane potentials in the presence of salt gradients across the membranes (Fig. 1, Table 2). The measurements were performed by creating a salt concentration gradient across a membrane where about 100 porin pores had been inserted. This created a driving force for movement of the ions through the porin pores towards the dilute side of the membrane. The ions moved

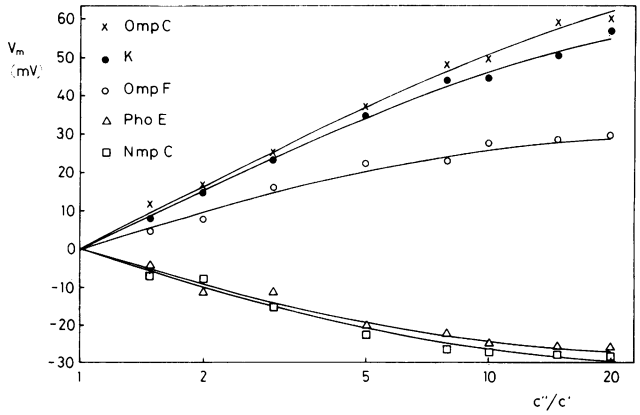

FIG. 1. Zero-current membrane potentials V_m (in millivolts) as a function of the KCl concentration gradient c''/c' across membranes where different porins of *E. coli* were inserted. c' was fixed to 10^{-2} M KCl and c'' was varied between 10^{-2} and 0.2 M KCl. The membranes were formed either from diphytanoyl phosphatidylcholine–*n*-decane or oxidized cholesterol–*n*-decane. V_m is the potential at the dilute side of the membrane; the temperature was 25°C. The lines were drawn according to the Goldman-Hodgkin-Katz equation (7), using the indicated values for P_c/P_a. The porins OmpF (B) and OmpF (K-12) showed the same selectivity. The data points represent mean values for at least four individual experiments.

TABLE 2. Zero-current membrane potentials[a]

Porin	V_m (mV)		
	KCl	LiCl	KCH_3COO
E. coli			
OmpF (B)	27	8.5	41
OmpF (K12)	26	8.4	39
OmpC	50	46	54
PhoE	−24[b]	−40[b]	−8.9
K	46	37	52
NmpC	−26	−47	−13
LamB[c]	30	—[d]	—
S. typhimurium			
OmpF	46	28	53
OmpC	54	42	56
OmpD	50	34	53
P. aeruginosa			
F[e]	20	—	—
P	−58	−57	−59
Y. pestis			
E	45	33	47

[a] Zero-current membrane potentials V_m in the presence of a 10-fold salt concentration gradient across membranes where different porins were inserted. V_m is the electrical potential of the dilute side (10^{-2} M) minus the potential at the concentrated side (10^{-1} M). The membranes were formed either from diphytanoyl phosphatidylcholine–*n*-decane or from oxidized cholesterol–*n*-decane. The pH of the aqueous solutions was 6; the temperature was 25°C.
[b] From reference 1.
[c] From reference 9.
[d] —, Not done.
[e] From reference 3.

TABLE 3. Permeability ratios of different porins as calculated by the Goldman-Hodgkin-Katz equation[a]

Porin	P_c/P_a		
	KCl	LiCl	KCH_3COO
E. coli			
OmpF (B)	3.9	1.5	9.5
OmpF (K-12)	3.6	1.5	8.2
OmpC	26	15	54
PhoE	0.30	0.11	0.65
K	15	7.6	34
NmpC	0.27	0.055	0.53
LamB	4.5	—[b]	—
S. typhimurium			
OmpF	14	4.2	37
OmpC	41	10	65
OmpD	23	5.9	36
P. aeruginosa			
F	2.7	—	—
P	<0.01	<0.01	<0.01
Y. pestis			
E	13	5.6	17

[a] Calculated from the data shown in Table 2.
[b] —, Data not available.

according to the selectivity of the porins (e.g., cation-selective channels preferentially allowed the passage of cations). Thus, the voltage increased across the membrane until the induced voltage balanced the salt concentration gradient and ions ceased to flow across the membrane. At this stage the voltage was the zero-current membrane potential.

Figure 1 shows the zero-current membrane potentials V_m in the presence of the different *E. coli* porins measured as a function of the salt gradient c''/c' across the membranes. V_m was positive on the dilute side for OmpF, OmpC, and protein K but negative for PhoE and NmpC. This indicated preferential movement of cations in the first case and preferential movement of anions in the latter case in response to the concentration gradient. The results (Fig. 1) could be reasonably well fitted to the Goldman-Hodgkin-Katz equation as described earlier (7) with the indicated ratios of the cation permeability P_c divided by the anion permeability P_a (Fig. 1). The zero-current membrane potentials measured as a result of a 10-fold salt concentration gradient across membranes doped with the different porins are given in Table 2.

These data were used to calculate the permeability ratios P_c/P_a by application of the Goldman-Hodgkin-Katz equation (Table 3). Four of the porins from the *E. coli* outer membrane were found to be cation selective. The same applied to the three porins of the outer membrane of *S. typhimurium*, to protein F of *P. aeruginosa*, and to protein E of *Y. pestis*. The permeability ratio was found to be dependent on the ions present in the aqueous phase. The pores generally showed greater cation selectivity if Cl^- was replaced by the less mobile anion acetate and less cation selectivity if K^+ was replaced by the less mobile cation Li^+. This clearly indicated that the ions move through the pores similarly to the way they move in the aqueous phase (i.e., in bulk solution).

The two anion-selective pores of *E. coli*, PhoE and NmpC, showed a greater anion selectivity for the less mobile cation and a smaller selectivity for the less mobile anion. In this respect protein P of the *P. aeruginosa* outer membrane represents a complete exception. Although the single-channel conductance in the presence of 1 M KCH_3COO was drastically reduced compared with that in 1 M KCl, the pore remained highly anion selective in KCH_3COO with a P_a/P_c of >100. This finding is in agreement with the earlier-published observation that cations cannot enter the protein P pore (2).

DISCUSSION

This paper clearly demonstrates that most porins of gram-negative bacteria form large water-filled channels in lipid bilayer membranes. The ions used in this study did not show any noticeable interaction with the pore interior, which could be related to the size of the pores or of the ions per se. On the other hand, it could be demonstrated that the channels were slightly selective. The normal porins, i.e., those present under most growth conditions, in the outer membrane of *E. coli*, *S. typhimurium*, *P. aeruginosa*, and *Y. pestis* were cation selective. This cation selectivity is apparently caused by an excess of negatively charged groups inside the pore or on the surface of the protein or both, as shown in some cases by chemical modification of the porin trimers (8, 13, 20) and by the dependence of the ionic selectivity on the pH of the aqueous phase (1, 7). The magnitude of the selectivity may be dependent on the number of excess charges. If this is true, it would seem that the OmpC and protein K pores have a larger excess of negatively charged groups than the OmpF pore. In any case, of the methods used to date to measure the permeability properties of the porin pores, the lipid bilayer technique is the only one which could be used to detect the magnitude of the selectivity of porin pores. For example, in a study with reconstituted vesicles, Nikaido and Rosenberg pointed out considerable theoretical problems in the interpretation of their experimental results (33). In contrast, the interpretation of the results obtained from lipid bilayer membranes is more straightforward because of the use of simple salt solutions containing ions with known aqueous mobilities.

All three porins of the *S. typhimurium* outer membrane were found to be cation selective. This result is in agreement with the data obtained earlier from single-channel experiments (5). It is, however, in disagreement with the results which have been derived from the vesicle permeability assay (30). In this study it was claimed that OmpD, named earlier the 34K (38) or 38K (5) protein, plays the same role in *S. typhimurium* as PhoE in the outer membrane of *E. coli*, i.e., it is a pore with some preference for the permeation of negatively charged solutes (30, 33, 39). In fact, OmpD was demonstrated here to be cation selective. Furthermore, a pore which is similar to that formed by PhoE has recently been observed in the outer membrane of phosphate-starved *S. typhimurium* cells (K. Bauer, R. Benz, J. Brass, and W. Boos, unpublished data). This clearly demonstrates, together with the results presented here, that OmpD does not have the same function as the PhoE protein.

E. coli B outer membrane contains under normal growth only one porin, OmpF (B) (11), whereas expression of the two porins in the outer membrane of *E. coli* K-12, OmpF (K-12) and OmpC, is regulated by the osmolarity of the growth medium (31), although the promoter exchange between the *ompF* and *ompC* genes has virtually no influence on the growth rate of the cells (25). The amino acid sequences of OmpF (B) and OmpF (K-12) differ in only a small number of positions (11, 22). The selectivity of both porins was similar, although the effective diameter of the OmpF (B) pore appears to be a little larger than that of the OmpF (K-12) pore. This result is in agreement with the data obtained from reconstituted vesicle experiments (30).

PhoE and NmpC of *E. coli* and protein P of *P. aeruginosa* all formed anion-selective pores in lipid bilayer membranes. PhoE and NmpC were only weakly anion selective, and the selectivity was dependent on the mobility of the ions (Table 3). In contrast, protein P showed, for all salts tested here, a greater than 100-fold preference for anions over cations even though the single-channel conductance was drastically reduced in the presence of acetate (Table 1). This substantial difference in selectivity of the three anion-selective channels can be largely explained by the difference in size of the PhoE and NmpC channels compared with that of the protein P channel. We have presented evidence that protein P has a constriction of about 0.6 nm diameter, with positively charged amino acids located near this constriction (2, 20). PhoE and NmpC apparently do not contain such a constriction, according to the data presented here and elsewhere (1, 13, 21). Thus, despite the presence of excess positively charged groups in the channel, cations are not completely repelled and can enter the channel. In agreement with this we have demonstrated that the protein P channel has a strong binding site for anions which results in saturation of the single-channel conductance at high salt concentrations, whereas no saturation was observed for the PhoE and NmpC pores (1, 21). From this we may conclude that the postulated binding site (34) inside the PhoE pore may be questionable. On the

other hand it is obvious that even a weak anion selectivity results in an increased penetration rate of negatively charged solutes through the PhoE pore as compared with that through OmpF and OmpC pores (Table 1).

The question whether porin trimers from *E. coli*, *S. typhimurium*, and *P. aeruginosa* contain one or three pores has been controversial (4, 6, 32, 36). Our own lipid bilayer experiment results have always been consistent with the assumption of only one pore per trimer, whereas Schindler and Rosenbusch (36) proposed the existence of three independent voltage-gated pores. Structural data have now been presented demonstrating that the OmpF trimers contain basically one pore with three openings on the external surface of the cell (14, 15). The three channels on the outside merge into one channel at approximately the middle of the outer membrane (15). This means that the structure of the OmpF pore is consistent with the results obtained from our lipid bilayer experiments. On the other hand, it cannot be excluded that in the case of other organisms porin trimers contain three pores, as has in fact been observed for porins from *Rhodopseudomonas sphaeroides* (40) and from *Anabaena variabilis* (1a).

Strong homology exists between the primary sequences of the OmpF, OmpC, and PhoE porins (26). Furthermore, protein K and NmpC have about the same molecular weight and show immunological cross-reaction with the other three porins (23, 37). This suggests that the arrangement of these three polypeptides in the pore-forming unit could be very similar for these five porins of *E. coli*, and the same pore structure may be expected for all of them. OmpF, OmpC, and OmpD of *S. typhimurium* show some chemical analogy to OmpF, OmpC, and NmpC of *E. coli* (23, 35), and we would expect a similar pore structure in the trimers of both these gram-negative bacteria. However, we would like to stress the point that OmpD is cation selective, whereas NmpC shows a weak selectivity for anions. Furthermore, some evidence exists that the pores of *S. typhimurium* generally have a larger cross-section than the *E. coli* porin pores (5, 29).

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